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FATTY ACID PROFILE AND HYDROCARBON CHARACTERIZATION OF *NITZSCHIA CLOSTERIUM (W.SM.)*

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ABSTRACT

Lipid productivity is a critical variable for evaluating algal species for biodiesel production. The pennate diatom *Nitzschia closterium* (W. Sm.) isolated from the local water body of Madurai was cultivated in four different media (WCg, Csi, F/2 and Diatom medium) and screened for the presence of neutral lipid content using Nile red fluorescent dye. The relative abundance of lipid content was estimated by fluorescence spectroscopy using Nile red. The numbers of cells exhibiting fluorescence were found to increase on 10th day in comparison to 5th day in the strain *Nitzschia closterium*. The maximum lipid production was noticed in *Nitzschia closterium* grown at pH -7. Green and orange color bands developed in the thin layer chromatography indicated the presence of chlorophyll a and fucoxanthin pigments. Thin Layer Chromatography of the extracted lipid fractions revealed the presence of Monoacylglycerols, Diacylglycerols and Triacylglycerols. Gas Chromatography confirmed the presence of fatty acids such as Myristic (14:0), Palmitic (16:0), Stearic (18:0) and other FAMES in the isolated strain. In addition, high molecular weight hydrocarbons were detected using GC-MS analysis. The higher percentage of lipid and fatty acids indicate the suitability of this indigenous organism as a potential source for algal bio-fuel.

KEY WORDS: ***Nitzschia closterium*, Lipid, Hydrocarbons, Fatty acids, GC, GC-MS.**

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INTRODUCTION

Biodiesel is a renewable alternative to petro-diesel that is currently produced from vegetable oils extracted from traditional oil-producing crop plants. Major crop based feedstock for biodiesel are soybeans, canola oil, animal fat, palm oil, corn oil, waste cooking oil and Jatropha oil [6]. These feedstock's have limitations such as low biomass productivity, requirements of large land area, non renewability and dissatisfaction towards meeting the existing demand for fuel [3]. In recent years, microalgae based bio-fuels are considered as viable alternative, in the context of food security and also requirement of large stocks to meet the growing fuel demand. Aquatic Species Program [National Renewable Energy Laboratory (NERL), Department of Energy (DOE), USA)], provided impetus to the global bio-fuel research.

Recently, microalgae, which have the ability to grow rapidly and synthesize and accumulate large amounts (20–50% of dry weight) of neutral lipid stored in cytosolic lipid bodies [5], [17] have re-emerged as a popular feedstock for the production of bio-fuel and present a promising alternative to highly controversial first generation bio-fuels [26]. The algal species that have been identified as the best candidates for oil production fall in the Bacillariophyta taxon, also known as diatoms [23]. Diatoms are one of the youngest algal groups and colonize a vast number of habitats and are known for their high productivity and accumulation of oils.

Lipid bodies are recognized as dynamic organelles existing in most eukaryotic cells that range greatly in size (diameter <1–100 μm) [8]. Their hydrophobic cores contain neutral lipids, most notably triacylglycerols (TAGs) and sterol esters, which serve as reservoirs of membrane lipid components [28]. An important characteristic for any biodiesel feedstock is the suitability of the fatty acid profile for biodiesel production. A few studies have only investigated the quality of micro-algal biodiesel [10], [22], [31]. Oil content in micro algae can exceed 80% by weight of dry biomass [19].

Diatoms have been regarded as useful neutral lipid sources of liquid-fuel precursors [1]. Triacylglycerols are a very important energy storage substance and have been used as a condition index for marine fauna [11]. In this study, the diatom *Nitzschia closterium* was isolated from a local water body of Madurai. Lipid extraction was carried out and analyzed by TLC and GC. The main goal of the study was to determine the fatty acid and hydrocarbon composition of *Nitzschia closterium*.

MATERIALS AND METHODS

Growth condition

The diatom, *Nitzschia closterium* was grown in Csi medium [21], Diatom medium [2], F/2 medium [15], [16] and Wcg medium [30], [14], [16]. The cultures were grown under controlled conditions of 12 hours light and 12 hours dark cycle at 24° C at a light intensity of 3000 Lux. The diatom culture, *Nitzschia closterium* was homogenized and the growth rate of each culture was recorded continuously at an interval of 2 days by measuring the absorbance at 665nm in a Hitachi U -2001 spectrophotometer. Dry weight was also determined.

