academicJournals

Vol. 8(1), pp. 66-74, 1 January, 2014
DOI: 10.5897/AJMR12.806
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

The biodiversity of oleaginous microalgae in Northern Qinghai-Tibet Plateau

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Accepted 3 July, 2013

Microalgae represent an exceptionally diverse but highly specialized group of micro-organisms that adapt to various ecological habitats. Due to their unique environment (extremely low temperature and anoxia), the Tibetan Plateau is among the regions with numerous rare ecotypes such as arid desert, salt marsh, alpine permafrost, hot spring and lawn. In this study, 52 microalgae from different environments in Qinghai-Tibet Plateau were isolated and divided into 11 different groups based on the results of restriction fragment length polymorphism (RFLP). Then, 2-3 strains of each group were selected for 18s rDNA molecular identification. The oil contents among the studied strains varied from 3.26 to 25.47%. The results indicate that the number and high lipid content of the strains isolated from the water were much more than those from the soil. This provides a direction for the isolation of high oleaginous microalgae.

Key words: Northern Qinghai-Tibet Plateau, microalgae, lipids, restriction fragment length polymorphism (RFLP), 18s rDNA.

INTRODUCTION

Conventional biodiesel mainly comes from soybean and vegetable oils (Huang et al., 2010; Bunyakiat et al., 2006), palm oil (Al-Widyan et al., 2002), sunflower oil (Antolinet al., 2002), rapeseed oil (Peterson et al., 1996) as well as restaurant waste oil (Bouaid et al., 2007). Producing lipids by microbial fermentation is a new approach of lipids. Oleaginous microorganisms are now used more and more widely for producing functional oil and biodiesel. Among them, microalgae have many advantages such as high photosynthetic efficiency, fast growth rate, no restriction of season and climate, easy realization of large-scale production. Thus, microalgae are considered as raw materials for biodiesel with a lot of potential (Chisti, 2007). Exploiting oil resource with microalgae has attracted more and more attention. Biodiesel production with microalgae has become one of the important research areas.

Microalgae are photosynthetic micro-organisms that convert sunlight, water and carbon dioxide to algal biomass. Many microalgae are exceedingly rich in lipid (Chisti, 2007; Banerjee et al., 2002) which can be converted to biodiesel with the current technology.

At present, the research on oil microorganism focuses on exploring oil microorganisms by screening the existent microorganisms, or optimizing the microbial fermentation conditions to improve the lipid content, yet there are few research for isolation and diversity of the oleaginous microorganisms in different environments, which has limited the oil microorganisms' species. Northern Qinghai-

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Tibet Plateau is extremely cold and anoxic, which makes it one of the regions with special ecotypes.

As a result, abundant extreme microorganisms accumulate there. This special ecological environment includes high direct solar radiation, low atmospheric temperature, large diurnal temperature range, lot of salt lakes (Li, 2008), arid desert, salt marsh, alpine permafrost, hotspring, and lawn. In order to provide basic and cheap raw materials for the industrial production of microalgae lipid, a preliminary investigation has been done on the biodiversity of the oleaginous microalgae in Northern Qinghai-Tibet Plateau.

This experiment is mainly to study the oleaginous microalgae biodiversity in this region through isolation, identification, classification of the microalgae and the determination of the lipid content.

MATERIALS AND METHODS

Collection of samples

The samples including soil and water were collected from hot springs, wetlands, sands, grasslands, crop environment and saline, high radiation environment. Soil samples were collected at 5 - 20 cm under the surface, and immediately stored at 4°C.

Isolation, preservation and cultivation of microalgae

SE medium: $NaNO_3$ 0.25 g/L, $K_2HPO_4{\cdot}3H_2O$ 0.075 g/L, $MgSO_4{\cdot}7H_2O$ 0.075 g/L, $CaCl_2{\cdot}2H_2O$ 0.025 g/L, $KH_2PO_4$0.1175 g/L, NaCl 0.025 g/L, soil extract 40-50ml/L, $FeCl_3{\cdot}6H_2O$ 0.005 g/L, Fe-EDTA 1 ml/L, As Solution 1 ml/L and dissolved in synthetic seawater (Wang et al., 2010).

