

DESCRIPTION

METHOD FOR PRODUCING 3,5-DIHYDROXY-4-METHOXYBENZYL ALCOHOL FROM PLANKTONS

TECHNICAL FIELD

[0001]

The present invention relates to a producing method for 3,5-dihydroxy-4-methoxybenzyl alcohol for producing 3,5-dihydroxy-4-methoxybenzyl alcohol as an antioxidant from plankton.

BACKGROUND ART

[0002]

The present inventor has already found 3,5-dihydroxy-4-methoxybenzyl alcohol (hereinafter referred to as DHMBA) as a new antioxidant from a heated oyster meat, and succeeded in its synthesis and identification. DHMBA has not been detected from a raw oyster meat.

Here, as ecological features of oyster, it has been generally known that an oyster inhales a large amount of seawater, and takes in plankton as a prey from the inhaled large amount of seawater.

[0003]

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DISCLOSURE OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0004]

However, the present inventor made a hypothesis that plankton contains DHMBA in the present invention.

Then, when the present inventor collected plankton, filtered them, and measured DHMBA in the plankton, DHMBA was not detected. Next, when the present inventor collected the plankton, filtered them, and heated them, DHMBA was detected. When the present inventor collected the plankton, filtered them, and applied a pressure to them, DHMBA was detected.

[0005]

In view of this, the present inventor considered that the DHMBA was a useful substance derived from plankton, and reached an invention of a method for producing the DHMBA from the plankton.

SOLUTIONS TO THE PROBLEMS

[0006]

The present invention is a method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton. The method includes: filtering collected seawater containing the plankton using a filter; taking out a cell content from the plankton remained on the filter; and subsequently heating the cell content thus taken out at a temperature of 60°C for one hour or more to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the heated material. The plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

Alternatively, a method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton includes: filtering collected seawater containing the plankton using a filter; crushing the plankton remained on the filter with an addition of an extracting solution; and extracting a cell content from the plankton and subsequently performing

heating at a temperature of 60°C for one hour or more to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the heated material. The plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

Alternatively, a method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton includes: filtering collected seawater containing the plankton using a filter; taking out a cell content from the plankton remaining on the filter; and subsequently heating the cell content thus taken out at a temperature of 80°C for 30 minutes or more to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the heated material. The plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

Alternatively, a method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton includes: filtering collected seawater containing the plankton using a filter; crushing the plankton remained on the filter with an addition of an extracting solution; and extracting a cell content from the plankton and subsequently performing heating at a temperature of 80°C for 30 minutes or more to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the heated material. The plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

Alternatively, a method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton includes: filtering collected seawater containing the plankton using a filter; taking out a cell content from the plankton remained on the filter; and

subsequently pressurizing the cell content thus taken out at 2 atmospheres for 30 minutes to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the pressurized material. The plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

Alternatively, a method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton includes: filtering collected seawater containing the plankton using a filter; crushing the plankton remained on the filter with an addition of an extracting solution; and extracting a cell content from the plankton and subsequently performing pressurization at 2 atmospheres for 30 minutes to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the pressurized material. The plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

Alternatively, a method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton includes: filtering collected seawater containing the plankton using a filter; taking out a cell content from the plankton remaining on the filter; and subsequently pressurizing the cell content thus taken out at 2.5 atmospheres for 30 minutes or more to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the pressurized material. The plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

Alternatively, a method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton includes: filtering collected seawater containing the plankton using a filter; crushing the plankton remained on the filter with an addition of an extracting

solution; and extracting a cell content from the plankton and subsequently performing pressurization at 2.5 atmospheres for 30 minutes or more to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the pressurized material. The plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

Alternatively, a method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton includes: filtering collected seawater containing the plankton using a filter; taking out a cell content from the plankton remaining on the filter; and subsequently pressurizing the cell content thus taken out at 3 atmospheres for 30 minutes or more to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the pressurized material. The plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

Alternatively, a method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton includes: filtering collected seawater containing the plankton using a filter; crushing the plankton remained on the filter with an addition of an extracting solution; and extracting a cell content from the plankton and subsequently performing pressurization at 3 atmospheres for 30 minutes or more to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the pressurized material. The plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

EFFECTS OF THE INVENTION

[0007]

The present invention can provide a method for collecting a seawater containing plankton and producing DHMBA as an antioxidant from the plankton included in the seawater, thus providing an excellent effect.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008]

Fig. 1 is a drawing for explaining a filtration of a collected seawater.

