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Full Length Research Paper

Isolation, identification and characterization of oleaginous fungi from the soil of Qinghai Plateau that utilize D-xylose

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We established a new screening method for oleaginous fungus, which was consistent with sudan black B stained but more convenient and faster. 11 strains of oleaginous fungi that utilize D-xylose were isolated from the soil of Qinghai Plateau by this method. These strains belong to 7 species by molecular identifying, they are *Tilletiopsis albescens*, *Backusella ctenidia*, *lectosphaerella sp.*, *Gibberella fujikuroi, Fusarium sp. Aspergillus fumigatus* and *Penicillium decumberns*; 4 of which were the first time found to produce lipids, they are *Tilletiopsis albescens*, *Backusella ctenidia*, *lectosphaerella* sp. and *Gibberella fujikuroi*. In addition, these oleaginous fungi could accumulate certain microbial oil by fermenting D-xylose and carboxymethyl cellulose (CMC), whose composition is similar to vegetable oil and can be converted to biodiesel. These microorganism species that may use cheap lignocellulose to produce microbial oil, it can pave lane for biodiesel production with low cost.

Key words: Oleaginous fungi, D-xylose, carboxymethyl cellulose, biodiesel.

INTRODUCTION

As fossil oil reserves decreases, the needs to seek for alternative energy source appear to be urgent on daily basis. Biodiesel, with the properties of low pollution, high fuel value which is safer than fossil diesel, is becoming the most promising alternative energy source for crude oil. And the research of biodiesel is also becoming a key direction in energy research (Knothe, 2006).

At present, 70-75% of biodiesel on the market, comes from vegetable oil, animal fat as well as waste dining oil (Pizarro and Enoch, 2003; Han et al., 2005). So the production of biodiesel consumes massive amount of vegetable oil and animal fat, which needs lots of farming land. On one hand, this will increase the prices of vegetable oil like soybean oil, the rapeseed oil and so on, on the other hand, this will also decreases the grain yields, increasing the pressure of grain supply and raising the grain price. So it is not sustainable (Ma and Hanna, 1999; Allen, 1999; Leunc, 2001). Microorganism with short life cycle has drawn people's attention, because it is easy to raise, suitable for high density fermentation, and easy to be mutated. Using oil producing microorganism to transform substrate to oil has become the best solution for sustainable development of biodiesel oil (Meng et al., 2009).

Nowadays, the research of oil producing micro-organism mainly focus on the optimization of fermentative conditions of the existing strain (Li et al., 2007), as well as screening functional oil producing microorganisms (Patnayak and Sree, 2005). However, very few studies had been done directly on isolating microorganisms from natural environment, which transform substrates to biodiesel. Pan Lixia et al. (2009) isolated 20 strains which belong to 13 different species of fat yeast from oil-rich soil and nut samples. This indicates there is abundant oil producing microorganisms in nature environment (Pan et al., 2009). Therefore, screening fat producing micro-organisms from natural environment, on one hand, it will expand the oil microorganism species resources, on the other hand, it also possibly obtains oil microorganism that have potential industrial application values.

In recent years, many studies had been done on producing biodiesel using oil producing microorganism. Moreover, microorganism fermentation had many advantages over oil-bearing plants and crops. However,

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the majority of oil producing microorganism known by now could only make use of glucose to produce oil (Li et al., 2007; Loffhagen et al., 2006; Papanikolaou, 2004). Producing biodiesel in this way raises concerns about the economic efficiency, which limits the development of microorganism fermentation. Therefore, the drawback of producing biodiesel with oil microorganism is the substrates used by microorganisms. Large progress about this question had been made. For example, isolated a strain of skinny silk spore yeast (As 2.1374) which could survive in organic acid, using the aldehyde and phenol derivatives produced by ignocellulose hydrolysate (Chen et al., 2009). (Hu Cuimin, 2009). screened a strain of red yeast which showed strong ability of fat production. They also studied to ferment glutanic acid sodium waste water to produce fat. However, most of these studies focus on fat yeast (Hu et al., 2009). Very few is about mycelial fungus. This limits the application of mycelial fungus in biodiesel production.

