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Isolation of Four Diatom Strains from Tidal Mud Toward Biofuel Production

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Abstract—**Development and utilization of bio-energy is an important way to relieve the pressure of global energy shortage. Biodiesel can be a focus of the bio-energy, because it is a cleaner-burning and renewable fuel. Microalgae have been considered to be an ideal source of biodiesel for its high growth rate and high lipid content. Benthic diatoms in tidal mud are considered to be new candidate for obtaining high lipid content microalgal strains although they have not been well noticed.**

The purpose of this study is getting the diatom strains with high-lipid content from tidal mud toward biofuel production. With microtubule method, four diatom strains were successfully isolated from the tidal mud located in Haicang, Xiamen. They are *Navicula halophila, Nitzschia frustulum* var. *symbiotica, Amphora coffeaeformis,* and *Nitzschia closterium*. The characteristics of morphology, ecology, and distribution of each species are described in the present paper with images of light microscope and electron microscope.

Diatoms were cultured in the laboratory and the lipid content of each strain was determined. The lipid content (% dried weight) of the four diatoms are: *Navicula halophila* 39.1%, *Nitzschia frustulum* var. *symbiotica* 35.7%, *Amphora coffeaeformis* 26.1%, and *Nitzschia closterium* 18.9%. The result indicates that the lipid content of diatoms from tidal mud is relatively higher compared to the strains from seawater which can be a good source candidate of microalgal biodiesel

Keywords- biodiesel; diatom;isolation; tidal mud

I. INTRODUCTION

Because of the increases in crude oil prices, the limited resources of fossil oil and the environmental concerns there have become a serious problem in recent years and bio-energy have been a focus. Development and utilization of bio-energy has been considered as an important way to relieve the pressure of global energy shortage and guarantee the security of energy. Among all kinds of bio-energy, biodiesel has attracted attention as a potential energy source because of the fact that it is a renewable and nontoxic, biodegradable and an excellent replacement for petroleum diesel. Biodiesel is a form of methyl or ethyl ester of fatty acids produced by the transesterification of oil plants and hydrophytes oil, animal fat and used cooking oils. Since the carbon in the oil or fat originated mostly from carbon dioxide in the air, biodiesel is considered to contribute much less to global warming than fossil fuels. Moreover, biodiesel is nontoxic and biodegradable and has low emission profiles, and so is environmentally beneficial.

Marine microalgae play an important role in providing energy and organic matter in marine ecosystem. Compared with other algae, microalga is considered as a good source of biodiesel for its high photosynthetic efficiency and environmental adaptability, short growth cycle, high biomass yield and high growth rate [1, 2]. It is reported that the lipid content of *Chlorella protothecoides* can be up to 57.2% of its dried weight by heterotrophic cultivation and bio-oil can be obtained by a fast heating [3].

Diatom is considered as one of the most ideal algae toward industrialized production of lipid for its high lipid content [4]. Diatom is a solid source of natural lipid no matter as a fuel or as a phytoplankton or bait for shrimp larvae [5], which is not only because of its high lipid content, but also for its containing a large amount of PUFAs[6].

Nowadays, the primary difficulty in massive production of biodiesel is the shortage of raw material resources. Though many woody oils can be the source of biodiesel, none of them are manufactured on a large scale. Herbal oil plants like soybeans and peanuts can also be a source of biodiesel, but they have to compete for agricultural land with crop plants such as rice and corn, so the potential to expand their production scale is limited. In this situation, to seek new and broad resources of raw materials is supposed to be a solution. Unlike terrestrial plants, microalgae do not require fertile land or irrigation. Therefore, we should keep our eyes on the marine microalgae with vast resources in order to make a new breakthrough.

Selecting microalgae with high lipid content is very important in the research of microalgal biodiesel production because microalgae with high lipid content reduce the cost.

A unique feature of many tidal mud areas is the concentration of benthic diatoms that appear on the surface as golden brown patches. These benthic diatoms mainly include *Navicula, Nitzschia, Amphora, Pleurosigma and Coscinodiscus*. They are often used to feed the commercial marine animals like *Periophthalmus cantonensis* and *Bullacta exarata* because they are rich in nutrition. But so far, the research on diatoms from the tidal mud is still very limited, particularly there is no report which has noticed the significance of utilization of diatoms from the tidal mud toward biofuel production.

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The goal of this study is to isolate the cultivable diatom strains with high lipid content from diatoms in the tidal mud and provide theoretical and technical support for the massive production of microalgal biodiesel.

II. MATERIALS AND METHODS

A. Sample collection, isolation, purification and cultivation of diatoms

Mud samples with golden and oily appearance were collected with a clean blade by scratching the tidal mud from muddy seaside in Haichang, Xiamen. The algal samples were transferred to a small amount of f/2 medium and taken back to the laboratory.