Fig. 2 is a drawing for explaining a sonication of plankton remaining on a filter used for the filtration and a subsequent heating extraction of DHMBA.

Fig. 3 is a drawing for explaining a sonication of plankton remaining on a filter used for the filtration and a subsequent pressure extraction of DHMBA.

Fig. 4 is an explanatory drawing for explaining whether or not DHMBA is produced when plankton existing in a seawater collected in a predetermined sea area of Hiroshima at a predetermined timing is heated.

Fig. 5 an explanatory drawing for explaining whether or not DHMBA is produced when a pressure is applied to plankton existing in a seawater collected in a predetermined sea area of Hiroshima at a predetermined timing.

Fig. 6 is an explanatory drawing (1) for explaining a result of a search with a search condition of global search excluding environmental sequences.

Fig. 7 is an explanatory drawing (2) for explaining a result of a search with a search condition of global search excluding environmental sequences.

Fig. 8 is an explanatory drawing (1) for explaining a result of a search with a search condition limited to sequences from type material.

Fig. 9 is an explanatory drawing (2) for explaining a result of a search with a

search condition limited to sequences from type material.

Fig. 10 is an explanatory drawing (3) for explaining a result of a search with a search condition of global search excluding environmental sequences.

Fig. 11 is an explanatory drawing (4) for explaining a result of a search with a search condition of global search excluding environmental sequences.

Fig. 12 is an explanatory drawing (5) for explaining a result of a search with a search condition of global search excluding environmental sequences.

Fig. 13 is an explanatory drawing (3) for explaining a result of a search with a search condition limited to sequences from type material.

Fig. 14 is an explanatory drawing (4) for explaining a result of a search with a search condition limited to sequences from type material.

Fig. 15 is an explanatory drawing (5) for explaining a result of a search with a search condition limited to sequences from type material.

Fig. 16 is an explanatory drawing (6) for explaining a result of a search with a search condition of global search excluding environmental sequences.

Fig. 17 is an explanatory drawing (7) for explaining a result of a search with a search condition of global search excluding environmental sequences.

Fig. 18 is an explanatory drawing (8) for explaining a result of a search with a search condition of global search excluding environmental sequences.

Fig. 19 is an explanatory drawing (6) for explaining a result of a search with a search condition limited to sequences from type material.

Fig. 20 is an explanatory drawing (7) for explaining a result of a search with a search condition limited to sequences from type material.

Fig. 21 is an explanatory drawing (8) for explaining a result of a search with a search condition limited to sequences from type material.

Fig. 22 is an explanatory drawing (9) for explaining a result of a search with a search condition of global search excluding environmental sequences.

Fig. 23 is an explanatory drawing (10) for explaining a result of a search with a search condition of global search excluding environmental sequences.

Fig. 24 is an explanatory drawing (11) for explaining a result of a search with a search condition of global search excluding environmental sequences.

Fig. 25 is an explanatory drawing (9) for explaining a result of a search with a search condition limited to sequences from type material.

Fig. 26 is an explanatory drawing (10) for explaining a result of a search with a search condition limited to sequences from type material.

Fig. 27 is an explanatory drawing (11) for explaining a result of a search with a search condition limited to sequences from type material.

DESCRIPTION OF PREFERRED EMBODIMENTS

[0009]

First, a seawater was collected in a sea area (for example, sea area of Hiroshima) where an oyster culture was mainly performed at a predetermined timing, the collected seawater was filtered, plankton 1 remaining on a filter used for the filtration were taken out, the plankton 1 was heated or pressurized, and then a search and an analysis of whether DHMBA was detected or not were performed.

[0010]

First, the detection of DHMBA by heating will be described.

A seawater was collected in a sea area as a field of oyster culture (for example, sea area of Hiroshima).

[0011]

(Collection of Seawater containing Plankton)

A seawater containing plankton in September is pumped up by a pump or the like in a sea area (for example, sea area of Hiroshima) where an oyster culture is performed.

While the seawater in the sea area of Hiroshima was collected in this embodiment, the seawater is not limited to the seawater of Hiroshima.

For the timing, it is not limited to the seawater in September.