Year after year, the Qinghai plateau is in extreme environment of low temperature, low nutrition, oxygen deficit, strong ultraviolet and so on. Its unique geographical environment has accomplished special ecological environment like the high salty, the high temperature, the low temperature, the high radiation and so on. These special ecological environments have also bred rich microorganism resources. Do the fat funguses living in Qinghai area have some special characteristics in species resources and substrate use?

D-xylose is one of the main derivatives in lignocellulose hydrolysates. It distributes widely in natural environment. In this article, we studied the fat fungus living in Qinghai plateau, some of which are able to use D-xylose. We also studied their potential to ferment xylose and carboxymethylcellulose to produce fat. This study laid foundation for the industrial application of biodiesel.

MATERIALS AND METHODS

Soil sample collection

10 soil samples had been collected from hot spring, wetland, sand, lawn, saline and alkali land, farmland of Qinghai Plateau. Bulk samples were collected 5-15 cm below the surface and then were stored at 4℃.

Reagents

Ex-Taq polymerase and PMD18-T Plasmid were obtained from TaKaRa (Japan). All other reference substances and chemicals were purchased from Sinopharm Chemical Reagent (China), and were of analytical grade unless otherwise specified.

Enrichment of oleaginous microorganisms

1 g sample was added into the 250 mL flask containing 50 mL sterilized enrichment medium. Enrichment medium formula was as follows (g/L): D-xylose 100, yeast extract 1, KH_2PO_4 2.0, $MgSO_4.7H_2O$ 0.75, Na_2HPO_4 1, $CaCl_2.2H_2O$ 0.2, $FeCl_3$ 0.01, $ZnCl_2$ 0.1, pH 7.0, Rose Bengal (4,5,6-Tetrachlorofluorescein) and 3.3 mL of streptomycin solution (10000 U/mL). The mixture was cultured for 48 h, at 28℃, 180 rpm. So the amount of oleaginous microorganisms and the content of lipid accumulated could reach a certain level.

Screening and isolation of oleaginous microorganisms

1 mL enriched sample was gradient diluted with sterilized water, then 0.1 mL of which was spread on screening medium. Screening medium was the medium lack of carbon, and the medium formula was as follows (g/L): yeast extract 1, KH_2PO_4 2.0, $MgSO_4.7H_2O$ 0.75, Na_2HPO_4 1, $CaCl_2.2H_2O$ 0.2, $FeCl_3$ 0.01, $ZnCl_2$ 0.1, agar 20, pH 7.0, Rose Bengal (4, 5, 6-Tetrachlorofluorescein) and 3.3 mL of streptomycin solution (10000 U/mL). The screening plates were kept in incubator at 28℃ for 1-3d. The strains that appeared the earliest and grew the fastest were picked for further study.

Sudan Black B staining method

The oleaginous fungus were stained with Sudan Black B technique and observed under a microscope on oil immersion for the presence of blue or grayish colored fat globules within the cell (Thakur, 1989).

Activation and fermentation of oleaginous microorganisms

Oleaginous fungus were activated on PDA for 72 h, then they were added into 250-mL flask containing 100 mL fermentation medium, the fermentation medium formula was as follows (g/L): D-xylose 60 or carboxymethyl cellulose (CMC) 40, yeast extract 1, KH_2PO_4 2.0, $MgSO_4.7H_2O$ 0.75, Na_2HPO_4 1, $CaCl_2.2H_2O$ 0.2, $FeCl_3$ 0.01, $ZnCl_2$ 0.1, pH 7.0, The mixture was cultured for 6d, at 28℃, 200 rpm. Duplicate samples were set in order to determine biomass, dry weight and lipid content.

Determination of dry weight of fungus biomass

Zymotic fluid was centrifugated at 6000 g in order to collect thalli, and then was washed twice with sterilized water and centrifugated again. The thalli were kept at 80℃ for 24 h, then biomass dry weigh was determined.