Isolation and preservation of microalgae

Petri dishes containing growth medium solidified with 1-1.5% agar medium were prepared. The agar was ½ to 2/3 the depth of the dish. 1-2 drops of mixed phytoplankton sample were placed near the periphery of the agar. A wire loop was flamed sterilize.

The sterile loop was used to make parallel streaks of the suspension on the agar by aseptic technique. 16 streaks (4 sets of 4) were made and the whole surface of the agar plate was used. The plate was covered and sealed with parafilm. And then was inverted and incubated under low light at constant temperature. Colonies free of other organisms were selected for further isolation. The sample was removed using a sterilized wire loop and placed in a drop of sterile culture medium on a glass slide. We checked microscopically to ensure that the desired species was isolated and is unialgal. The streaking procedure was repeated with the algal cells from a single colony and colonies were allowed to develop. This second streaking reduces the possibility of bacterial contamination and of colonies containing more than one algal species. Selected colonies were transferred to liquid or agar medium.

Cultivation of microalgae

Each microalgae strain was grown in 1 L Erlenmeyer flask containing 500 ml SE medium (pH 6.8) in a temperature controlled incubator at 25±2°C and 14 h light/10 h dark photoperiod.

Determination of lipid content

The isolated algae were inoculated into the SE medium, and then their lipid contents were determined under the same conditions.

Estimation of dry weight

Dry cell weight was determined gravimetrically according to Hu et al. (2008). A known volume of algal culture was centrifuged for 10 min at 5,000 rpm and the harvested biomass was dried in vacuum at 60°C; a constant weight was obtained.

Extraction of lipid from algal biomass

Extraction of lipid was done following the protocol of Bligh and Dyer (1959). Calculation of lipid content is as follows:

Lipid content = (Lipid weight/Dry cell weight) ×100% (Cao et al., 2004).

Restriction fragment length polymorphism (RFLP) analysis of the microalgae Extraction of DNA and PCR amplification of 18s rDNA

A microalga-specific forward primer18sF (5′GTCAGAGGTGAAATTCTTGGATTTA-3′) was used with reverse primer 18sR (5′-AGGGCAGGGACGTAATCAACG-3′ (Sara et al., 2009)) in order to obtain a 740 bp PCR product. The DNA was extracted using the cetyltrimethylammonium bromide method (Bellstedt, 2010); about 50 ng of DNA was used as template for amplification in PCR. Each PCR reaction consisted of a total volume of 50 µl containing 20 pmol of each primer, 200 µmol/L concentrations of each deoxynucelotide triphosphate, 1.5 mmol/L magnesium chloride, 0.15 mmol/L 10× PCR buffer, and 2.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The PCR conditions were as follows: initial denaturation at 94°C for 5min, 30 cycles of amplification consisting of a 30 s denaturation step at 94°C, a 30 s annealing step at 56°C, a 1-min extension step at 72°C, and a final 10 min extension at 72°C.

RFLP analysis

PCR products (approximately 100 ng) were digested at 37°C for 2 h. Each digest contained 7 µl cleaned PCR product, 0.5 µl restriction enzyme *Hha* I, and 0.5 µl *Hae* III, 1µl restriction buffer and filled up to a final volume of 10 µl with ultrapure water. The reaction mixtures were electrophoresed at 3.5% agarose gel in a 0.5×TBE buffer system. Marker of 100 bp ladder (TaKaRa, Biotech. Co., Ltd., Dalian) was used as a size marker. The reaction mixtures were recorded with the digital imager (Bio-rad, USA) after staining with ethidium bromide (0.5 µg mL^{-1}). After analysis of each restriction map, a matrix analysis was done with the Ntrys software.

Cloning of 18s rDNA and sequencing

18s rDNA gene from each microalgae sample was amplified as described above and used for direct cloning with the TOPO-TA cloning system (Invitrogen, Shanghai) following the manufacturer's instructions. The ligated plasmids were transformed into *Escherichia coli* JM109 with high transformation efficiency. The transformed cells

Table 1. Isolation and lipid contend determination of microalga.