[0012]

(Filtration of Seawater containing Plankton)

After collecting the seawater containing the plankton 1, the seawater is filtered by, for example, a GF/C filter 4 formed of a nonwoven fabric or the like (see Fig. 1).

Here, while the amount of seawater to be filtered is not limited, the seawater was collected by about 3100 liters for each in the sea area of Hiroshima in this time, and filtered.

A large amount of the plankton 1 adhere to the filter after the filtration by the above-described method, and the filter to which the plankton 1 adhere can be kept frozen until the extraction work is performed.

[0013]

(Ultrasonic Treatment)

Next, for example, the filter to which the plankton 1 adhere is frozen, and the frozen filter is put in a container 5 such as a centrifuge tube, an ultrapure water or the like is added in the container 5, and further, the plankton 1 is subjected to a sonication by ultrasonic sound wave for about one hour, a ball milling, or the like to break cell walls, thus causing DHMBA and the like to be easily extracted.

[0014]

(Extraction)

After breaking the cells of the plankton 1 by the method such as the sonication, heating is performed to take out contents of the cells. That is, the plankton and the like are subjected to a hot water extraction at about 60°C or about 80°C or the like (see Fig. 2).

Here, because of the heating by hot water extraction, the plankton 1 in the container 5 is not directly heated. That is, a heated hot water is reserved in a beaker or the like, and the container 5 is put in the hot water, thereby performing what is called indirect heating. This provides an advantage of a stable heating extraction by the heating at a constant temperature or the like.

[0015]

(Sampling)

Then, for the hot water extraction, sampling was performed at each of 30 minutes, one hour, two hours, three hours, four hours, and five hours.

[0016]

(Analysis of Useful Substance)

The concentration of DHMBA of the sampled plankton 1 was measured by LC-MS/MS.

[0017]

(Result of Extraction Experiment by Hot Water Extraction Performed at About 60°C)

DHMBA was not detected in the plankton before heating, and after 30 minutes and after five hours from the hot water extraction.

However, it was confirmed that DHMBA was detected by an extraction amount of 0.0053 (ng/L) after the heating, that is, after one hour from the hot water extraction.

It was confirmed that DHMBA was detected by the extraction amount of 0.0064 (ng/L)

after two hours from the hot water extraction, and DHMBA was detected by the extraction amount of 0.0059 (ng/L) after three hours from the hot water extraction. Furthermore, it was confirmed that DHMBA was detected by the extraction amount of 0.0063 (ng/L) after four hours from the hot water extraction (see Fig. 4).

[0018]

(Result of Extraction Experiment by Hot Water Extraction Performed at About 80°C)

DHMBA was not detected in the plankton before heating and after two hours from the hot water extraction.

However, it was confirmed that DHMBA was detected by the extraction amount of 0.0035 (ng/L) after the heating, that is, after 30 minutes from the hot water extraction, and DHMBA was detected by the extraction amount of 0.0053 (ng/L) after one hour from the hot water extraction. It was confirmed that DHMBA was detected by the extraction amount of 0.0035 (ng/L) after three hours from the hot water extraction, and DHMBA was detected by the extraction amount of 0.0045 (ng/L) after four hours from the hot water extraction. Furthermore, it was confirmed that DHMBA was detected by the extraction amount of 0.0036 (ng/L) after five hours from the hot water extraction (see Fig. 4).

[0019]

Note that, the heating period is not limited to a period until the elapse of one hour. There is a possibility that DHMBA is detected after the heating period shorter than one hour.

In any case, it was found that while DHMBA was not detected in plankton when the plankton was not heated, DHMBA was promptly detected in the heated plankton when the plankton was heated.

[0020]

Next, the detection of DHMBA by pressurizing will be described.

First, a seawater was collected in a sea area as a field of oyster culture (for example, sea area of Hiroshima).

[0021]

(Collection of Seawater Containing Plankton)

A seawater in a predetermined timing in a sea area (for example, sea area of Hiroshima) where an oyster culture is performed, that is, a seawater containing plankton in September 2019 in a sea area of Hiroshima prefecture is pumped up by a pump or the like.

While the seawater in the sea area of Hiroshima was collected in this embodiment, the seawater is not limited to the seawater of Hiroshima. For the timing, it is not limited to the seawater in September.

[0022]

(Filtration of Seawater Containing Plankton)

After collecting the seawater containing the plankton 1, the seawater is filtered by, for example, a GF/C filter 4 formed of a nonwoven fabric or the like (see Fig. 1).