Extraction of lipid compounds

Lipid was extracted by advanced Bligh and Dyer method (Bligh and Dyer, 1959) and then it was dried. Operations were as follows: 100 mL zymotic fluid was centrifugated a t 5000 g for 10 min, the thalli collected was kept in 50 mL centrifugal tube and washed twice with sterilized water. 15 mL 4 M HCl was added to the thalli and the mixture was kept at room temperature for 30 min. Then the whole centrifugal tube with the mixture in was kept at -80℃ for 20 min, then was immediately transferred into boiling water for 10 min. This step was repeated 3 times in order to cleavage cells. 30 mL chloroform/methanol (1:1) was added in, and the mixture was centrifugated at 5000 g for 10 min after being vibrated. Chloroform layer the lower layer was dried in decompression device, and then was weighted to get the content of lipid.

Fatty acid composition determination

After fatty acid armor esterification, gas chromatography (Japan, GCMS-QP2010) was used to determine its composition and indicate the percentages of all components.

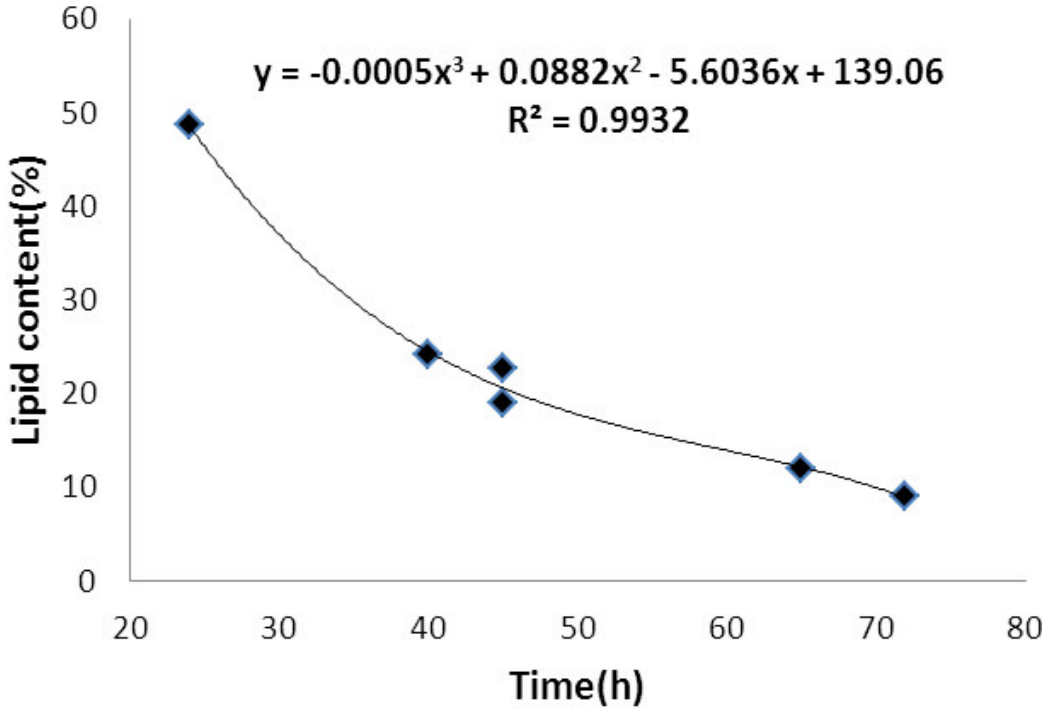

Figure 1. The relationship between colonies appearing time and oil content.

Molecular analyses of the 18S rDNA

The genome DNA of the strains for the PCR was prepared by the SDS-proteinaseK-CTAB method (Sambrook and Russel, 2001). All DNA samples were treated with RNase A, and examined by ethidium bromide-stained agarose gels. The sequences of primes for 18S rDNA were 5'-CCTCTAAATGACCAAGTTTG-3' (EF3) and 5'-GGAAGGG (G/A)TGTATTTATTAG-3' (EF4) (Smit, 1999).The amplified sequences were linked with PMD18-T. Sequencing was done by invitrogen corporation (ShangHai). Results were blast in NCBI.

