BATCH FERMENTATION WITH *NITZSCHIA. SING1* DIATOMS CONVERTS ALGINATE INTO OILS

FIELD OF THE INVENTION

This invention relates to a method for producing oil for food and/or fuel application from apochlorotic *Nitzschia sp.* diatoms grown in batch fermentation using supplemented or synthetic seawater and alginate as feedstock. More particularly, the invention relates to the use of an isolated *Nitzcshia* sp., designated *N. sing1-1* (or *N. sing1*), deposited under the terms of the Budapest Treaty with CCAP accession number CCAP-1052/27.

BACKGROUND OF THE INVENTION

In recent years, massive *Sargassum* blooms occurring in the Atlantic Ocean (up to 20 million metric tons annually) have had a devastating impact on coastal environments and economies in the Caribbean and southern coastal US. The precise cause of these *Sargassum* blooms remains unknown. However, they are likely fed by ocean currents transporting nutrients derived from terrestrial sources related to global warming and the overuse of agricultural fertilizers, in particular nitrogen. Thus, it is likely that these blooms will persist and increase in frequency and location of occurrence over time. This is further supported by recent reports of abnormal *Sargassum* blooms in the East China and Yellow Sea.

There is currently an international effort to valorize Sargassum biomass. Sargassum production in Singapore local waters is estimated to be about 1,100 metric tonnes per year.

There is a need to provide methods to produce useful products from Sargassum and other seaweed biomass.

SUMMARY OF INVENTION

There are a variety of photosynthetic microalgae employed as oil production platforms.

Alginate is an abundant biopolymer produced by the invasive brown seaweed Sargassum, amounting to 25% of the dry seaweed biomass.
The present invention provides non-photosynthetic *Nitzschia sp.* diatoms, isolated from local Singapore waters and shown to metabolize the brown seaweed (Sargassum) cell wall polysaccharide alginate.

One of several *Nitzschia* clades isolated, designated *N. sing1-1* (or *N. sing1*) was deposited under the terms of the Budapest Treaty with the Culture Collection of Algae and Protozoa (CCAP), SAMS

Ltd., Scottish Marine Institute, OBAN, Argyll PA37 Lqa, United Kingdom on 1 July 2024 and given the accession number CCAP 1052/27 on 11 September 2024.

When grown on alginate as a feedstock, oils account for a high proportion of *N. sing1* cell biomass (up to ~50% dry weight), suggesting that the invention can be employed locally to produce carbon neutral foods and fuels.

This invention is also distinguished from the prior art in being scalable in batch fermentation with no requirement for light. A comparison of estimated yields suggests that the first manifestation of the invention is already competitive with a leading company in this area: Viridos, Inc., CA, USA. To the best of the inventor's knowledge, this is the first account of the ability of diatoms to utilize alginate as feedstock. Figure 1 shows features of *N. sing1*.

The recent emergence of massive Sargassum blooms in the Atlantic Ocean further suggests that the invention can have a global impact.

According to a first aspect, there is provided an isolated apochlorotic diatom strain of *Nitzschia sp*, having alginate-lytic activity in the presence of a synthetic seawater medium (ESAW) or a naturally occurring seawater collected from Singapore local waters that has been supplemented with nutrients as described below, supplemented seawater medium (SSW+) containing alginate to produce oil bodies.

In some embodiments, the isolated apochlorotic diatom strain is selected from the group comprising *N. sing1-1, N. sing1-2, N. sing1-3, N. sing2-1, N. sing2-2, N. sing2-3, N. sing2-4, N. sing2-5, N. sing2-6, N. sing3-1, N. sing3-2* and *N. sing3-3.*

In some embodiments, the isolated apochlorotic diatom strain designated *N. sing1-1* was deposited with Culture Collection of Algae and Protozoa (CCAP), SAMS Ltd., Scottish Marine Institute, OBAN, Argyll PA37 Lqa, United Kingdom (CCAP) and assigned deposit accession number CCAP 1052/27.

In some embodiments, the diatom strain is a non-photosynthetic strain.

According to a second aspect, there is provided an alginate-metabolizing and oil body-producing composition, comprising the isolated apochlorotic diatom strain of the first aspect.