Here, while the amount of seawater to be filtered is not limited, the seawater was collected by about 3100 liters in the sea area of Hiroshima in this time, and filtered.

A large amount of the plankton 1 adhere to the filter after the filtration by the above-described method, and the filter to which the plankton 1 adhere can be kept frozen until the extraction work is performed.

[0023]

(Ultrasonic Treatment)

Next, for example, the filter to which the plankton 1 adhere is frozen, and the frozen filter is put in a container 5 such as a centrifuge tube, an ultrapure water or the

like is added in the container 5, and further, the plankton 1 is subjected to a sonication by ultrasonic sound wave for about one hour, a ball milling, or the like to break cell walls, thus causing DHMBA and the like to be easily extracted.

[0024]

(Extraction)

Then, after breaking the cells of the plankton 1 by the method such as the sonication, a pressure is applied to them to extract contents of the cells. That is, the plankton and the like are pressurized to about 2 atmospheres, about 2.5 atmospheres, or about 3 atmospheres, thus performing the extraction (see Fig. 3).

Here, the pressurizing method is not limited. The pressurization may be performed using an autoclave as a general method. The pressurization may be performed by another method.

[0025]

(Sampling)

Then, for the pressure extraction, sampling was performed at each of 30 minutes, one hour, three hours, and five hours.

[0026]

(Analysis of Useful Substance)

The concentration of DHMBA of the sampled plankton 1 was measured by LC-MS/MS.

[0027]

(Result of Extraction Experiment by Pressurization to About 2 Atmospheres)

Fig. 5 illustrates the detection result of DHMBA by the sampling in the sea area of Hiroshima.

Fig. 5 illustrates the change in the extraction amount of DHMBA for each time.

DHMBA was not detected in the plankton before the pressurization. It was confirmed that DHMBA was detected by the extraction amount of 0.0125 (ng/L) after the pressurization at 2 atmospheres, that is, after the pressurization at 2 atmospheres for 30 minutes.

[0028]

(Result of Extraction Experiment by Pressurization to About 2.5 Atmospheres)

DHMBA was not detected in the plankton before the pressurization (see Fig. 5). It was confirmed that DHMBA was detected by the extraction amount of 0.197 (ng/L) after the pressurization at 2.5 atmospheres, that is, after the pressurization at 2.5 atmospheres for 30 minutes, and DHMBA was detected by the extraction amount of 0.355 (ng/L) after the pressurization for one hour. It was confirmed that DHMBA was detected by the extraction amount of 0.483 (ng/L) after the pressurization at 2.5 atmospheres for three hours, and DHMBA was detected by the extraction amount of 0.683 (ng/L) after the pressurization at 2.5 atmospheres for five hours (see Fig. 5).

[0029]

(Result of Extraction Experiment by Pressurization to About 3 Atmospheres)

DHMBA was not detected in the plankton before the pressurization (see Fig. 5). It was confirmed that DHMBA was detected by the extraction amount of 0.282 (ng/L) after the pressurization at 3 atmospheres, that is, after the pressurization at 3 atmospheres for 30 minutes, and DHMBA was detected by the extraction amount of 0.938 (ng/L) after the pressurization for one hour. It was confirmed that DHMBA was detected by the extraction amount of 1.925 (ng/L) after the pressurization at 3 atmospheres for three hours, and DHMBA was detected by the extraction amount of 1.341 (ng/L) after the pressurization at 3 atmospheres for five hours (see Fig. 5).

[0030]

In any case, it was found that while DHMBA was not detected in plankton when the plankton was not pressurized, DHMBA was promptly detected in the pressurized plankton when the plankton was pressurized.

[0031]

(Identification of Plankton 1 in Which DHMBA Can Be Detected)

Many kinds of the plankton 1 exist in the seawater, and it has been unknown what kind of the plankton 1 DHMBA is detected in. Therefore, the present inventor decided to identify the plankton 1 in which DHMBA was detected.

[0032]

The present inventor collected the seawater as described above, and collected the plankton 1 in the seawater.

About 200 kinds were selected from the collected large number of kinds of the plankton 1, and the about 200 kinds of the plankton 1 were each cultured.

Then, the above-described extraction process of DHMBA was performed for each of the about 200 kinds of the cultured plankton. That is, the ultrasonic treatment, the extraction process by heating or pressurizing, and the like were performed.