Statistical analysis

Experiments were performed in triplicates and the dates were analyzed using one way analysis of variance (ANOVA).Differences with $p<0.05$ were considered statistically significant.

RESULTS

The establishment of oleaginous fungus separation

The method of current Isolation of oil fungi is Sudan black stain, which is cumbersome and time-consuming, and the result is greatly influenced by the staining. Therefore, rapid isolation method is needed. Oil as an energy storage material, grows rapidly on the medium without nitrogen The isolated fungus with different fat content after being cultured on enrichment medium was inoculated on the medium without nitrogen, to determine the relationship between the time when the colonies appeared and the fat content (Figure 1), the regression equation: $y = -0.0005x^3 + 0.0882x^2 - 5.6036x + 139.06$ ($R^2 = 0.9932$). According to the regression equation, that the colonies appeared within 45 hours, the oil content of them was more than 20%. Compared with the Sudan black strain (Figure 2), the method has good reproducibility. The oil content of the 11 strains isolated by the established method was than 20% (Table 1).

Characterization of oleaginous fungi fermenting D-xylose

Biomass, oil content, and oil yield are important indicators to evaluation of microbial oils, cellulose as the major hydrolysate of agricultural waste. The result of fermentation of the 11 isolated strains using cellulose as the carbon source was showed (Figure 3), there were 7 strains the oil content of which was more than 20%, and 6-2 reached 32.1%; 3 strains the biomass of which was more than 15 g/L, D6-2, D10-1 and D10-2, reached 15.38, 15.27 and 16.45 g/L, respectively. The oil yield of D6-1, D6-2 and D10-2 were 4.28, 4.93 and 3.74 g/L, respectively. Therefore, D6-1, D6-2 and D10-2 can utilize cellulose as carbon source to product oil well.

