

DESCRIPTION

**Title of Invention: WHITE CHLORELLA PROTOTHECOIDES STRAIN HAVING HIGH
CRUDE PROTEIN CONTENT AND METHOD FOR PRODUCING CHLORELLA
BIOMASS USING SAME**

TECHNICAL FIELD

The present invention relates to a white *Chlorella protothecoides* strain having a high crude protein content and a method for producing a *Chlorella* biomass from a culture solution of the strain.

BACKGROUND ART

Chlorella, which is one of the freshwater green microalgae, contains nutrients such as carbohydrates, proteins, fats, as well as chlorophyll (i.e., a green pigment referred to as chlorophyll), vitamins, minerals, and the like, which are beneficial to the human body. Thus, chlorella is known as a raw material for foods with excellent nutritional value and has been selected even as a space food. In addition, chlorella, which is widely used as a raw material for health functional foods, not only has a high antioxidant effect, but can also help improve skin health, immune function, and blood cholesterol. Chlorella is cultured by autotrophic culture, heterotrophic culture, or a combination thereof and may be cultured in a medium containing a carbon source, a nitrogen source, a trace metal, a trace element, and the like. Green chlorella containing chlorophyll is unstable to pH and/or heat and thus has a characteristic that is easily changed into brown color.

Chlorella-derived crude proteins, i.e., the crude proteins contained in *Chlorella* strains, comprise pure proteins, nucleic acids, amide compounds, urea, and the like. The present inventors have developed a *Chlorella* strain having high crude protein productivity [e.g., *Chlorella protothecoides* DS-NCRC7 (KCTC 18633P)] and a method for producing *Chlorella*-derived crude proteins using the same (Korean Patent No.

10-2026681).

Meanwhile, International Patent Publication No. WO 2010/120923 discloses a microalgal food composition comprising microalgal biomass. The microalgal biomass is a biomass of the white color mutant selected from the *Chlorella protothecoides* stains through treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) [i.e., *Chlorella protothecoides* 33-55 (PTA-10396)]. However, the cell body (biomass) of *Chlorella protothecoides* 33-55 (PTA-10396) disclosed in International Patent Publication No. WO 2010/120923 has a high lipid content (approximately 68%) and thus a relatively low crude protein content (e.g., up to 32% even if the amounts of carbohydrates, moisture, and ash are assumed to be 0%).

Accordingly, there is a need in the art to discover a *Chlorella protothecoides* strain providing a biomass capable of solving the problem of color development caused when added to foods and the like, as well as having a high crude protein content within the biomass.

DISCLOSURE

Technical Problem

The present inventors have carried out various studies to develop a white *Chlorella protothecoides* strain having high crude protein productivity. Especially, the present inventors obtained various mutants through N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment on *Chlorella protothecoides* DS-NCRC7 (KCTC 18633P), which had been developed by the present inventors, and evaluated whiteness and crude protein productivity of the obtained mutants. As a result, the present inventors have found that the selected specific strain not only exhibits high crude protein productivity but also provides a biomass having white color.

Therefore, it is an object of the present invention to provide the novel *Chlorella protothecoides* strain selected as in the above.

It is another object of the present invention to provide a method for producing a *Chlorella* biomass comprising culturing the novel *Chlorella protothecoides* strain and

recovering a biomass therefrom.

Technical Solution

5 In accordance with an aspect of the present invention, there is provided a *Chlorella protothecoides* strain, having a whiteness index (WI) of 70 or more as calculated from the following formula and a crude protein content of 55 wt% or more in a biomass thereof.

$$\text{WI} = 100 - \sqrt{(100 - L)^2 + a^2 + b^2}$$

10 wherein, L, a, and b are Hunter L, a, and b values.

In the *Chlorella protothecoides* strain according to the present invention, the crude protein content in the biomass may be 55 to 80 wt%. A chlorophyll content may be 3.0 or less mg/g dry cell weight, e.g., 0.1 to 3.0 mg/g dry cell weight. In an embodiment, the strain may be *Chlorella protothecoides* DS-NCRC7W (KCTC 15163BP).