Nile red assay [4]

Nile red is a lipophilic fluorescent dye used for intracellular lipid determination in prokaryotic and eukaryotic cells, capable of detecting neutral lipids. The relative abundance of intracellular triacylglycerols present in *Nitzschia closterium* was estimated by fluorometric assay using the dye Nile red. Nile red dissolved in acetone was added to a final concentration of 1μg mL^{-1} to 2mL of cells.

Pigment analysis [18]

The cell pellets of *Nitzschia closterium* was obtained through centrifugation. The pigments were transferred from chloroform: methanol (2:1) extraction to diethyl ether for thin layer chromatography. The best resolution was obtained when purified with petroleum ether

before use. Chromatographic plates coated with silica gel were used for separation of pigments and rapid development. The solvent system for the development of plates was n-propanol: light petroleum (60°C – 80°C) – 2.5: 97.5 (v/v) for approximately 20-30 minutes.

Thin layer chromatography [20]

Lipid extract samples were spotted onto silica gel TLC plates. The mobile phase consisted of a solvent mixture of hexane/ diethyl ether/ acetic acid (70:30:1) by volume. The plates were developed by exposing the vapors of iodine crystals to stain the plates for visualizing the lipids. Extracted lipids were also analyzed by High performance thin layer chromatography on silica coated plates.

Fatty Acid Methyl Ester analysis by Gas Chromatography

A Shimadzu 2010 plus series equipped with a FID detector and capillary column (105 meter, 0.32mm ID, 0.20 μm film thickness) was used. The lipid fraction from *Nitzschia closterium* was prepared with Chloroform-Methanol (1:1) solvent mixtures. The samples were injected into the gas chromatograph where nitrogen was used as the carrier gas. Injector and detector temperatures were maintained at 280°C. The column temperature was programmed from 160° to 240°C. Peak areas and retention times were measured by electronic integration with computer. Fatty acids were identified by comparison with retention times of known standards [24].

Hydrocarbon analysis by GC-MS [13]

For quantification and identification of hydrocarbons, GC-MS was used [GC Clarus 500 PerkinElmer MS Turbo Mass gold with mass detector]. 100ml of algal culture was taken and centrifuged at 10,000rpm for 20 minutes. The pellet was taken and to it 5ml of hexane was added and mixed. The sample was centrifuged at 10,000rpm for 15 minutes and the supernatant was recovered.

RESULTS AND DISCUSSION

Selection of culture medium

The relative growth rate of *Nitzschia closterium* in Diatom medium was three times higher than the Csi, Wcg and F/2 media. Low growth rate was recorded in Wcg medium compared to Csi and F/2 medium. The growth rate of *Nitzschia closterium* started increasing after 2 days of cultivation and reached the highest peak on the 8 – 10th day in the Diatom medium [Figure 2]. Diatom medium serves as the potential source for the higher biomass productivity of *Nitzschia closterium*.