Characterization of oleaginous fungi to utilize CMC

The current research of oil production is mainly

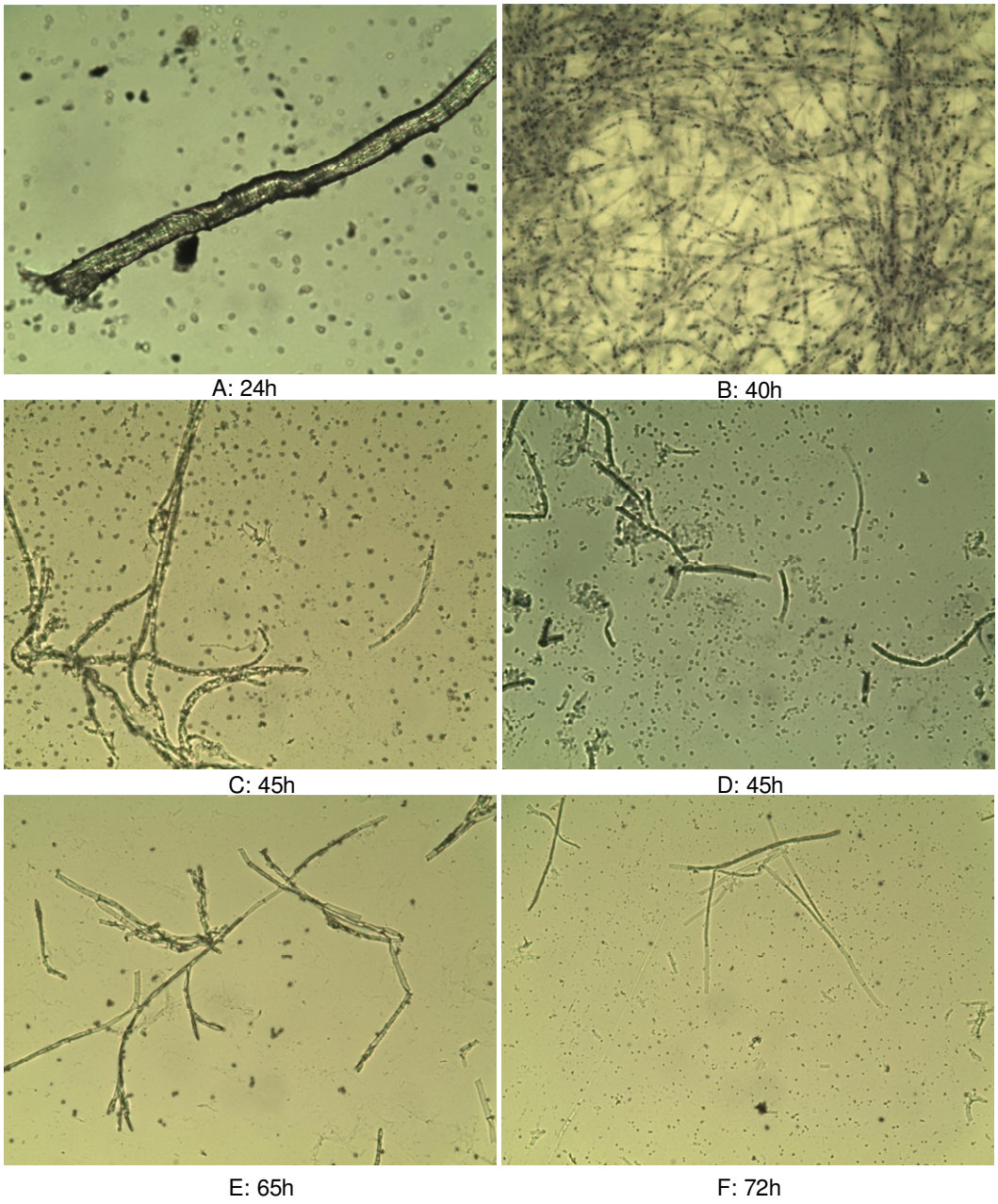

Figure 2. The results of oleaginous fungi stained by Sudan Black B.

concentrated on the high concentration of glucose as carbon source, which little has done on hemicellulose, cellulose. The results of fermentation using CMC as carbon source is showed (Figure 4), the oil content of strain D1-2, D3-1and D10-2 were 13.5, 12.5 and 13.9%, respectively. The highest biomass strains were D3-1, D6-2 and D10-2, which were 6.48, 7.31 and 7.53 g/L, respectively. Therefore, stain D3-1, D6-2 and D10-2 can utilize CMC as carbon source to product oil well. Although the biomass, the oil content and the oil yield were lower than that of xylose, it laid a foundation for the comprehensive utilization of cellulose.

Fatty acid composition and percentage

Lipid samples produced by fermented were analyzed by gas chromatography. Relative fatty acid content is shown in Table 1. It is clear that the oleaginous fungus from Qinghai Plateau is composed mainly of long-chain fatty acids with 16 and 18 carbon atoms.

Evaluation of microbial oils composition is an important indicator that whether it is suitable for biodiesel. Oil composition of the 11 strains (Table 1) is mainly C18:1, C16:0, C18:0 and C18:2, close to vegetable oil, unsaturated fatty acids, oleic acid and linoleic acid are

Table 1. Fatty acid composition and relative amount of total fatty acids.

Strain No.	Time(h)	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	Others
D1-1	36	25.7	0.8	6.1	55.2	9.8	0.6	1.8
D1-2	36	21.3	2.4	8.2	54.2	8.4	2.6	2.9
D2-1	24	17.9	1.8	9.6	58.4	9.1	1.7	1.5
D3-1	32	20.2	1.4	10.6	49.8	13.2	2.4	3.4
D4-1	48	17.8	3.2	4.9	60.5	8.3	1.6	3.7
D6-1	24	23.4	2.3	8.3	53.7	9.4	1.5	1.4
D6-2	24	19.3	0.9	17.0	48.9	8.5	2.1	3.3
D7-1	48	24.3	0.6	8.6	52.6	11.2	0.8	1.9
D9-1	48	17.0	1.7	12.5	56.3	9.1	1.2	2.2
D10-1	42	24.2	0.5	8.8	50.9	12.3	1.4	1.9
D26-1	36	16.7	3.6	7.5	57.4	10.6	2.7	1.5