15 In accordance with another aspect of the present invention, there is provided a method for producing a *Chlorella* biomass, comprising culturing said *Chlorella protothecoides* strain; and recovering a biomass from the resulting culture solution.

In the method of the present invention, the strain may be *Chlorella protothecoides* DS-NCRC7W (KCTC 15163BP). The culturing may be carried out in an aqueous
20 medium having a pH of 6.5 to 7.5, containing 2-15 g/L of MgSO₄, 0.5-5 g/L of KH₂PO₄, 0.0001-0.1 g/L of ZnSO₄, 0.0001-0.01 g/L of CuSO₄, 0.001-0.1 g/L of CaCl₂, and 0.001-0.1 g/L of FeSO₄.

ADVANTAGEOUS EFFECTS

25 The *Chlorella protothecoides* strain obtained according to the present invention, particularly *Chlorella protothecoides* DS-NCRC7W (KCTC 15163BP), can not only exhibit high crude protein productivity but also provide a biomass having white color. Therefore, the strain and the *Chlorella* biomass obtained therefrom can effectively solve
30 the problem of color development caused when added to foods and the like.

DESCRIPTION OF DRAWINGS

FIG. 1 shows the appearances of the culture solutions obtained by culturing under shaking the *Chlorella protothecoides* mutant strains obtained according to the present invention.

FIG. 2 shows the appearance of the powder obtained by spray drying a culture concentrate of the novel *Chlorella protothecoides* strain obtained according to the present invention.

FIG. 3 shows the results obtained by measuring the appearance of the powder obtained by spray drying a culture concentrate of the novel *Chlorella protothecoides* strain obtained according to the present invention, with a colorimeter.

BEST MODE

As used herein, Whiteness Index (WI) refers to the degree of whiteness calculated according to a known method (e.g., Chin-Lin Hsu et al., Food Chemistry 83 (2003) 85-92). Specifically, the whiteness index (WI) is a value calculated by the following formula from the Hunter values obtained by measuring from three spots of the sample with a colorimeter (colour differential meter), i.e., Hunter L, a, and b values (average values for five samples each).

<Formula 1>

$$WI = 100 - \sqrt{(100 - L)^2 + a^2 + b^2}$$

wherein, L, a, and b are Hunter L, a, and b values.

The present invention provides a *Chlorella protothecoides* strain, having a whiteness index (WI) of 70 or more as calculated from the above formula and a crude protein content of 55 wt% or more in a biomass thereof.

The present inventors carried out random mutation by treating the known *Chlorella protothecoides* DS-NCRC7 (KCTC 18633P) twice with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and selected a mutant strain exhibiting high

cell mass and crude protein content as well as exhibiting white color. And, the present inventors confirmed that the selected mutant strain could be effectively cultured through fed-batch cultivation in a 5 L jar fermenter. The selected mutant strain was named *Chlorella protothecoides* DS-NCRC7W and deposited at the Korean Collection for Type Culture (KCTC) of Korea Research Institute of Bioscience and Biotechnology on October 31, 2022, and was assigned the accession number KCTC 15163BP.

In the *Chlorella protothecoides* strain according to the present invention, the crude protein content in the biomass may be preferably 55 to 80 wt%. And, the *Chlorella protothecoides* strain according to the present invention has a low chlorophyll content. For example, the chlorophyll content in the *Chlorella protothecoides* strain according to the present invention may be 3.0 or less mg/g dry cell weight (DCW), e.g., 0.1 to 3.0 mg/g dry cell weight. In an embodiment, the strain may be *Chlorella protothecoides* DS-NCRC7W (KCTC 15163BP).

The present invention also provides a method for producing a *Chlorella* biomass, comprising culturing said novel *Chlorella protothecoides* strain; and recovering a biomass from the resulting culture solution.