In some embodiments, the alginate-metabolizing and oil body-producing composition is for use as an oil producer from seaweed biomass.

In some embodiments, the oil bodies comprise oils, such as triglycerides, diglycerides and polyunsaturated fats (PUFAs).

According to a third aspect, there is provided the use of the isolated apochlorotic diatom strain of the first aspect, or the alginate-metabolizing and oil body-producing composition of the second aspect to produce oil.

According to a fourth aspect, there is provided a method of producing oil using an isolated apochlorotic diatom, comprising culturing the isolated diatom strain of the first aspect, or a composition of the second aspect in medium under conditions for oil production, wherein the medium comprises synthetic seawater medium or supplemented seawater medium, and alginate as carbon source.

In some embodiments, the medium comprises alginate in the range of 0.5-2.0 wt%,

In some embodiments, the medium further comprises glucose.

In some embodiments, the method comprises:

i) culturing the isolated diatom strain of the first aspect, or a composition of the second aspect in medium comprising synthetic seawater medium or supplemented seawater medium, and alginate for 2-3 days at 30°C with aeration;

ii) inoculating the culture from i) into fresh medium comprising synthetic seawater medium or supplemented seawater medium, and alginate, at 1:10 ratio, and culturing for 2-4 days at 30°C with aeration.

In some embodiments, the method further comprises:

a) harvesting the cultured apochlorotic diatoms through flocculation and/or filtration/centrifugation;

b) extracting oil bodies from the harvested diatoms; and

c) purifying the extracted oil body contents.

In some embodiments, the isolated apochlorotic diatom is selected from the group comprising *N. sing1-1, N. sing1-2, N. sing1-3, N. sing2-1, N. sing2-2, N. sing2-3, N. sing2-4, N. sing2-5, N. sing2-6, N. sing3-1, N. sing3-2* and *N. sing3-3.*

In some embodiments, the isolated apochlorotic diatom is *N. sing1-1* with CCAP deposit accession number CCAP 1052/27.

Advantageously, the novel strain of the present disclosure is able to solubilize seaweed cell wall alginate to produce oil bodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a comparison of *N. sing1* to *N. gaditana*. (**A**). The two strains are shown at the same magnification. Note the large oil bodies seen in *N. sing1*. (**B**). An example of how *N. gaditana* is grown in shallow ponds. (**C**). Historical Viridos oil yields from *N. gaditana*. (**D**). Comparison of oil productivity of *N. gaditana* and *N. sing1*. Note that for this calculation it was assumed a pond depth of 20 cm for

N. gaditana growth, *N. sing1* dry mass yield of 0.5 g / L and an *N. sing1* oil yield of 0.35 g / L. All data for *N. gaditana* are from Viridos Inc.

Figure 2 shows the isolation of apochlorotic *Nitzschia* sp. (**A**) Maximum-likelihood phylogeny of apochlorotic *Nitzschia* (shaded box) and Bacillariales photosynthetic outgroup taxa. The Singaporean isolates are identified as clade 1, clade 2 and clade 3. The material from which they were isolated is indicated as Sargassum, Bryopsis, twig, or leaf. Filled circles show simplified bootstrap support values. Scale bar = 0.1 nucleotide substitutions. Arrowheads identify apochlorotic species with sequenced genomes. (**B**) The image shows cultures of diatoms on seawater alginate (1.5% w/v) medium. The tubes are tilted approximately −80° to show alginate gel liquefaction in the top and bottom three tubes. Alginate lyase enzyme and medium alone serve as positive and negative controls, respectively.

Figure 3 shows a Lipid Production Workflow.

Figure 4 shows yields of *N. sing1* diatoms (dry cell biomass) upon growth to saturation in a synthetic seawater medium (ESAW) or supplemented seawater medium (SSW+). Alginate is present at a concentration of 0.5% (w/v).

Figure 5 shows the speed of *N. sing1* radial colony expansion on alginate in the presence (+) or absence (-) of 0.5% glucose. The measurements were made in triplicate. Standard deviation is indicated.

DETAILED DESCRIPTION OF THE INVENTION

Bibliographic references mentioned in the present specification are for convenience listed in the form of a list of references and added at the end of the examples. The whole content of such bibliographic references is herein incorporated by reference.