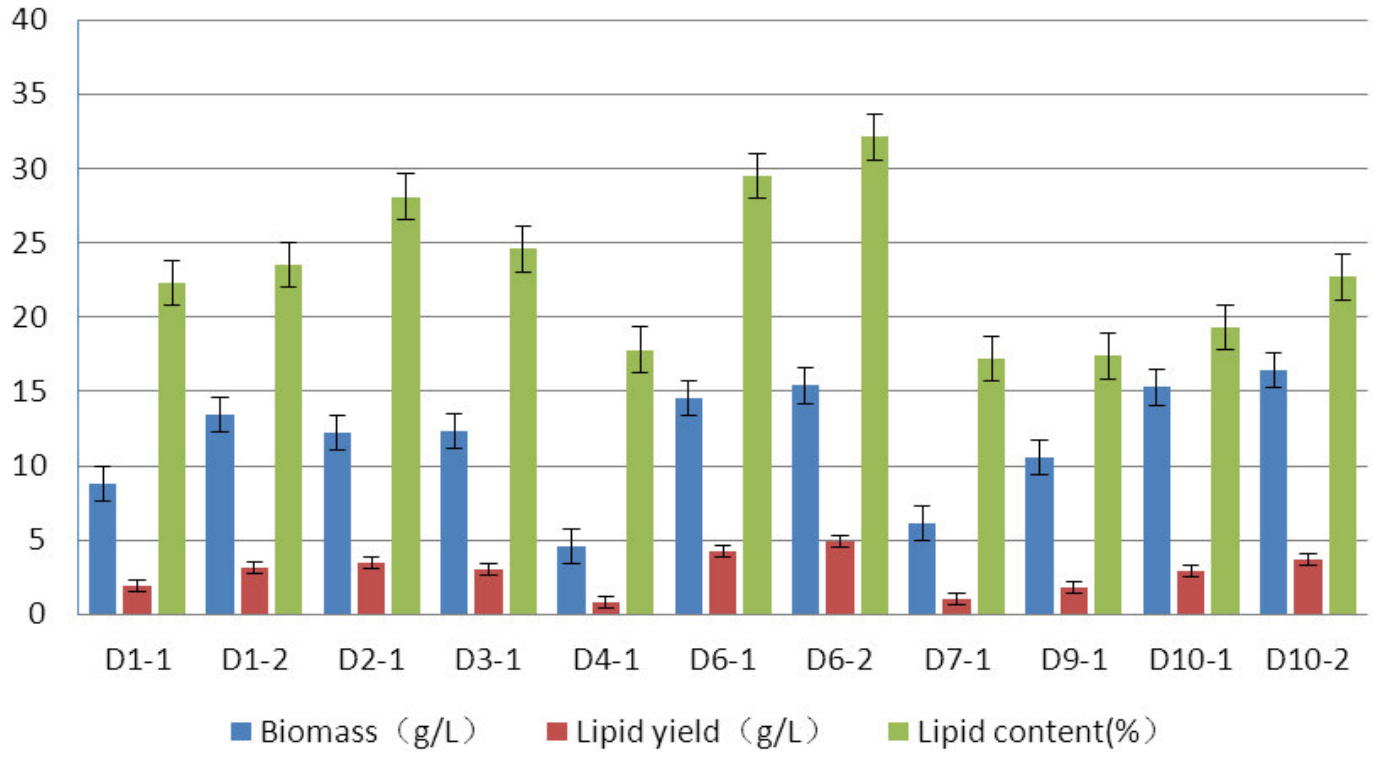

Figure 3. The biomass, oil content and oil yield of D-xylose fermentation by different strains.

the major components of microbial oils. So it is a better stock of biodiesel.