Strain no.	isolation medium	Environment	Content of lipids (% of the dry weight)	Strain No.	isolation medium	Environment condition	Content of lipids (%)
2	water	Wetland	17.67	34	water	Shaliu River	23.32
3	water	Wetland	17.81	35	soil	Wetland	15.74
5	water	Wetland	24.02	36	soil	Wetland	17.94
6	water	Wetland	9.84	37	soil	Grassland	16.08
7	water	Qinghai Lake	18.31	38	soil	Grassland	7.33
8	water	Qinghai Lake	16.84	39	soil	Firm ground	12.87
9	water	Wetland	14.98	40	soil	Rhizosphere soil	18.01
10	water	Qinghai Lake	16.35	41	soil	Sand	20.43
11	water	Qinghai Lake	8.62	42	soil	Wetland	17.96
12	water	QinghaiLake	20.83	44	soil	Wetland	25.45
13	water	Wetland	17.84	45	soil	Grassland	19.98
15	water	Qinghai Lake	25.17	46	soil	Rhizosphere soil	15.45
17	water	Qinghai Lake	25.47	47	soil	Salinate fields	17.42
18	water	Fairy Cove	16.57	48	soil	Grassland	21.88
19	water	Fairy Cove	14.29	49	soil	Wetland	8.53
20	water	Sewage treatment plant	12.41	50	soil	Wetland	13.24
21	water	Shaliu River	19.72	51	soil	Wetland	21.57
22	water	Shaliu River	22.89	52	soil	Firm ground	10.74
23	water	Wetland	14.99	53	soil	Hot spring soil	6.29
24	water	Wetland	3.83	56	soil	Frozen soil	14.89
26	water	Sewage treatment plant	11.37	57	soil	Firm ground	10.06
27	water	Fairy Cove	3.26	58	soil	Hot spring silt	22.02
28	water	Shaliu River	14.73	60	soil	Rape fields	6.47
29	water	Shaliu River	18.2	61	soil	Grassland	15.27
31	water	Sewage treatment plant	8.88	62	soil	Firm ground	12.02
32	water	Fairy Cove	13.14	63	soil	Lake side	20.96

were plated on Luria-Bertani agar plates containing 50 mg/μl Ampicillin. The positive recombinant clones were picked for direct PCR amplification with 18sF and 18sR primers. The clones were sequenced in company (Invitrogen, Shanghai) and the sequences were analyzed in NCBI database.

RESULTS

Isolation and lipid content determination of selected microalgae strains

Fifty two strains of oleaginous microalgae from a variety of habitats in Qinghai Province had been isolated; among them 27 strains were isolated from water samples and 25 strains from soil samples. The lipid contents of 52 strains were determined in the fermentation medium (Table 1).

There was a significant difference in the lipid content of all 52 strains. The minimum lipid content of 3.26% was strain No. 27 isolated from the fairy cove; the maximum was 25.47% corresponding to strain No.17 isolated from Qinghai Lake.

Among all analyzed microalgae samples, there were 12 strains (accounting for 23.1% of the 52 strains) with lipid content higher than 20%; and there are 19 strains (accounting for 36.5% of the 52 strains) with lipid content between 15 and 20%.

Diversity analysis of microalgae

Validation by restriction fragment length polymorphism (RFLP) method

According to the results of RFLP (Figure 1), all of the microalgae are divided into two groups. The first group contains the microalgae strain No. 17, 22, 26 and 34; the second group contains the microalgae strain No. 2, 8 and 28. The PCR products of the above mentioned seven microalgae strains were digested by the restriction enzyme Hha I and Hae III.