The culturing may be carried out according to conventional culture methods of a *Chlorella* strain (e.g., Republic of Korea Patent No. 10-2026681). For example, the culturing may be carried out in a medium containing glucose as a sugar source, phosphates (KH_2PO_4 , K_2HPO_4 , etc.), ammonium salts ($(\text{NH}_4)_2\text{SO}_4$, etc.), calcium salts (CaCl_2 , etc.), metal salts (MgSO_4 , ZnSO_4 , CuSO_4 , FeSO_4 , etc.), etc., in water (e.g., purified water, etc.). In an embodiment, the culturing may be carried out in an aqueous medium having a pH of 6.5 to 7.5, containing 2-15 g/L of MgSO_4 , 0.5-5 g/L of KH_2PO_4 , 0.0001-0.1 g/L of ZnSO_4 , 0.0001-0.01 g/L of CuSO_4 , 0.001-0.1 g/L of CaCl_2 , and 0.001-0.1 g/L of FeSO_4 . And, if necessary, the medium may further include an antifoaming agent or the like. The culturing in the medium described above may be carried out according to conventional culture methods, for example, according to fed-batch culture in a sterile tank, under 0.3-1.0 vvm of air and 200-500 rpm of agitation.

The recovering a biomass may be carried out by recovering a biomass, e.g., through centrifuging the culture solution. The obtained biomass may be concentrated to an appropriate concentration through a plurality of concentration and washing steps

as needed. The *Chlorella* biomass may be obtained in a powder form through conventional methods, for example, spray drying or drum drying.

The present invention will be described in further detail with reference to the following examples. These examples are for illustrative purposes only and are not intended to limit the scope of the present invention.

Example 1: Selection of mutant strains through random mutation

(1) Primary selection of mutant strains

For performing random mutation, *Chlorella protothecoides* DS-NCRC7 (KCTC 18633P) was treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Specifically, *Chlorella protothecoides* DS-NCRC7 (KCTC 18633P) was treated with NTG for about 30 minutes, spread on the plates containing a medium (glucose 15 g/L, KH₂PO₄ 1 g/L, ZnSO₄ 0.001 g/L, and agar 20 g/L), and then cultured for about 15 days. After about 60 strains exhibiting a white color were selected from the plates, the strains were pooled in distilled water and then two mutants exhibiting a white color were selected.

(2) Secondary selection of mutant strains

The two mutant strains selected in (1) above were treated with NTG for about 30 minutes, spread on plates containing a medium (glucose 15 g/L, MgSO₄ 2 g/L, KH₂PO₄ 1 g/L, ZnSO₄ 0.001 g/L, FeSO₄ 0.03 g/L, and agar 20 g/L) in the same manner as in (1), and then cultured for about 15 days. After about 40 mutants exhibiting a white color were obtained from the plates, the mutants were pooled in distilled water and then two mutants exhibiting a white color were selected.

In order to confirm the protein contents and growths of the secondary-selected mutants, a medium [an aqueous solution (pH 7.0) containing glucose 10 g/L, MgSO₄ 0.3 g/L, KH₂PO₄ 0.7 g/L, ZnSO₄ 0.001 g/L, and FeSO₄ 0.03 g/L] was added to a 500 mL baffled flask and then cultured under shaking. The above shaking culture was carried out for 48 hours under the conditions of a starting liquid volume of 100 ml, a culture temperature of 28°C, and an agitation speed of 140 rpm. After performing the shaking culture for 48 hours as described above, the cell mass and crude protein content from each culture flask were measured. The cell mass was obtained by centrifuging the culture solution to remove the medium, washing the pellet twice with distilled water, drying the resulting cell suspension at 105°C for 24 hours, and then measuring the

weight of the resultant. The crude protein content was obtained by centrifuging the culture solution to remove the medium, washing the pellet twice with distilled water, adding sulfuric acid (H₂SO₄) and a decomposition accelerator, Kjeltabs S-3.4 (manufactured by Foss) thereto for heat-decomposition, and then measuring with the Kjeldahl nitrogen quantitative assay method. The results are shown in Table 1 below. And, the appearances of the culture solutions obtained through the shaking culture are as shown in Figure 1.