[0033]

Consequently, the plankton 1 in which DHMBA was detected were four strains of microalgae. By the observation of the four strains of microalgae based on morphology in detail, they were identified as diatoms.

The diatoms are most abundant in phytoplankton, and many kinds of diatoms exist.

When the present inventor observed the morphology of the four strains of microalgae in which DHMBA was detected in detail by a microscope, the four strains were all diatoms. Here, the magnification of the used microscope is 1,000 times

(eyepiece: 10 times, objective lens: 100 times).

The four strains of microalgae, that is, diatoms are families of marine diatoms widely distributed in seawater of bays, and known as diatoms producing abundant unsaturated fatty acids. They are also known as super planktonic diatoms.

[0034]

Next, the present inventor performed a DNA analysis of the four strains of microalgae, that is, diatoms, and intended to further specifically identify the diatoms.

The DNA analysis is performed to obtain which class in classification the diatoms belong to, and the class in classification is classified into "phylum," "subphylum," "class," "subclass," "order," "family," "genus," and "species." In the order from the "phylum" toward the "species," the classification levels become more specific.

The following describes the process of the DNA analysis.

[0035]

Classification of Diatom (Strain H-7-09) in Which DHMBA Is Detected

It seems a diatom of subclass Bacillariophycidae. However, classification was not made at the level of "order" or below.

[0036]

(Method)

Performing megaBLAST search with Genbank (NCBI, NHI)

Search Condition (1) global search excluding environmental sequences (Fig. 6, Fig. 7)

→ The examination is performed based on this result.

Search Condition (2) Search limited to sequences from type material (Fig. 8, Fig. 9)

→ Since the information amount of sequence data of comparison targets were insufficient, the examination based on this result is not performed.

Site	Sequence name	Base sequence length used for analysis	Sequence assembly	Blast analysis result
18S	H7-09-18S	1223 bp	Performed	Fig. 6
28S	H7-09-28S-D2R2	474 bp	Not performed	Fig. 7

[0037]

(megaBLAST Search Result)

· 18S

The homology with the sequence of *Humidophila schmassmannii* isolate HYU-D030 strain was approximately 96.2%.

→ The homology is low at approximately 96%.

However, in consideration of sequences hit in the second and subsequent orders, it is supposed to be a diatom of class Bacillariophyceae, subclass Bacillariophycidae.

· Sequence of 28S D2R2

The homology with the sequence of *Pseudo-nitzschia multistriata* strain HAB-132 strain was approximately 96.1%.

→ The homology is low at approximately 96%. Since the sequence is short, and both the score and the cover rate are low, the degree of reliability on the classification result is low compared with the result of 18S.

However, it is consistent with the possibility of being a diatom of subclass Bacillariophycidae supposed from the result of 18S.

[0038]

(Classification Result)

They are considered to be diatoms of phylum Bacillariophyta, subphylum

Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae.

However, classification cannot be made at the level of "order" or below.

[0039]

Classification of Diatom (Strain H-9-05) in Which DHMBA was Detected

It seems a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

However, classification was not made at the level of "species."

[0040]

(Method)

Performing megaBLAST search with Genbank (NCBI, NHI)

Search Condition (1) global search excluding the environmental sequences (Fig. 10, Fig. 11, Fig. 12)

→ The examination is performed based on this result.

Search Condition (2) Search limited to sequences from type material (Fig. 11, Fig. 12, Fig. 13)

→ Since the information amount of sequence data of comparison targets were insufficient, the examination based on this result is not performed.

Site	Sequence name	Base sequence length used for analysis	Sequence assembly	Blast analysis result
18S	H9-05-18S	1204 bp	Performed	Fig. 10
28S	H9-05-28S-D2C2	480 bp	Not performed	Fig. 11
28S	H9-05-28S-D2R2	458 bp	Not performed	Fig. 12

[0041]

(megaBLAST Search Result)

· 18S

The homology with the sequence of *Entomoneis paludosa* L431 strain was

98%.

→ While the homology is slightly low at 98%, in consideration that the second and subsequent orders hit the genus *Entomoneis*, it is supposed to be a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus *Entomoneis*.

· Sequence of 28S D2C2

The homology with the sequence of *Entomoneis ornata* 27D strain was 92%.

→ Since the homology is considerably low at 92%, it is not very useful as a reference. Since the sequence is short, and both the score and the cover rate are low, the degree of reliability on the classification result is considerably low compared with the result of 18S.