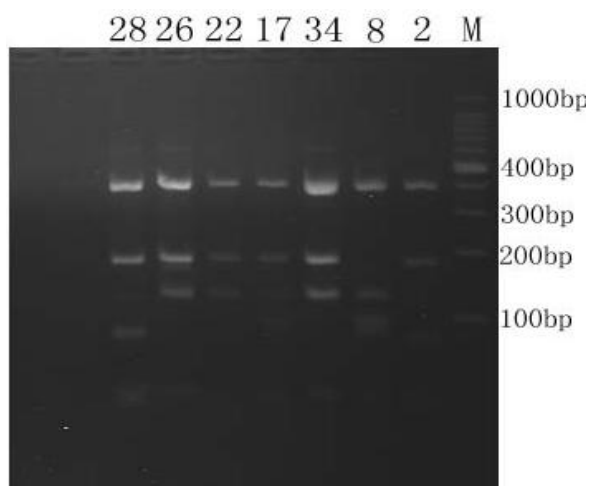

Figure 1. 18s rDNA fragment bidigestion results of seven microalgae LaneM:100bp DNA Ladder Marker.

There were differences between electrophoresis bands in the fingerprint of 18s rDNA of each microalgae by double enzyme digests, and every lane was easily distinguished. Therefore, this method could be used to analyze different categories (Figure 2).

After analysis of each restriction map, a matrix analysis could be done with the Ntrys software. The evolutionary tree showed that all the microalgae could be divided into 11 different groups (Figure 3), and 2-3 strains of each group were selected randomly for 18s rDNA molecular identification.

In order to identify microalgae species suitable for lipid production and their environmental distribution, RFLP method combined with microalgae 18s rDNA sequence analysis was employed to determine all strains' taxonomy position. The results of molecular identification showed that all of the 52 strains belong to 11 different genera (Table 2).

DISCUSSION

The aim of this experiment is to realize an extensive study on biodiversity of microalgae strains suitable for lipid production through isolation, identification, classification of the wide set of microorganisms and the determination of their lipid content. The traditional microbial classification and identification methods are based on the microorganisms phenotype characteristics, such as growth, morphology, nutrition, physiological and biochemical characteristics etc. However, these methods are very time-consuming, tedious and easily influenced by operation approach. In recent years, with the development of molecular biology technology, more and more applications such as RFLP, random amplified polymorphic DNA (RAPD), enterbacterial repetitive intergenic consensus-PCR (ERIC-PCR) have been introduced in the research fields of the microbial population structure, system diversity and dynamic change. These techniques overcome the limitations of traditional methods, and are playing a greater role in the development and advances of the microbial classification, identification and diversity analysis.

Validation of RFLP method

The analysis of RFLP method depends largely on how to choose the ideal nucleic acid enzymes. We used double enzyme *Hha* I and *Hae* III to digest the 18s rDNA fragments in this experiment. Combining the enzyme cut map and sequence alignment results, it was found that the above two enzymes could be used to correctly distinguish different microalgae, and their genus taxonomic position.

Through the 18s rDNA Molecular Identification approach, we found that among all 34 strains the microalgae No.17, 22, 26 belonged to the same Genus *Scenedesmus*, and No.2, 8, 28 were different from the above four in taxonomic position. After the analysis of the enzyme cut map, it was found that among the 18s rDNA some of the digesting products of microalgae No.17, 22, 26, 34 were very similar, and others were significantly different. The results had been confirmed by repeated trials. Thus we could infer that the classification of different microalgae could effectively be done through restriction fragment length polymorphism (RFLP) analysis with the extinction enzymes *Hha* I and *Hae* III; and the taxonomic position can be accurate to Genus. The results show that the method of RFLP is simple and relatively