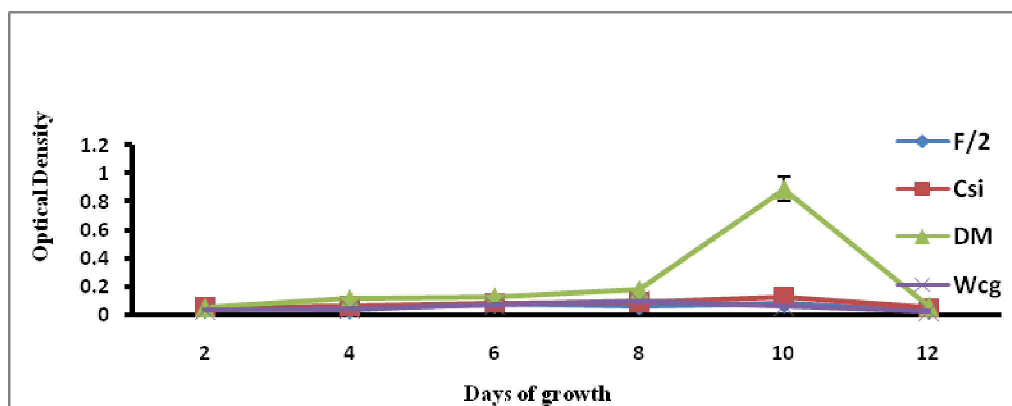

Figure 2

Growth response of Nitzschia closterium in different media

Pigment analysis

Green and orange color bands were developed which indicated the presence of chlorophyll a and fucoxanthin pigments [Figure 4]. The R_f value was 0.58 for fucoxanthin and 0.29 for chlorophyll a. An absorption maximum of 449nm was achieved which is the characteristic absorption range for fucoxanthin pigment [Figure 5]. Confirmation of the identification of fucoxanthin was obtained by comparison with published maxima of absorption spectra [9].