Deposit of Biological Material

N. sing1-1 has been deposited on 1 July 2024 under the terms of the Budapest Treaty with Culture Collection of Algae and Protozoa (CCAP), SAMS Ltd., Scottish Marine Institute, OBAN, Argyll PA37 Lqa, United Kingdom (CCAP), and given the accession number CCAP 1052/27.

Definitions

For convenience, certain terms employed in the disclosure, examples and appended claims are collected here.

In general, technical and scientific terminologies used herein have the same meaning as understood by those skilled in the art to which this invention belongs. Further, the following technical comments

and definitions are provided. These definitions should in no way limit the scope of the present invention to those terms alone, but are put forth for a better understanding of the following description.

As used herein, "a" or "an" may mean one or more than one unless indicated to the contrary or otherwise evident from the context.

As used herein, the term "comprising" or "including" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. However, in context with the present disclosure, the term "comprising" or "including" also includes "consisting of". The variations of the word "comprising", such as "comprise" and "comprises", and "including", such as "include" and "includes", have correspondingly varied meanings.

The term "isolated" is herein defined as a biological component (such as a diatom) that has been substantially separated, produced apart from, or purified away from other biological matter in its natural environment, such as seaweed and/or seawater, in which the diatom naturally occurs.

As used herein, the term "medium" is used in the context of the present invention to refer to any substrate which may conventionally be used to enable diatom growth. In some embodiments, the medium is synthetic seawater medium (ESAW), or supplemented seawater medium (SSW+), as described in the Examples.

A person skilled in the art will appreciate that the present invention may be practiced without undue experimentation according to the method given herein. The methods, techniques and chemicals are as described in the references given or from protocols in standard biotechnology textbooks.

EXAMPLES

Example 1

Medium

A synthetic seawater medium (ESAW) was used as described in Harrison et al. 1980, modified in Berges et al. 2001. This is an enriched artificial seawater medium designed for coastal and open ocean phytoplankton. The medium composition is from Berges et al. (2001) and has been modified from the earlier version (Harrison et al. 1980). Modifications include adding borate only in the salt solution (not in trace metals), an inorganic phosphate in place of glycerophosphate, and the silicate stock solution is made at half strength without acidification to facilitate dissolution. Three trace elements have been added: $Na_2MoO_4 \cdot 2H_2O$, Na_2SeO_3 and $NiCl_2 \cdot 6H_2O$. The iron is now added, solely as chloride (to remove ammonium), from a separate stock with EDTA.

The anhydrous and hydrated salts must be dissolved separately; masses assume specific gravity = 1.021 at 20°C. Dissolve the specified quantity of the anhydrous salts in 600 mL of deionized water (d H_2O) and dissolve the hydrated salts in 300 mL d H_2O. Combine salt solutions I and II, and then add 1 mL of the nitrate and phosphate solutions, 2 mL of the silicate solution, 1 mL of the Iron-EDTA solution, 1 mL of the Trace Metals Solution II and 1 mL of the vitamin stock solution. Bring the final volume to 1 liter with d H_2O. Filter sterilization is recommended, e.g., a 147 mm Millipore GS filter (pore size 0.22 µm) with a Gelman A/E prefilter. Autoclaving the final medium often causes precipitates to form. If autoclaving is necessary, autoclave the two salt solutions separately, and when they are completely cooled, aseptically combine them. The 1 or 2 mL nutrient additions should be added using a 0.2 µm pore size sterile filter and a syringe. The medium should be bubbled with filtered air for 12 hours before use. The final pH is 8.2.