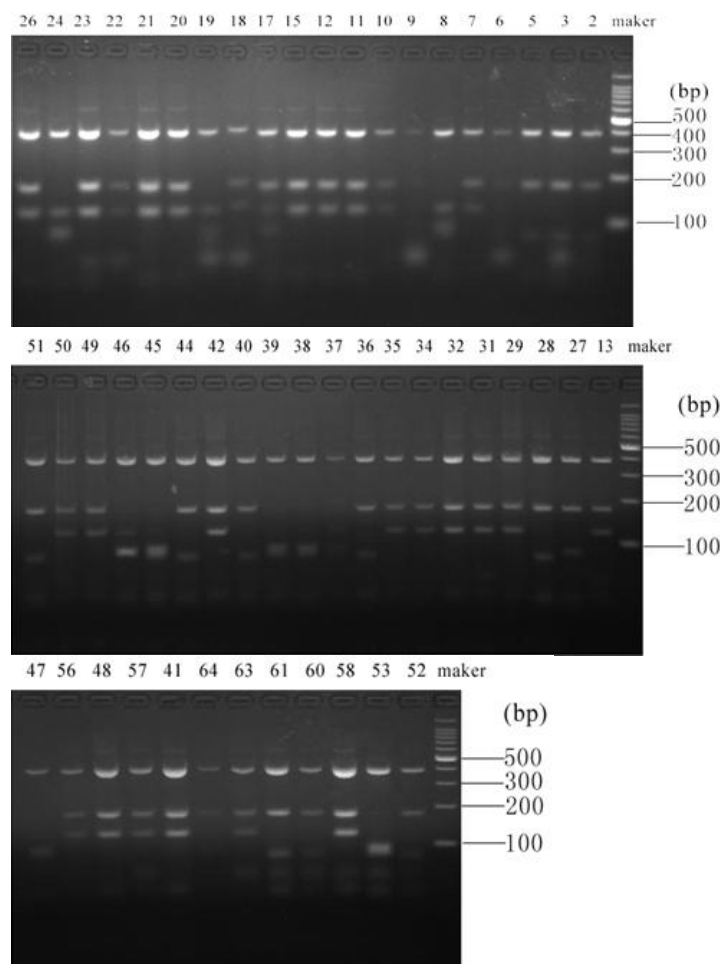

Figure 2. The electrophoresis maps of the fragments of 18srDNA by double enzyme digest.

cheap in species identification (Thaenkham et al., 2011).

Biodiversity of oleaginous microalgae

The lipid contents of all microalgae were quite different in Northern Qinghai-Tibet Plateau. The range of the lipid contents were between 3.26 to 25.47% (Figure 4). The results show that the oleaginous microalgae were extremely rich in the Northern Qinghai-Tibet Plateau. Microalgae of high quality could be separated from these special habitats, which could provide efficient and more economical microalgae species for industrial production of microalgae grease.

The results of molecular identification showed that all of the 52 strains belonged to 11 different Genera. 22 strains of them belonged to *Scenedesmus* Genus accounted for 42.31%, and other strains belong to *Chlorella*, *Nannochloris*, *Dictyosphaerium*, *Ankistrodesmus*, *Micractinium*, *Botryococcus*, *Pseudomuriella*, *Muriella*, *Chlamydomonas*, and*Graesiella*, respectively (Figure 5). Thus, it could be seen that the oleaginous microalgae in the Northern Qinghai-Tibet Plateau special habitat showed extremely rich diversity.

Relationship between habitat and distribution of the oleaginous microalgae

The lipid contents of microalgae were very different because of their different growth environments. The numbers of microalgae whose lipid contents were higher than 15% (including 15%) isolated from water and soil samples were 18 and 15, respectively. Obviously, the amount and species of microalgae in water samples were richer than those in soil samples (Figure 6).