However, it is consistent with the possibility of being a diatom of genus *Entomoneis* supposed from the result of 18S.

· Sequence of 28S D2R2

The homology with the sequence of *Pseudo-nitzschia multistriata* HAB-132 strain was 96%.

→ Since the homology is low at 96%, it is not very useful as a reference. Since the sequence is short, and the score and the cover rate are low compared with D2C2, the degree of reliability on the classification result is considerably low.

However, it is consistent with the possibility of being a diatom of subclass Bacillariophycidae supposed from the result of 18S.

[0042]

(Classification Result)

They are considered to be diatoms of phylum Bacillariophyta, subphylum

Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

However, classification was not made at the level of "species."

[0043]

Classification of Diatom (Strain H-9-06) in Which DHMBA IS Detected

It seems a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus Entomoneis.

However, classification was not made at the level of "species".

[0044]

(Method) Performing megaBLAST search with Genbank (NCBI, NHI)

Search Condition (1) global search excluding the environmental sequences (Fig. 16, Fig. 17, Fig. 18)

→ The examination is performed based on this result.

Search Condition (2) Search limited to sequences from type material (Fig. 19, Fig. 20, Fig. 21)

→ Since the information amount of sequence data of comparison targets were insufficient, the examination based on this result is not performed.

Site	Sequence name	Base sequence length used for analysis	Sequence assembly	Blast analysis result
18S	H9-06-18S	1204 bp	Performed	Fig. 16
28S	H9-06-28S-D2C2	440 bp	Not performed	Fig. 17
28S	H9-06-28S-D2R2	609 bp	Not performed	Fig. 18

[0045]

(megaBLAST Search Result)

· 18S

The homology with the sequence of *Entomoneis paludosa* L431 strain was

99%.

→ In consideration that the homology is 99%, and the second and subsequent orders hit the genus *Entomoneis*, it is supposed to be a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus *Entomoneis*.

· Sequence of 28S D2C2

The homology with the sequence of *Entomoneis ornata* 27D strain was 91%.

→ Since the homology is considerably low at 91%, it is not very useful as a reference. Since the sequence is short, and the score is low, the degree of reliability on the classification result is considerably low compared with the result of 18S.

However, it is consistent with the possibility of being a diatom of genus *Entomoneis* supposed from the result of 18S.

· Sequence of 28S D2R2

The homology with *Vannella septentrionalis* (ameba) was 88%.

The homology with fungi forming arbuscular mycorrhiza was 79% or less.

→ The homology is less than 90%. Since the sequence is short, and the score and the cover rate are low compared with D2C2, the degree of reliability on the classification result is considerably low.

Therefore, it is excluded from this examination on the classification.

[0046]

(Classification Result)

They are considered to be diatoms of phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, genus *Entomoneis*.

[0047]

Strain H-9-09 Classification

Undetermined

[0048]

(Method) Performing megaBLAST search with Genbank (NCBI, NHI)

Search Condition (1) global search excluding the environmental sequences (Fig. 22, Fig. 23, Fig. 24)

→ The examination is performed based on this result.

Search Condition (2) search limited to sequences from type material (Fig. 25, Fig. 26, Fig. 27)

→ Since the information amount of sequence data of comparison targets were insufficient, the examination based on this result is not performed.

Site	Sequence name	Base sequence length used for analysis	Sequence assembly	Blast analysis result
18S	H9-09-18S	1228 bp	Performed	Fig. 22
28S	H9-09-28S-D2C2	511 bp	Not performed	Fig. 23
28S	H9-09-28S-D2R2	441 bp	Not performed	Fig. 24

[0049]

(megaBLAST Search Result)

· 18S

The homology with the sequence of *Caecitellus paraparvulus* HFCC320 strain (*bicosoeca*) was 97%.

For each of the second and subsequent orders, the homology with the diatom in the same genus was 97%.

→ While the homology is slightly low at 97%, the possibility that the sequence of *bicosoeca* was amplified cannot be denied.

However, in the microscopic examination performed in advance, it is confirmed that the specimen has a shape like a diatom.

· Sequence of 28S D2C2

The homology with the sequence of *Caecitellus paraparvulus* HFCC71 strain (bicosoeca) was 88%.

→ The homology is less than 90%. Since the sequence is short, and both the score and the cover rate are low, the degree of reliability on the classification result is considerably low compared with the result of 18S.