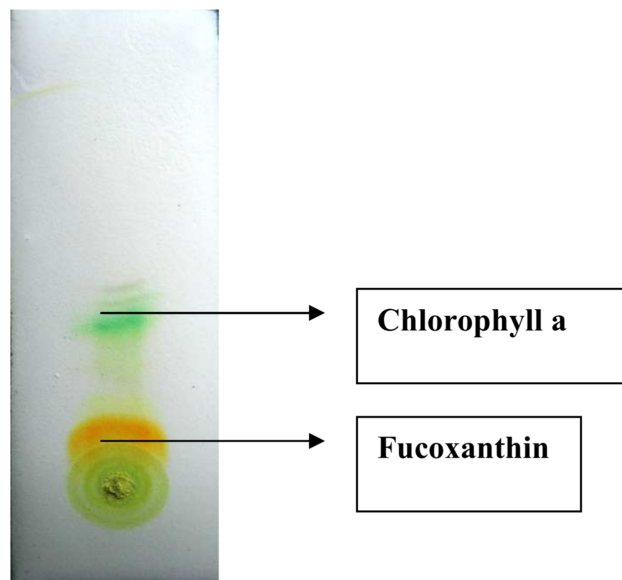

Figure 4
Separation of Fucoxanthin pigment by TLC

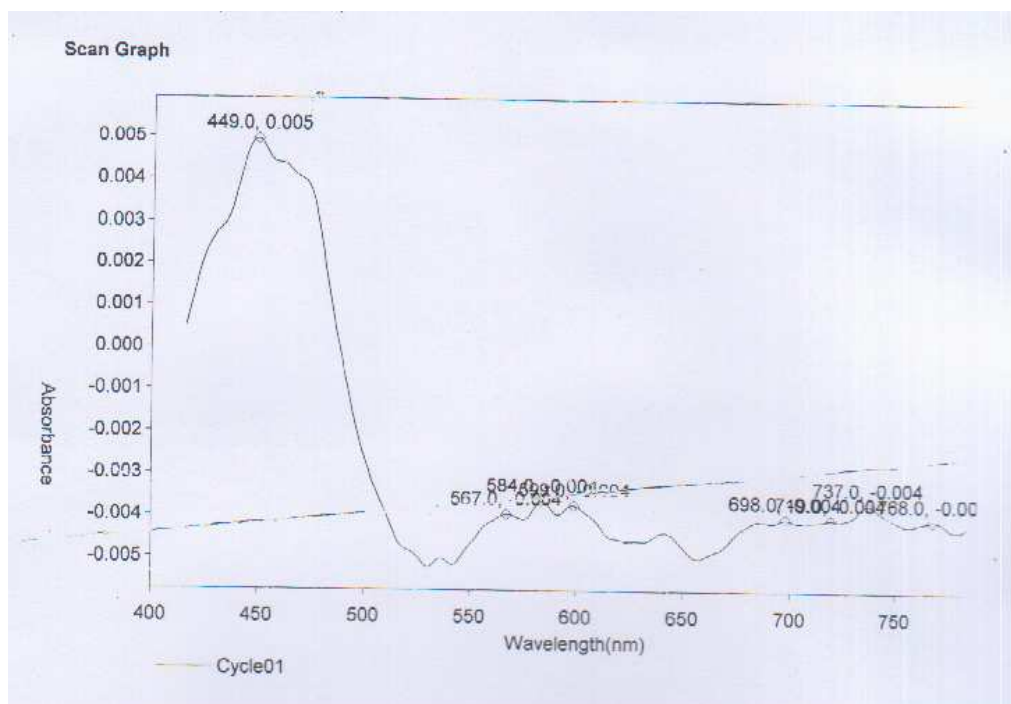

Figure 5
Absorption spectra of Fucoxanthin in Nitzschia closterium

Neutral lipid accumulation in Nitzschia closterium

The number of cells exhibiting fluorescence on 10th day was high in comparison to the 5th day of growth [Table 1]. After staining with Nile red, the lipid bodies in *Nitzschia closterium* had a characteristic yellow fluorescence [Figure 1] and could be clearly identified. Neutral lipids including hydrocarbons and triglycerides were stained in yellow color [25]. *Nitzschia closterium* exhibited fluorescence emission in the wavelength range of 600- 800nm [Figure 3]. The quantum yield was 0.37.

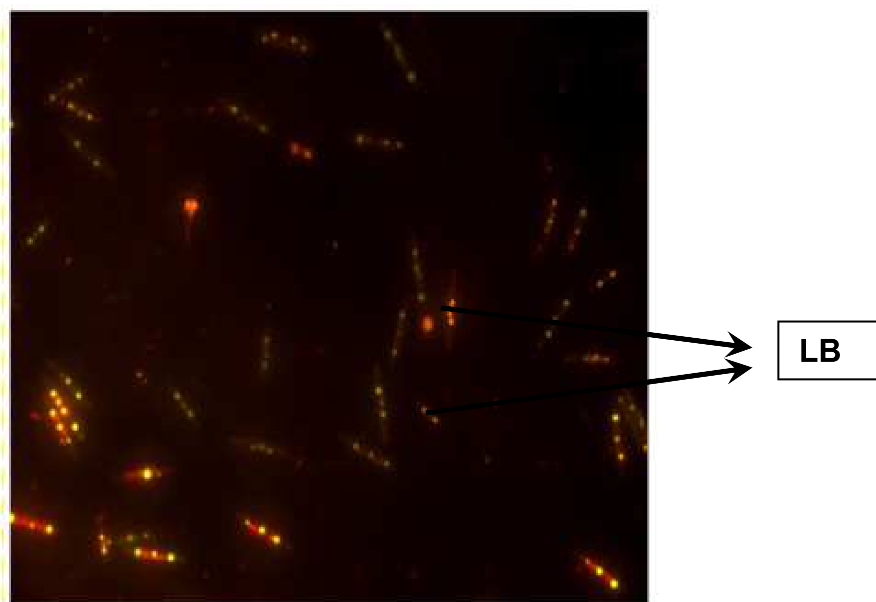

Figure 1
Fluorescence Microscopic image of *Nitzschia closterium* stained by Nile red. The lipid bodies (LB) are yellow in color.

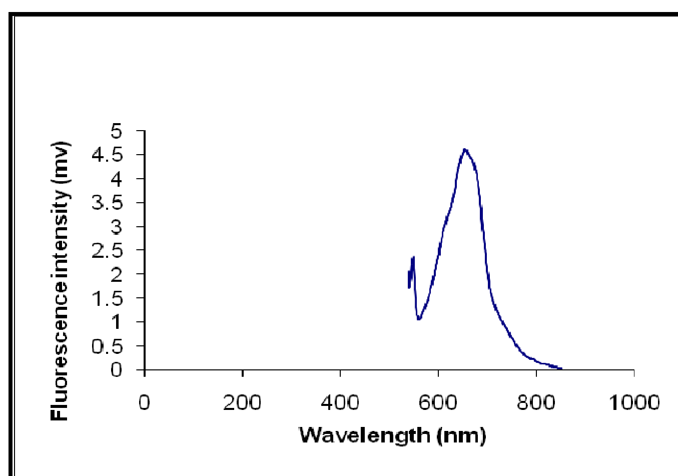

Figure 3
Fluorescence emission spectra of Nitzschia closterium

Table1
Nile red staining of Nitzschia closterium

No	No of days	Cells emitting fluorescence	
		0.5 mg	1 mg
1	5	+	++
2	10	++	+++

+++ - Cells exhibiting maximum fluorescence
++ - Cells exhibiting moderate fluorescence
+ - Cells exhibiting minimum fluorescence

Thin Layer Chromatography
Figure 6 shows a developed plate which includes the Monoacylglycerol, diacylglycerol and triacylglycerol [7]. Diacylglycerols and Triacylglycerols were found to be present in small quantity. TLC separation of lipids showed monoacylglycerols (Propanoic acid, tetradecanoic acid, hexadecanoic acid and octadecanoic acid) was present in large quantities.