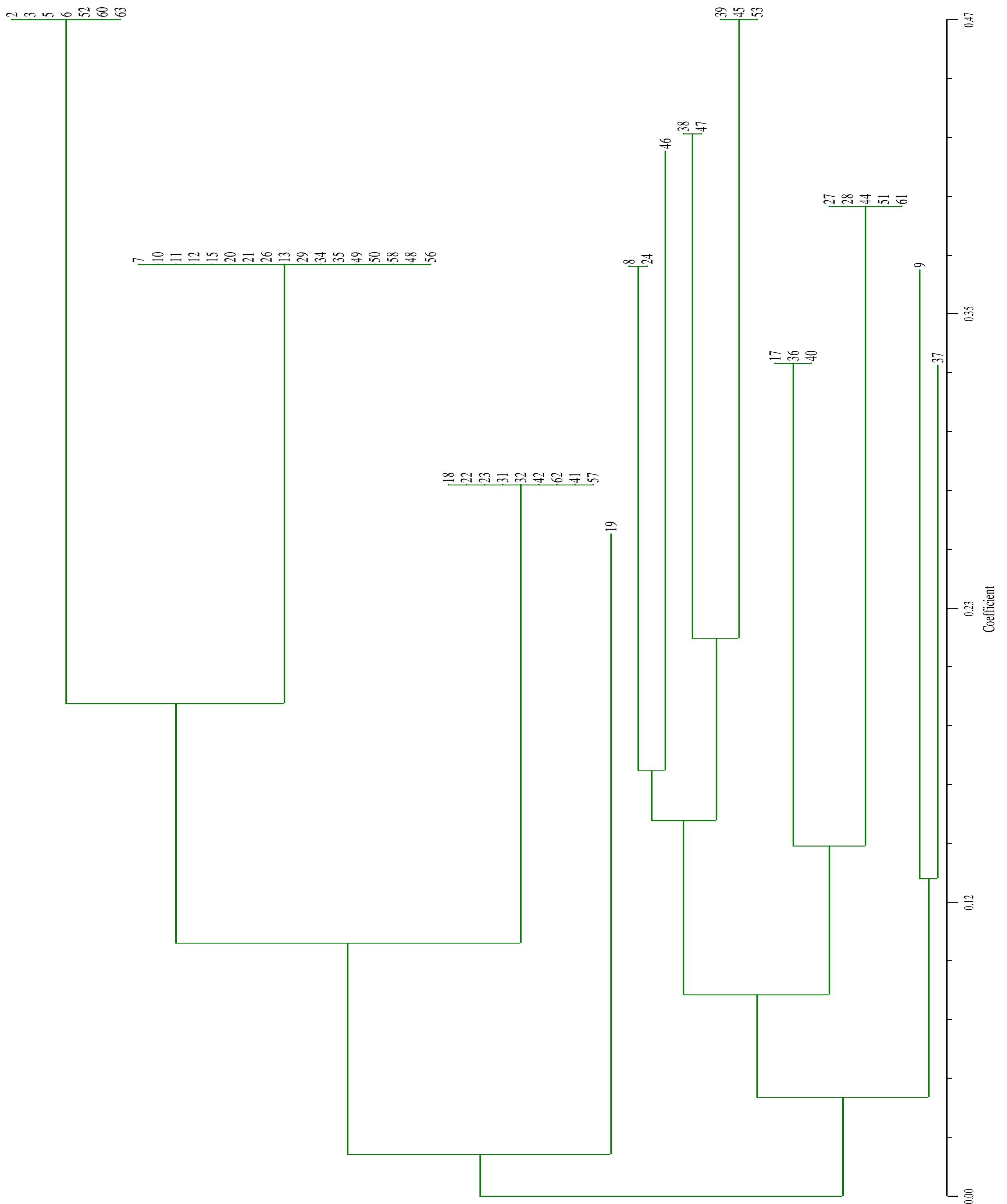

Figure 3. Classification cladogram of 52 microalgae constructed with Ntrys software