The diatom strains were isolated in the laboratory by microtubule method [7]. The isolated diatom cell was cultivated with f/2 medium [7] under appropriate light illumination for 5 days and was observed under microscopy for the confirmation of the culture result. If the culture was monospecies, the strain was selected and transformed to a new culture medium and was cultivated using f/2 at 20±1℃ , 4000 lux illumination for 12h L:12h D light cycle. During the exponential phase of algal growth, the algal cultures was transferred into a 5mL tubes and was preserved with Lugol's solution in order to preparing samples for morphological obserbvation and species identification under light microscope (LM) and transmission electron microscope (TEM)

B. Determination method of the lipid content of diatom cells

The lipid content was determined with the methods of lipid extraction and weighting in reference to Folch's method[8]. The algal culture at the late exponential growth phase was harvested with centrifugation (5000g, 10min), the sediment is then freeze-dried. 20% silica was added to the algal pellet and grinded in a mortar. After that, the algae powder was put into a 5mL centrifuge tube, 3mL chloroform / methanol (2:1 v/v) was added and vibrated for 20min at room temperature, centrifugated at 8000rpm for 20min and the supernatant was obtained. The above steps were repeated twice, all the supernatant was combined into a rotary bottle which was pre-weighed and evaporated in the rotary evaporator (temperature 50℃, rotate speed 90r/min) to get acquired total lipid. Neutral lipids and phospholid were separated using liquid-liquid separation: in methanol of 95% water for phospholipids and petroleum ether for neutral lipids. The solvent was evaporated under a stream of nitrogen or reduced pressure. The lipid residue was precisely weighed and stored at -20℃.

III. RESULT

A. Isolation of diatom strains and morphological observation

Mud samples with golden and oily appearance were collected from tidal from the tidal mud of the seaside, Shuitou Dock, Haichang, Xiamen and were brought back to the laboratory. A large amount of benthic diatom cells were found under the light microscope. Four species of benthic diatoms were isolated with their morphological observation and identification which were listed in Table 1. Two of the benthic diatoms belong to dominant species: *Navicula halophila* and *Nitzchia frustulum* var. symbiotica while the other two are *Amphora coffeaeformis* and *Nitzschia closterium*.

The morphological structure, ecology and distribution of each species was described in the following part together with LM and TEM photographs.

1) *Navicula halophila* (Grun.) Cleve Fig. 1

Valve navicular to long elliptical shape, 11-22μm in length, 3.7-5.4μm in width. Striae in centre radial arrangement, transversal striae parallel and sparser arrangement in central part, striae at the end of epitheca narrow, reach to 15-17 in 10μm. Central axis area narrow, central area distinct or slightly wider than axial area, terminal nodule distinct.

Ecology: Marine and brackish, benthic.

Distribution: Recorded in Xiamen Harbor. Also recorded in UK, Netherland, Denmark, Belgium, Sweden and Germany.

2) *Nitzchia frustulum* var. *symbiotica* Lee et Reimer (Fig. 2)

Valves long elliptical, 4.4-8 μm in length, 2.3-2.7μm in width. Transversal striae parallel, 35-50 in 10μm. Raphe in valve margin, density of keel puncta 15 in 10 μm.

Ecology: Marine species

Distribution: Recorded in Xiamen Harbor. Firstly discovered by Lee and Reimer (1982)[9] symbiosing in the body of *Amphistegina lobifera* at Great Barrier Reef, Australia.

TABLE 1 Diatoms isolated from tidal mud at Haicang, Xiamen

Strain No.	Species Name
MMDL5114	*Navicula halophila*
MMDL50314	*Nitzchia frustulum* var. *symbiotica*
MMDL5104	*Amphora coffeaeformis*
MMDL50313	*Nitzschia closterium*

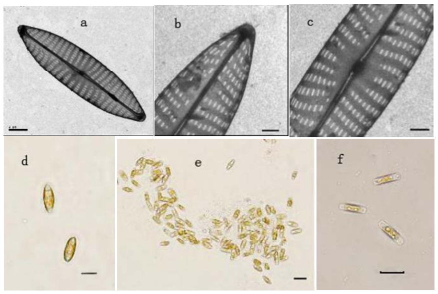

Figure 1 LM and TEM micrographs of *Navicula halophila*
a: Valve view, b: Valve end, c: Central area of the valve, d: Valve view, e: Colony valve view, f: Girdle view
(Scale bar: a=2μm, b, c=1μm, d=10μm, e, f=20μm)