Table 1: Composition of Synthetic Seawater Medium (ESAW)

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
Salt solution I Anhydrous salts			
NaCl		10.600 g	1.81×10^{-1} M
Na_2SO_4		3.550 g	2.50×10^{-2} M
KCl		0.599 g	8.03×10^{-3} M
$NaHCO_3$		0.174 g	2.07×10^{-3} M
KBr		0.0863 g	7.25×10^{-4} M
H_3BO_3		0.0230 g	3.72×10^{-4} M
NaF		0.0028 g	6.67×10^{-5} M
Salt solution II Hydrated salts			
$MgCl_2 \cdot 6H_2O$		8.375 g	4.12×10^{-2} M
$CaCl_2 \cdot 2H_2O$		1.344 g	9.14×10^{-3} M
$SrCl_2 \cdot 6H_2O$		0.0220 g	8.25×10^{-5} M
Major Nutrients			
I) $NaNO_3$	46.67 g L^{-1} dH_2O	1 mL	5.49×10^{-4} M
II) $NaH_2PO_4 \cdot H_20$	2.52 g L^{-1} dH_2O	1 mL	2.10×10^{-5} M
III) $Na_2SiO_3 \cdot 9H_2O$	30 g L^{-1} dH_2O	10 mL	1.06×10^{-3} M

Iron-EDTA Stock Solution			
$Na_2EDTA{\cdot}2H_2O$	---	3.09 g	8.30×10^{-6} M
$FeCl_3{\cdot}6H_2O$	1.77 g L^{-1} dH_2O	1 mL	6.55×10^{-6} M
Trace Metals II Stock Solution	(see recipe below)	1 mL	
Vitamin Stock Solution	(see recipe below)	1 mL	

Trace Metals Solution II

Prepare primary stock solutions. To prepare final Trace Metals Solution II, begin with 900 mL of dH_2O and add the indicated quantities of trace elements. Bring the final volume to 1 liter with dH_2O. For each liter of ESAW, add 1 mL of the Trace Metals Solution II.

Table 2: Trace Metals Solution components

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
$Na_2EDTA{\cdot}2H_2O$	---	2.44 g	6.56×10^{-6} M
$ZnSO_4{\cdot}7H_2O$	---	0.073 g	2.54×10^{-7} M
$CoSO_4{\cdot}7H_2O$	---	0.016 g	5.69×10^{-8} M
$MnSO_4{\cdot}4H_2O$	---	0.54 g	2.42×10^{-6} M
$Na_2MoO_4{\cdot}2H_2O$	1.48 g L^{-1} dH_2O	1 mL	6.12×10^{-9} M
Na_2SeO_3	0.173 g L^{-1} dH_2O	1 mL	1.00×10^{-9} M
$NiCl_2{\cdot}6H_2O$	1.49 g L^{-1} dH_2O	1 mL	6.27×10^{-9} M

Vitamin Stock Solution

To prepare, begin with 80 mL dH_2O, add 10 mL of thiamine and biotin stock solution and 100 µl cyanocobalamin solutions (given below). This will prepare 100 mL. Filter sterilize and store frozen in small volumes.

Table 3: Vitamin Stock Solution components

Component	To prepare subcomponents	Molar Concentration in Final Medium
thiamine · HCl (vit. B1)	10 mg in 10 mL dH_2O	2.96 x 10^{-7} M
biotin (vit. H)	20 mg in 100 mL dH_2O	8.19 x 10^{-8} M
Cyanocobalamin (vit. B12)	100 mg in 10 mL DMSOs	7.38 x 10^{-10} M

A supplemented seawater medium (SSW+) was also used, comprising natural seawater with the addition of Major Nutrients, Solutions II and III (phosphate and silicate, respectively), and vitamin stock solution, as described in Table 3.

Example 2

2.1 *The diatom strains used in this invention:*

Apochlorotic diatoms can be isolated from the nutrient-rich waters of the intertidal zone where they occur as epiphytes on seaweeds, on decaying plant matter and in the surrounding waters (Kamikawa R., 2014; Lewin and Lewin, 1966). *N. putrida* was obtained from National Institute for Environmental Studies, Microbial Culture Collection, (NIES-MCC), Japan [Accession no. NIES-4239].

Figure 1 shows a comparison of *N. sing1* to *N. gaditana*. (**A**). The two strains shown at the same magnification. Note the large oil bodies seen in *N. sing1*. (**B**). An example of how *N. gaditana* is grown in shallow ponds. (**C**). Historical Viridos oil yields from *N. gaditana*. (**D**). Comparison of oil productivity of *N. gaditana* and *N. sing1*. Note that for this calculation it was assumed a pond depth of 20 cm for *N. gaditana* growth, *N. sing1* dry mass yield of 0.5 g / L and an *N. sing1* oil yield of 0.35 g / L. All data for *N. gaditana* are from Viridos Inc.