Therefore, it is excluded from this examination on the classification.

However, it is consistent with the result of 18S (being bicosoeca).

· Sequence of 28S D2R2

The homology with the sequence of *Pseudo-nitzschia multistriata* HAB-132 strain (subclass Bacillariophycidae) was 92%.

→ The homology is low at 92%. Since the sequence is short, and both the score and the cover rate are low, the degree of reliability on the classification result is low compared with the result of 18S.

However, it is consistent with the morphology observation performed in advance (being a diatom).

[0050]

(Classification Result)

Undetermined

DESCRIPTION OF REFERENCE SIGNS

[0051]

- | | |
|---|-------------|
| 1 | Plankton |
| 4 | GF/C filter |
| 5 | Container |

CLAIMS

1. A method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton, the method comprising:

filtering collected seawater containing the plankton using a filter;

taking out a cell content from the plankton remained on the filter; and

subsequently heating the cell content thus taken out at a temperature of 60°C for one hour to four hours to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the heated material, wherein

the plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, and genus Entomoneis.

2. A method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton, the method comprising:

filtering collected seawater containing the plankton using a filter;

crushing the plankton remained on the filter with an addition of an extracting solution;

and

extracting a cell content from the plankton and subsequently performing heating at a temperature of 60°C for one hour to four hours to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the heated material, wherein

the plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, and genus Entomoneis.

3. A method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton, the method comprising:

filtering collected seawater containing the plankton using a filter;

taking out a cell content from the plankton remaining on the filter; and

subsequently heating the cell content thus taken out at a temperature of 80°C for 30 minutes to one hour, or three hours to five hours to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the heated material, wherein

the plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, and genus Entomoneis.

4. A method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton, the method comprising:

filtering collected seawater containing the plankton using a filter;

crushing the plankton remained on the filter with an addition of an extracting solution; and

extracting a cell content from the plankton and subsequently performing heating at a temperature of 80°C for 30 minutes to one hour, or three hours to five hours to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the heated material, wherein

the plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, and genus Entomoneis.

5. A method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton, the method comprising:

filtering collected seawater containing the plankton using a filter;
taking out a cell content from the plankton remained on the filter; and
subsequently pressurizing the cell content thus taken out at 2 atmospheres for 30 minutes to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the pressurized material, wherein

the plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, and genus Entomoneis.

6. A method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton, the method comprising:

filtering collected seawater containing the plankton using a filter;
crushing the plankton remained on the filter with an addition of an extracting solution;
and

extracting a cell content from the plankton and subsequently performing pressurization at 2 atmospheres for 30 minutes to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the pressurized material, wherein

the plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, and genus Entomoneis.

7. A method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton, the method comprising:

filtering collected seawater containing the plankton using a filter;
taking out a cell content from the plankton remaining on the filter; and

subsequently pressurizing the cell content thus taken out at 2.5 atmospheres for 30 minutes or more to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the pressurized material, wherein

the plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, and genus Entomoneis.

8. A method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton, the method comprising:

filtering collected seawater containing the plankton using a filter;

crushing the plankton remained on the filter with an addition of an extracting solution;

and

extracting a cell content from the plankton and subsequently performing pressurization at 2.5 atmospheres for 30 minutes or more to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the pressurized material, wherein

the plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, and genus Entomoneis.

9. A method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton, the method comprising:

filtering collected seawater containing the plankton using a filter;

taking out a cell content from the plankton remaining on the filter; and

subsequently pressurizing the cell content thus taken out at 3 atmospheres for 30 minutes or more to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the pressurized

material, wherein

the plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, and genus Entomoneis.

10. A method for producing 3,5-dihydroxy-4-methoxybenzyl alcohol from plankton, the method comprising:

filtering collected seawater containing the plankton using a filter;

crushing the plankton remained on the filter with an addition of an extracting solution;

and

extracting a cell content from the plankton and subsequently performing pressurization at 3 atmospheres for 30 minutes or more to produce 3,5-dihydroxy-4-methoxybenzyl alcohol from the pressurized material, wherein

the plankton is a diatom belonging to phylum Bacillariophyta, subphylum Bacillariophytina, class Bacillariophyceae, subclass Bacillariophycidae, order Surirellales, family Entomoneidaceae, and genus Entomoneis.