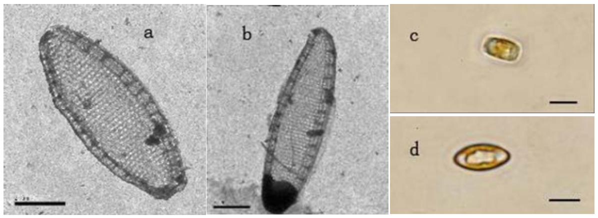

Figure 2 LM and TEM micrographs of *Nitzchia frustulum* var. *symbiotica*
a, b: Valve view, c: Girdle view, d: Valve view (scale bar: a, b=2μm, c, d=10μm)

3) *Amphora coffeaeformis* (Agardh) Kuetzing Fig.3

Frustules narrowly elliptical, ends truncate. Valves lunate, ends elongated or capitate. 10-46μm in length, 4-8μm in width. Striae sparse at the center of dorsal, striae at the ends narrow, reach to 14-26 in 10μm. A striae formed by two rows of puncta are visible in EM, tow puncta near raphe always connected each other. Puncta fine indistinct. Striae short and narrow on the dorsal side. No striae at central nodule. Raphe straight, central area dilated to dorsal side.

Ecology: Marine, brackish and fresh waters.

Distribution: Frequently distributed species in coastal waters of China. Also recorded in Hawaii, Australia and coastal waters of Atlantic of North America.

4) *Nitzschia closterium* (Ehrenberg) W. Smith Fig.4

Cell small, live individually. The valve of cells long, centre enlarge, with fairly elongated poles. The polar parts slightly twisted. Two discoidal, chloroplasts in each cell.

Ecology: Marine species, benthic in the intertidal flat, but always occurs in plankton samples.

Distribution: Cosmopolitan species. Recorded in Bohai Sea, Yellow Sea and East China Sea.

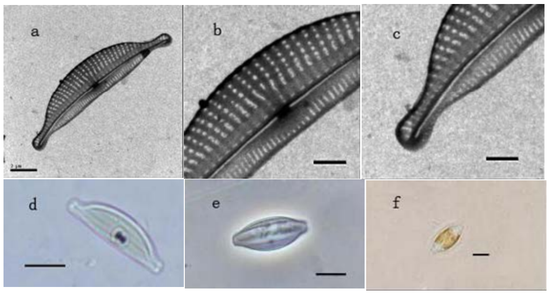

Figure 3 LM and TEM micrographs of *Amphora coffeaeformis*
a: Valve view, b: Central area of the valve view, c: Valve end, d-f: valve view (scale bar: a =2μm, b,c=1μm ,d-f=10μm)

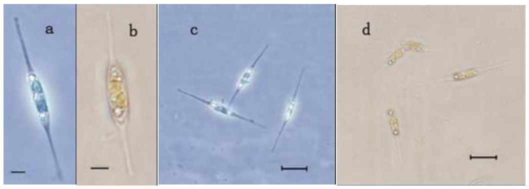

Figure 4 LM micrographs of *Nitzschia closterium*
a-d: Valve view (scale bar: a, b=5μm, c, d=20μm)

B. Determination of the total lipid content in the diatom strains

The total lipid content of the four different diatom strains were determined with the methods mentioned above. The result is showed in Table 2

As indicated in Table 2, diatoms from the tidal mud have relatively higher lipid content compared with those from waters. The highest value of 39.1% (dried weight) was found in *Navicula halophila*; followed by *Nitzschia frustulum* var. *symbiotica* which has the lipid content value of 35.7%. The lipid content of *Amphora coffeaeformis* was lower than the previous two but it was also up to 26.1%; the lipid content of *Nitzschia closterium* was relatively lower (18.9%).

IV. CONCLUSION

In this study, we isolated four benthic diatom strains from the tidal mud. Based on the experimental work on axenic cultivation, morphological observation and total lipid content determination of these diatoms, we suggested that the diatoms from the tidal mud be an ideal source for biodiesel production due to their high content of lipid and suitability for cultivation.

In the case of *Navicula halophila* and *Nitzschia frustulum* var. *symbiotica*, a promising result of over 35% lipid content is obtained which is much higher than most of the other diatom species and takes more advantages over most diatoms. Meanwhile, we have observed that these two species grew well in laboratory culture. Therefore, diatom strains isolated from the tidal mud could be considered as the ideal source of biodiesel. Ultimately, more and more diatom strains with high lipid content are expected to be discovered through isolation and axenic cultivation experiments in the future.

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TABLE2 Total lipid content of different diatom strains

Latin Name	Strain No.	Total Lipid Content （% DW）
Navicula halophila	MMDL5114	39.1
Nitzschia frustulum var. *symbiotica*	MMDL50314	35.7
Amphora coffeaeformis	MMDL5104	26.1
Nitzschia closterium	MMDL50313	18.9

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