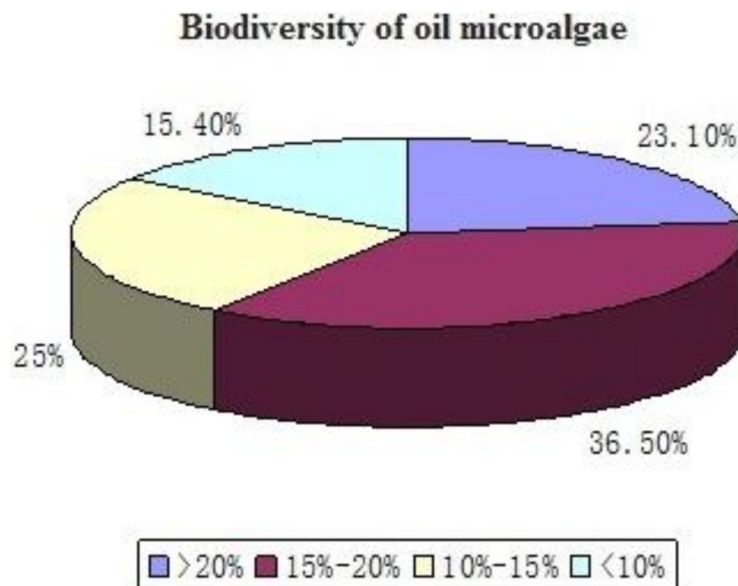

Figure 4. Biodiversity of lipid content in microalgae.

Table 2. The results of molecular identification.

Strain no.	Name	Strain no.	Name
2	*Chlorella* sp.	34	*Scenedesmus regularis*
3	*Chlorella* sp.	35	*Scenedesmus regularis*
5	*Chlorella* sp.	36	*Dictyosphaerium* sp.
6	*Chlorella* sp.	37	*Botryococcus* sp.
7	*Scenedesmus communis*	38	*Nannochloris* sp.
8	*Ankistrodesmus gracilis*	39	*Nannochloris* sp.
9	*Pseudomuriella aurantiaca*	40	*Dictyosphaerium* sp.
10	*Micractinium* sp.	41	*Scenedesmus* sp.
11	*Scenedesmus pectinatus*	42	*Graesiella emersonii*
12	*Scenedesmus deserticol*	44	*Dictyosphaerium* sp.
13	*Scenedesmus regularis*	45	*Nannochloris* sp.
15	*Scenedesmus* sp.	46	*Muriellaterrestris*
17	*Scenedesmus* sp.	47	*Nannochloris* sp.
18	*Scenedesmus subspicatus*	48	*Scenedesmus regularis*
19	*Chlamydomonas* sp.	49	*Scenedesmus regularis*
20	*Scenedesmus* sp.	50	*Scenedesmus regularis*
21	*Scenedesmus* sp.	51	*Dictyosphaerium* sp.
22	*Scenedesmus subspicatus*	52	*Chlorella sorokiniana*
23	*Scenedesmus subspicatus*	53	*Nannochloris* sp.
24	*Ankistrodesmus* sp.	56	*Scenedesmus* sp.
26	*Scenedesmus* sp.	57	*Scenedesmus* sp.
27	*Dictyosphaerium* sp.	58	*Scenedesmus regularis*
28	*Dictyosphaerium tetrachotomum*	60	*Chlorella sorokiniana*
29	*Scenedesmus regularis*	61	*Pseudomuriella aurantiaca*
31	*Graesiella* sp.	62	*Scenedesmus* sp.
32	*Graesiella* sp.	63	*Chlorella sorokiniana*

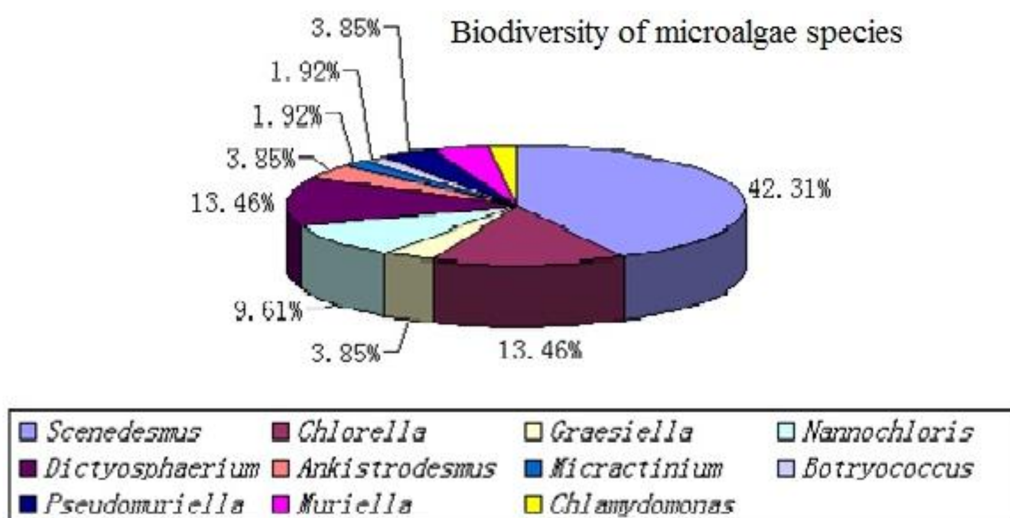

Figure 5. Biodiversity of microalgae species.