Apochlorotic *Nitzschia* isolates were obtained from the wild using an alginate gel liquefaction assay (Figure 2).

2.2 *Growth on alginate*

The growth of *N.* sing1 was examined on ESAW media solidified with the brown algal polysaccharide alginate. The rate of radial colony expansion had a concentration optimum and tended to decrease with increasing concentration (Figure 5). The medium underwent liquefaction and browning indicative of polysaccharide hydrolysis.

2.3 *Lipid production*

A schematic of the lipid production workflow is shown in Figure 3, and the method for lipid production is as follows:

Crude Alginate Preparation from Brown Seaweed Biomass

- Brown seaweed biomass, preferably Sargassum was harvested and the seaweed biomass was cleaned and dried.
- Dried biomass was homogenized into a fine powder.
- Powder was resuspended in water (3 g / 100 mL) and autoclaved.
- Resuspension was acidified with HCl to 0.2 M and incubated overnight at room temperature with stirring (magnetic stir bar).
- Resuspension was centrifuged and the precipitate washed twice with water.
- Washed powder was pelleted and resuspended in 2% sodium carbonate solution and incubated for 3h at 90°C, after which the mixture was centrifuged and the supernatant collected.
- 100% ethanol was added to the supernatant in a 2:1 ratio. The resultant precipitated alginate was collected.
- Solid alginate was washed in 100% ethanol by soaking. Washed alginate was freeze-dried and powdered for downstream use.
- Alginate was dissolved in water at 65°C. A further acidification step with HCl was required for diatom culture.
- In the current laboratory conditions, the inventors achieved a 25% yield of alginate (i.e. 0.25 kg/kg dried biomass).

Interestingly, diatom growth was not inhibited by crude seaweed alginate which may comprise contaminants such as polyphenolics. It would be understood that there are alternative alginate extraction methods available, some of which may achieve a higher yield (for example, Bojorges H., et al., 2023).

2.4 *N. sing1 Batch Fermentation*

- A suitable medium for *N. sing* batch fermentation was prepared using either ESAW or SSW+ containing 0.5% alginate (w/v) as carbon source. Both ESAW and SSW+ based media were used and worked well.
- Starter culture: the fermentation medium was inoculated with *N. sing1* culture; growth for 2-3 days at 30°C with aeration.
- Primary fermentation: the starter culture was inoculated into fresh medium at 1:10 ratio; growth for 2-4 days at 30°C with aeration.

- Laboratory-scale yield was exemplified in Figure 4. Oil bodies account for up to about 70% of *N. sing1* mass by dry weight.

2.5 *Processing of N. sing1 Oil Body to Extract Lipid Contents*

- the *N. sing1* culture was harvested after the fermentation period; cells were separated from medium through flocculation and/or filtration/centrifugation.
- the oil bodies were extracted from *N. sing1* cells using a suitable extraction method (e.g., centrifugation, solvent extraction).
- the extracted lipid contents were purified to remove impurities.

The lipid composition for the non-photosynthetic species *Nitzschia alba* is available in the art, which predominantly comprises triglycerides and diglycerides (Anderson R. et al., 1978). Some *Nitzschia* sp. also show the ability of producing polyunsaturated fatty acid (PUFA) (Anderson R. et al., 1978; Renaud, S.M., et al., 1995). For this particular strain *N. sing1*, similar lipids may be produced.

References:

Anderson R, et al., 1978; The lipid composition of the non-photosynthetic diatom *Nitzschia alba*. Biochim Biophys Acta. 528(1): 77-88.

Berges, J.A., Franklin, D.J. and Harrison, P.J. 2001. Evolution of an artificial seawater medium: improvements in enriched seawater, artificial water over the past two decades. J. Phycol. 37: 1138-1145.

Bojorges H., et al., 2023; Overview of alginate extraction processes: Impact on alginate molecular structure and techno-functional properties. Trends in Food Science & Technology 140: 104142: doi:10.1016/j.tifs.2023.104142.