Identifying oleaginous fungus

By sequencing their 18S rDNA, 11 oleaginous funguses were identified as 7 different strains, 4 of which are reported for the first time as lipid-producing micro-organism, such as *Tilletiopsis albescens, Backusella ctenidia, Plectosphaerella* sp. and *Gibberella fujikuroi*. Results from the sequencing as listed in Table 2 confirm their genetic differences, thus supporting their identification.

DISCUSSION

Isolation of oleaginous fungi

The basic mechanism of lipid accumulation in microorganisms has been well studied (Botham and

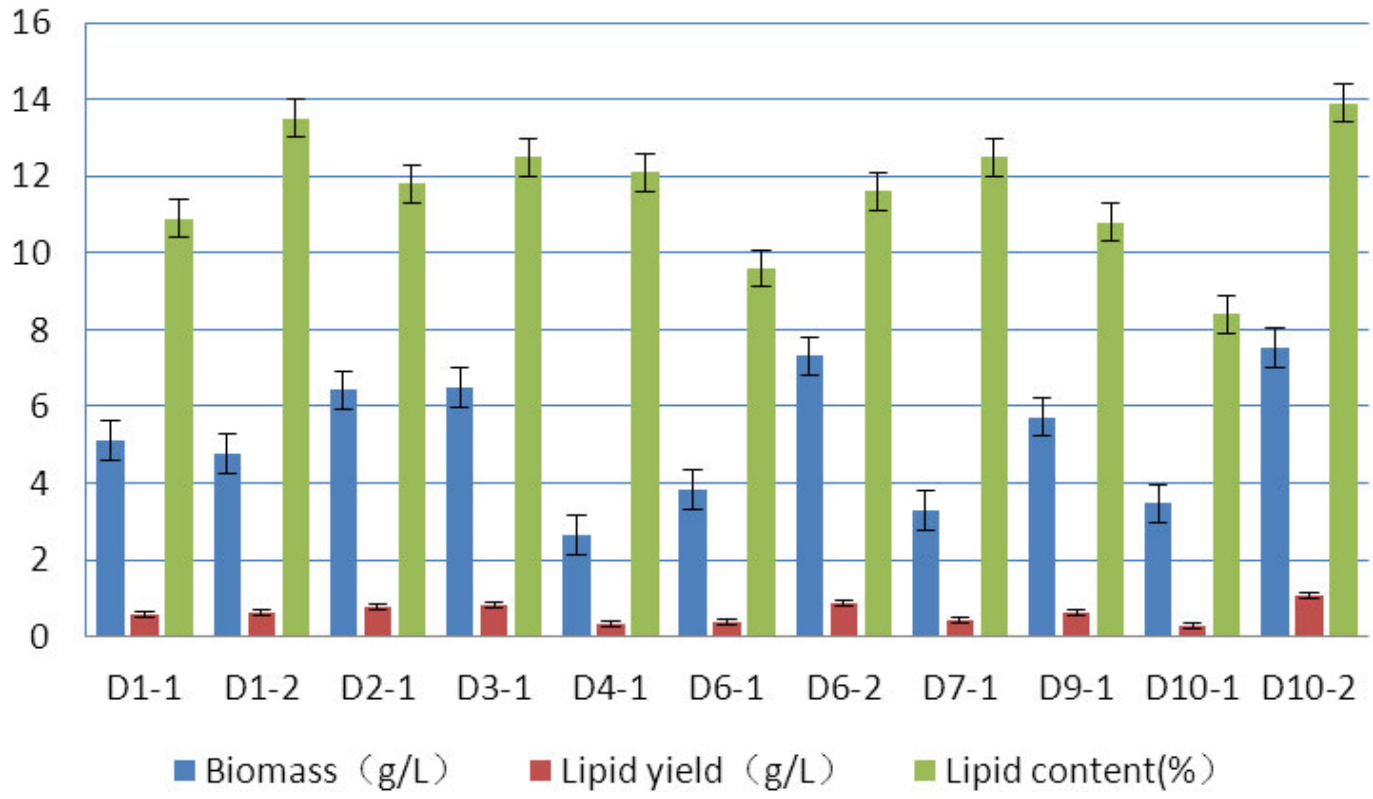

Figure 4. The biomass, oil content and oil yield of CMC fermentation by different oleaginous fungi.