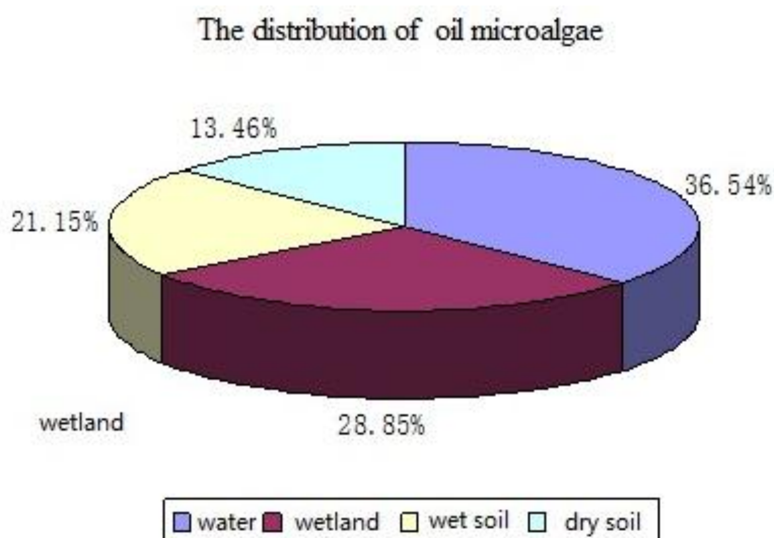

Figure 6. The distribution of oleaginous microalgae.

Our experiment results showed that it was easier to find more microalgae with high lipid content in water and wet soft soil (such as wetland, grass, hot spring mouth) than in the permafrost, hard court and sand hill soil samples. In addition, the last demonstrated lower average lipid content.

For example, in *Scenedesmus*, there were 13 strains isolated from the water, of which 10 strains' lipid contents were higher than 15% (including 15%), and there was the highest strain with the lipid content of 25.47%. However, there were only nine strains isolated from the soil samples, and there were only five strains of microalgae whose lipid contents were higher than 15% (including 15%); the highest one was 22.02%. Furthermore, among the microalgae separated from the soil samples, No. 51 and 58 had higher lipid contents than others separated from the river mouth grass and hot springs silt, respectively.

Obviously, microalgae with high lipid contents are easier to grow in the water environment. Therefore, we can infer that, moist environment is more suitable for the isolation of oleaginous microalgae.

Finding new energy resources to replace petroleum has

been a hot topic worldwide since the energy crisis. Microbial lipid is a new resource after animal fats and plant oils because it has many advantages over the conventional energy resources. Although, at present, the research on oleaginous microorganism focuses on exploring oleaginous microorganisms by screening the existing microorganisms or optimizing the microbial fermentation conditions to improve the lipid content; yet there are few researches for separation and diversity of the oleaginous microorganisms in different environments, which has limited the oleaginous microorganisms species. This experiment is mainly to study oleaginous microalgae biodiversity in Northern Qinghai-Tibet Plateau in order to provide the basis and cheap raw materials for the industrial production of microalgae lipid. We believe that, with the growing concern on microalgae lipid and the ceaseless improvement of technology and method, microalgae lipid will become a new direction of the lipid industry development.

ACKNOWLEDGEMENTS

This work was supported by National twelfth five-year science and technology support program (2011BAD14B05, 2013BAD10B01) and National Special Basic Research of china (SB2007FY400-4), Sichuan Science and Technology Bureau (2013GZ0058, 2012GZ0008), NSFC (J1103518).

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