Table 1

Mutant	Cell mass (g/L)	Crude protein (%)
DS-NCRC7W	4.8	67.21
DS-NCRC7W03	4.4	66.04

As can be seen from the results of Table 1 above, it can be confirmed that DS-NCRC7W exhibits high cell mass and crude protein content. The above-selected mutant strain, DS-NCRC7W, was named *Chlorella protothecoides* DS-NCRC7W and deposited at the Korean Collection for Type Culture (KCTC) of Korea Research Institute of Bioscience and Biotechnology on October 31, 2022, and was assigned the accession number KCTC 15163BP.

Example 2: 5L JF culture of *Chlorella protothecoides* DS-NCRC7W

A medium [an aqueous solution (pH 7.2) containing glucose 40 g/L, MgSO₄ 3 g/L, KH₂PO₄ 2 g/L, ZnSO₄ 0.05 g/L, CuSO₄ 0.0005 g/L, CaCl₂ 0.04 g/L, and FeSO₄ 0.01 g/L] was added to a 5 L jar fermenter and the DS-NCRC7W strain [i.e., *Chlorella protothecoides* DS-NCRC7W (KCTC 15163BP)] was cultured under conditions of an agitation speed of 500 rpm and aeration of 1.6 vvm for approximately 3 days. After completion of the culture, a certain amount was taken, and the dry cell weight (DCW) and crude protein content were measured. The cell mass was obtained by centrifuging the culture solution to remove the medium, washing the pellet twice with distilled water, drying the resulting cell suspension at 105°C for 24 hours, and then measuring the weight of the resultant. The crude protein content was obtained by centrifuging the culture solution to remove the medium, washing the pellet twice with distilled water, adding sulfuric acid (H₂SO₄) and a decomposition accelerator, Kjeltabs S-3.4

(manufactured by Foss) thereto for heat-decomposition, and then measuring with the Kjeldahl nitrogen quantitative assay method. The results are shown in Table 2 below.

Table 2

Mutant	Cell mass (g/L)	Crude protein (%)
DS-NCRC7W	43.4	60.12

In addition, the culture solution was centrifuged to collect the supernatant, which was concentrated approximately twice to obtain a culture concentrate. 1 L of the obtained culture concentrate was spray-dried to obtain a powder, and the appearance of the obtained powder is shown in FIG. 2.

Example 3: Measurement of chlorophyll content and colorimetric measurement

The DS-NCRC7W strain obtained according to the present invention [i.e., *Chlorella protothecoides* DS-NCRC7W (KCTC 15163BP)], *Chlorella protothecoides* DS-NCRC7 (KCTC 18633P), and *Chlorella vulgaris* (dsv77) were cultured in a 5 L jar fermenter, respectively. Each powder obtained therefrom was used as a sample. Each total chlorophyll content was measured through the test method according to the standards and regulations of health functional food of Ministry of Food and Drug Safety (MFDS) [i.e., Total chlorophyll measurement protocol (First method)]. Specifically, distilled water was added to each powder, allowed to stand for 30 minutes, placed in a centrifugal tube, mixed with an alkaline pyridine solution, allowed to stand for 15 minutes in a 60°C water bath, and then centrifuged to obtain a supernatant. The total chlorophyll contents therein were quantified by measuring the absorbance at wavelengths of 419 nm and 454 nm against an alkaline pyridine solution. The results are shown in Table 3 below.

Table 3

Strain	Total chlorophyll (mg/g)
DS-NCRC7W [<i>Chlorella protothecoides</i> DS-NCRC7W (KCTC 15163BP)]	0.72
DS-NCRC7	0.21

[<i>Chlorella protothecoides</i> DS-NCRC7 (KCTC 18633P)]	
<i>Chlorella vulgaris</i> (dsv77)	28.64

In addition, Hunter L, a, and b values (mean and SD) were obtained for each powder (five samples per powder) using a colorimeter (manufactured by Konica Minolta), and the whiteness indexes (WI) were calculated from these values using the following formula. The results are shown in Table 4.