Harrison, P.J., Waters, R.E., and Taylor, F.J.R. 1980. A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton. J. Phycol. 16: 28-35.

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Lewin, J., and Lewin, R.A., 1967. Culture and Nutrition of Some Apochlorotic Diatoms of the Genus *Nitzschia*. Microbiol. 46, Issue 3: doi:10.1099/00221287-46-3-361.

Renaud, S.M., Zhou, H.C., Parry, D.L. et al., 1995. Effect of temperature on the growth, total lipid content and fatty acid composition of recently isolated tropical microalgae *Isochrysis* sp., *Nitzschia closterium, Nitzschia paleacea*, and commercial species *Isochrysis* sp. (clone T.ISO). *J Appl Phycol* **7**, 595–602.

Claims

1. An isolated apochlorotic diatom strain of *Nitzschia sp*, having alginate-lytic activity in the presence of synthetic seawater medium or supplemented seawater medium, to produce oil bodies.

2. The isolated apochlorotic diatom strain of claim 1, wherein the isolated apochlorotic diatom strain is selected from the group comprising *N. sing1-1, N. sing1-2, N. sing1-3, N. sing2-1, N. sing2-2, N. sing2-3, N. sing2-4, N. sing2-5, N. sing2-6, N. sing3-1, N. sing3-2* and *N. sing3-3.*

3. The isolated apochlorotic diatom strain of claim 2, wherein the isolated apochlorotic diatom strain designated *N. sing1-1,* deposited with the Culture Collection of Algae and Protozoa (CCAP) and assigned deposit accession number CCAP 1052/27.

4. The isolated apochlorotic diatom strain of any one of claims 1 to 3, wherein the diatom strain is a non-photosynthetic strain.

5. An alginate-metabolizing and oil body-producing composition, comprising the isolated apochlorotic diatom strain of any one of claims 1 to 4.

6. The alginate-metabolizing and oil body-producing composition of claim 5, for use as an oil producer from seaweed biomass.

7. The alginate-metabolizing and oil body-producing composition of claim 5 or 6, wherein the oil bodies comprise oils, such as triglycerides, diglycerides and polyunsaturated fats (PUFAs).

8. Use of the isolated apochlorotic diatom strain of any one of claims 1 to 4, or the alginate-metabolizing and oil body-producing composition of any one of claims 5 to 7 to produce oil.

9. A method of producing oil using an isolated apochlorotic diatom, comprising culturing the isolated diatom strain of any one of claims 1 to 4, or a composition of any one of claims 5 to 7 in medium under conditions for oil production, wherein the medium comprises synthetic seawater medium or supplemented seawater medium, and alginate as carbon source.

10. The method of claim 9, wherein the medium comprises alginate in the range of 0.5-2.0 wt%,

11. The method of claim 9 or 10, wherein the medium further comprises glucose

12. The method of any one of claims 9 to 11, wherein the method comprises:

    i) culturing the isolated diatom strain of any one of claims 1 to 4, or a composition of any one of claims 5 to 7 in medium comprising synthetic seawater medium or supplemented seawater medium, and alginate for 2-3 days at 30°C with aeration;

ii) inoculating the culture from i) into fresh medium comprising synthetic seawater medium or supplemented seawater medium, and alginate, at 1:10 ratio, and culturing for 2-4 days at 30°C with aeration.

13. The method of any one of claims 9 to 12, further comprising:

a) harvesting the cultured apochlorotic diatoms through flocculation and/or filtration/centrifugation;

b) extracting oil bodies from the harvested diatoms; and

c) purifying the extracted oil body contents.

14. The method of any one of claims 9 to 13, wherein the isolated apochlorotic diatom is selected from the group comprising *N. sing1-1, N. sing1-2, N. sing1-3, N. sing2-1, N. sing2-2, N. sing2-3, N. sing2-4, N. sing2-5, N. sing2-6, N. sing3-1, N. sing3-2* and *N. sing3-3.*

15. The method of claim 14, wherein the isolated apochlorotic diatom is *N. sing1-1,* deposited with the Culture Collection of Algae and Protozoa (CCAP) and assigned deposit accession number CCAP 1052/27.