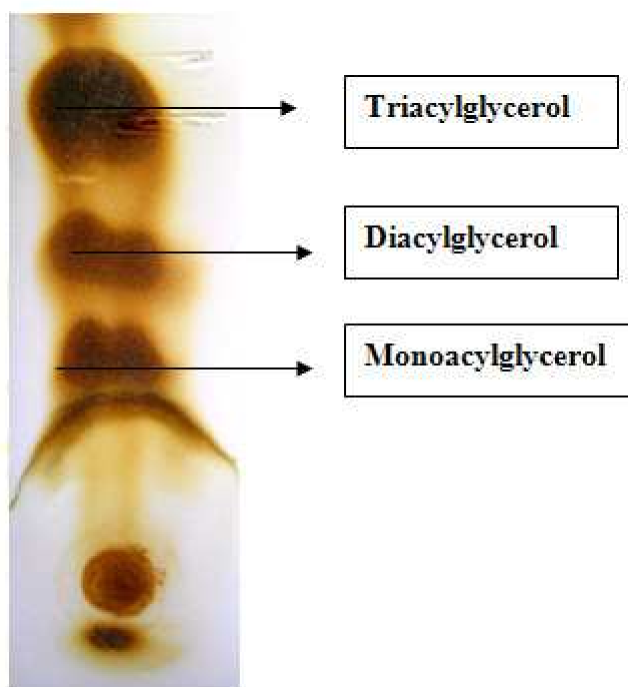

Figure 6
Separation of lipid fractions by TLC

HPTLC analysis revealed the presence of lipids such as Glycolipid- I, Digalactosyl diglyceride, Phosphatidylcholine, Phosphatidylethanolamine, and Monogalactosyl diglyceride [Figure.7]