Table 2. Result of the identification of 11 oleaginous fungus.

Strain No.	Accession No.	Name	Strain No.	Accession No.	Name
D1-1	HQ871880	*Fusarium sp.*	D6-2	HQ871893	*Aspergillus fumigatus*
D1-2	HQ871885	*Tilletiopsis albescens*	D7-1	HQ871895	*Fusarium* sp.
D2-1	HQ871886	*Plectosphaerella* sp.	D9-1	HQ871898	*Aspergillus fumigatus*
D3-1	HQ871888	*Backusella ctenidia*	D10-1	HQ871900	*Penicillium decumberns*
D4-1	HQ871889	*Fusarium* sp.	D10-2	HQ871901	*Gibberella fujikuroi*
D6-1	HQ871890	*Tilletiopsis albescens*			

Ratledge, 1979; Evans et al., 1981). When the culture medium lacks the nitrogen source, the isocitric dehydrogenase (ICDH) was suppresses, therefore the tricarboxylic acid circulation (TCA) was blocked. Extra carbon source was transformed to triglyceride (TAG) by a series of enzymes like the citric acid lytic enzyme, the malic acid enzyme, the fatty acid synzyme, thus completed the fat accumulation. In this article, fat microorganisms were screen by the principle of high fat contents and fast growth on carbon limited culture medium.

Firstly, microorganisms were cultured on rich culture medium with high C/N ratio to accumulate certain fat; afterward they were transferred to carbon limited culture medium for screening. As shown in Tables 1 and 2, the colonies appeared on carbon limited medium after 48 h culture contained more than 20% of fat after xylose fermentation. Our data also demonstrated that the earlier the colonies appeared on carbon limited medium, the higher they contained fat. This method of isolating fat microorganism gives less false positive results, when compared with the results got form Sudan Black B dyeing separation. Moreover, it is faster and simpler (Pan et al., 2009; Chen et al., 2009).

Biodiversity of the oleaginous fungi

At present, researches about mycelial fungus mainly focus on the functional fat production; very few are on the

oleaginous fungus screening and the substrate use. This has not only limited mycelial fungus's species resources, but has also limited its application in biodiesel production. In this study, we obtained 11 strains of fat producing microorganism those are able to use the xylose from Qinghai plateau. These strains belonged to 7 different species, 4 of which are firstly reported to be fat producing microorganism. They are *Tilletiopsis albescens, Backusella ctenidia, Plectosphaerella sp.* and *Gibberella fujikuroi*. The results of this study indicate that there are abundant microorganism resources which make use of xylose to produce fat in the special habitat of Qinghai plateau.

Composition of microbial oil

Diesel is about 15 molecules of carbon chains, study found that vegetable oil molecule is generally composed of 14-18 carbon chains, which is close to diesel, and the composition of fungal oil is similar to vegetable oil, mainly composed of 14-18 carbon atoms. Microbial diesel is a new kind of fuel made from microbial oil. By chemical analysis, Bio-diesel fuel is High fatty acid methane, acquired from the decomposition of glycerol, C18 unsaturated oleic acid as the main component, which is more environmentally friendly than conventional fossil diesel fuel.

Also it is known that fatty acids distribution impacts on the iodine value and saponification number of the particular lipid (Kalayasiri et al., 1996), which can also determine the cetane number (CN) of the corresponding biodiesel product (Krisnangkura, 1986). With the percentage distribution shown in Table 1, all the lipid samples could produce biodiesel with CN value higher than 50. Minimal CN values have been set at 47 and 49, by biodiesel standards ASTMD 6751 (USA) and DIN 51606 (Germany), respectively. Therefore, the FAMES produced from the microbial lipids of oleaginous fungus meet these standards. Results shown in Figures 3 and 4 indicate that these oleaginous funguses can not only ferment the xylose to accumulate fat, but also transform CMC into fat. In this study we found microorganism species that may use cheap lignocellulose to produce fat, which pave lane for biodiesel production with low cost.

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