<Formula 1>

$$WI = 100 - \sqrt{(100 - L)^2 + a^2 + b^2}$$

wherein, L, a, and b are Hunter L, a, and b values.

Table 4

Sample	Value	Mean	SD	Whiteness Index (WI)	Appearance
DS-NCRC7W [<i>Chlorella protothecoides</i> DS-NCRC7W (KCTC 15163BP)]	L	87.62	0.02	79	FIG. 3 (A)
	a	0.54	0.02		
	b	16.72	0.09		
DS-NCRC7 [<i>Chlorella protothecoides</i> DS-NCRC7 (KCTC 18633P)]	L	75.76	0.09	31	FIG. 3 (B)
	a	2.88	0.08		
	b	64.18	0.23		
<i>Chlorella vulgaris</i> (dsv77)	L	21.25	0.14	21	FIG. 3 (C)
	a	-3.75	0.13		
	b	6.06	0.17		

From the results of Table 3 and Table 4 (Fig. 3), it can be confirmed that the DS-NCRC7W strain selected according to the present invention [i.e., *Chlorella protothecoides* DS-NCRC7W (KCTC 15163BP)] has a chlorophyll content of 3.0 mg/g or less dry cell weight and exhibits a white color.

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

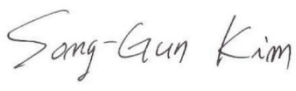
issued pursuant to Rule 7.1

TO: **Daesang Corporation**

Daesang Corporation

178 Magokjungang-ro, Gangseo-gu, Seoul

Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Chlorella protothecoides</i> DS-NCRC7W	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 15163BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on October 31, 2022 .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) 181, Ipsin-gil, Jeongeup-si, Jeollabuk-do 56212 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  KIM, Song-Gun, Director Date: November 30, 2022

Form BP/4 (KCTC Form 17)

sole page

(Reissue)

CLAIMS

[Claim 1] A *Chlorella protothecoides* strain, having a whiteness index (WI) of 70 or more as calculated from the following formula and a crude protein content of 55 wt% or more in a biomass thereof.

$$WI = 100 - \sqrt{(100 - L)^2 + a^2 + b^2}$$

wherein, L, a, and b are Hunter L, a, and b values.

[Claim 2] The *Chlorella protothecoides* strain according to claim 1, wherein a crude protein content in the biomass is 55 to 80 wt%.

[Claim 3] The *Chlorella protothecoides* strain according to claim 1, wherein a chlorophyll content is 3.0 or less mg/g dry cell weight.

[Claim 4] The *Chlorella protothecoides* strain according to claim 3, wherein a chlorophyll content is 0.1 to 3.0 mg/g dry cell weight.

[Claim 5] The *Chlorella protothecoides* strain according to any one of claims 1 to 4, wherein the strain is *Chlorella protothecoides* DS-NCRC7W (KCTC 15163BP).

[Claim 6] A method for producing a *Chlorella* biomass, comprising culturing a *Chlorella protothecoides* strain according to any one of claims 1 to 4; and recovering a biomass from the resulting culture solution.

[Claim 7] The method according to claim 6, wherein the strain is *Chlorella protothecoides* DS-NCRC7W (KCTC 15163BP).

[Claim 8] The method according to claim 6, wherein the culturing is carried out in an aqueous medium having a pH of 6.5 to 7.5, containing 2-15 g/L of MgSO₄, 0.5-5 g/L of

KH_2PO_4 , 0.0001-0.1 g/L of ZnSO_4 , 0.0001-0.01 g/L of CuSO_4 , 0.001-0.1 g/L of CaCl_2 , and 0.001-0.1 g/L of FeSO_4 .