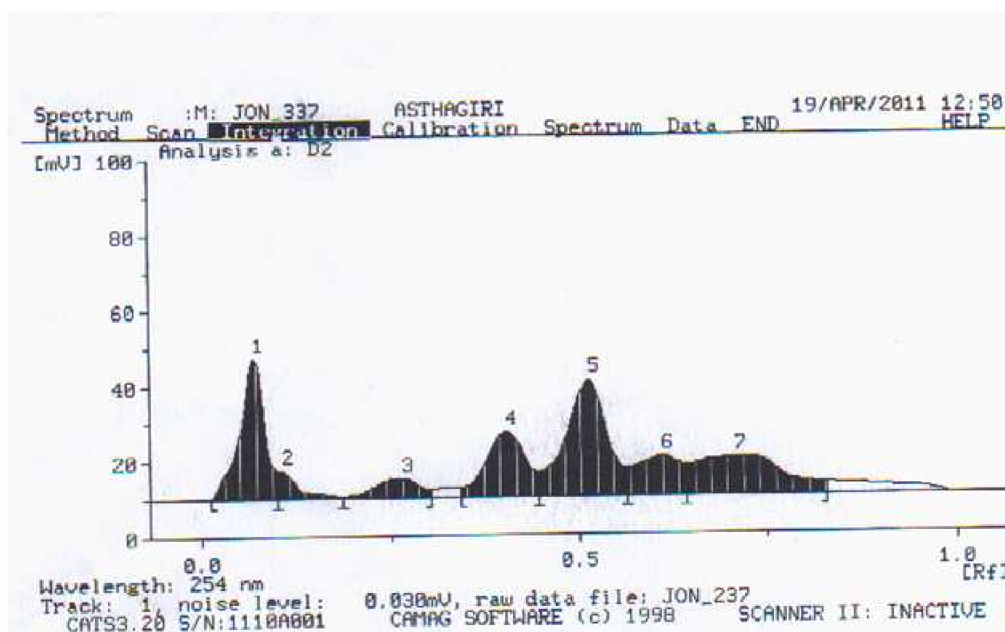

Figure 7
HPTLC Separation of lipids

Fatty acid Composition

The major fatty acids in *Nitzschia* species are 14:0, 16:0, 16:1(n-7), 16:3(n-4) and 20:5(n-3). The saturated fatty acids such as Caprylic acid (C8:0), Myristic acid (C14:0), Lignoceric acid (C24:0), Lauric acid (C12:0), Stearic acid (C18:0) and Behenic acid (C22:0). Behenic acid (C22:0) was present in *Nitzschia closterium*. Some previous studies explored the production of Behenic acid (C22:0) in Diatoms [12], [27], [29]. The presence of Myristic acid (C14:0) in GC analysis revealed the presence of neutral lipid in *Nitzschia closterium* [Table 2]. Caprylic acid was present in higher amount on the 5th day (97.16%) and 10th day (94.8%), followed by lignoceric acid on both 5th and 10th day of growth. The percentage of saturated fatty acids in *Nitzschia closterium* is 98.6% and unsaturated fatty acid is 2.01% on 5th day and the percentage of saturated fatty acids are 99.94% on 10th day. The utility of algal oil as biodiesel are largely dictated by the composition of fatty acids in triacylglycerols.

Table 2
Fatty acid profile of Nitzschia closterium

Days of growth	Fatty acid	Molecular Formula	Type	Area %
5	Caproic acid	C6:0	Unsaturated	2.01
	Caprylic acid	C8:0	Saturated	97.16
	Myristic acid	C14:0	Saturated	0.22
	Lignoceric acid	C24:0	Saturated	1.22
10	Caprylic acid	C8:0	Saturated	94.5
	Lauric acid	C12:0	Saturated	0.82
	Stearic acid	C18:0	Saturated	1.41
	Behenic acid	C22:0	Saturated	0.09
	Lignoceric acid	C24:0	Saturated	3.1

GC-MS characterization of hydrocarbons

High molecular weight hydrocarbons were identified with the help of GC-MS and they are represented in Table 3. Hexane fractions are separated by GC-MS and compounds were identified by comparing their retention time and mass weight with that of literature and by interpretation of mass spectra [13]. 1, 2-Benzenedicarboxylic acid, butyl 2- methyl propyl ester was found to present in higher quantity (92.96%).

Table 3
GC-MS spectral analysis of hydrocarbons

No	Component	Molecular formula	Mass weight g/mol	Mass %
1	Tridecane	$C_{13}H_{28}$	184	0.05
2	Ethanone, 1-(3-ethyloxyranyl)-	$C_6H_{10}O_2$	114	0.01
3	Hexadecane	$C_{16}H_{34}$	226	0.10
4	1-iodo-2-methylundecane	$C_{12}H_{25}I$	296	0.03
5	Tridecane,2-methyl-	$C_{14}H_{30}$	198	0.13
6	Tetradecane, 1-iodo	$C_{14}H_{29}I$	324	0.05
7	Dodecane,2,7,10,-trimethyl	$C_{15}H_{32}$	212	0.06
8	1,2- Benzenedicarboxylic acid, butyl 2- methyl propyl ester	$C_{16}H_{22}0_4$	278	92.96
9	Ethaneperoxoic acid, 1- cyano-1-(2-(2- phenyl-1,3- dioxolan-2-yl)ethyl)pentyl ester	$C_{19}H_{25}N0_5$	347	0.31
10	Nonadecane,2-methyl-	$C_{20}H_{42}$	282	0.45
11	Heptacosane	$C_{27}H_{56}$	380	0.69
12	Heneicosane	$C_{21}H_{44}$	296	0.94
13	Eicosane	$C_{20}H_{42}$	282	0.89
14	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390	1.13
15	Octadecane, 2-methyl-	$C_{19}H_{40}$	268	0.66
16	Eicosane, 2-methyl	$C_{21}H_{44}$	296	0.42

CONCLUSION

The Nile red fluorometric assay provided evidence of lipid content in *Nitzschia closterium.* The TLC plate methodology is a quick and easy technique to initially screen the presence of lipids, (Monoacylgycerol, Diacylglcerol and Triacylglcerol). In order to achieve the resolution necessary for absolute identification of the lipids present, Gas chromatography was employed. GC-MS add a high fidelity in identifying the high molecular weight hydrocarbons present in *Nitzschia closterium.* These characteristics indicate that *Nitzschia closterium* can be used for future exploitation as an alternative renewable fuel source.

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