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Isolation and identification of oleaginous endophytic fungi

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Endophytic fungi are a rich source of novel organic compounds with interesting biological activities and a high level of biodiversity. A total of 76 endophytic fungus strains were isolated from 13 species of plants. Through a preliminary screening and fermentation assay, a fungi named ML-GEN.1 isolated from *Strobilanthes cusia* was found and the lipid content reached 59%. This is the first report of oleaginous microorganism which is isolated from *S. cusia*. ML-GEN.1 was identified as *Fusarium* sp.ML-GEN.1 through morphological and molecular methods. Similar to vegetable oils, the fatty acid composition of lipid from *Fusarium* sp. ML-GEN.1 contained oleic acid (41.66%), palmitic acid (23.26%), linoleic acid (19.18%), and the unsaturated fatty acids amounted to about 61%. In waste molasses fermentation, *Fusarium* sp. ML-GEN.1 accumulated lipid to 29% of biomass when various sugars in waste molasses were utilized as the carbon source. The biomass was 22.8 g/L, which was much higher than the original value (12.7 g/L).

Key words: *Oleaginous* endophytic fungi, biodiesel, waste molasses.

INTRODUCTION

Bio-diesel fuels defined as fatty acid methyl esters derived from various renewable lipid resources (for example rapeseed oil, soybean oil, palm oil, etc.) is an appropriate alternative to petroleum-based diesel (Hirschmann et al., 2005). The employ of oleaginous microbial for the production of biodiesel (fatty acid methyl esters; FAME) has been described as one of the most promising ways with the potential to meet fossil diesel replacement targets without encroaching on arable land suitable for food production. Microbes accumulate more than 20% lipids in their cells in the form of triacylglycerols which were called oleaginous microorganism (Certik, 1999). Various agro-industrial residues, molasses (Zhu et al., 2008), methanol (Rupčić et al., 1996), orange peel (Gema et al., 2002), monosodium glutamate (Xue et al., 2006), sewage sludge (Angerbauer et al., 2008), starch (Papanikolaou et al., 2007) and pectin (Papanikolaou et al., 2007), were used for lipid production by different oleaginous micro-organisms.

Endophytes are microorganisms that reside within internal tissues of living plants without visibly harming the host plant (Fisher and Petrini, 1987). Some endophytic fungi have been found to produce similar chemical compounds to those produced by its host (Aly et al., 2010). Others have been shown to be potential source of novel natural products useful in medicine, agriculture and industry (Schulz et al., 2002; Rodriguez et al., 2009; Aly et al., 2010; Lin et al., 2010a; Flor et al., 2011).

We will focus on endophytic fungi as sources of lipid resources, including aspects of their interaction with the host plants, methods for isolation, preservation, identification and culturing, as well as endophytic fungi and recently isolated novel structures.

MATERIALS AND METHODS

Oil plants, peanut plant, rape, soybean, sunflower, sesame, cotton plant, and *Perilla, Jatropha*, were collected from Sichuan and Hebei of China. Chinese medicinal plants, *Rabdosia nervosa*, *Vitex trifolia*,

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Platycodon grandiforus, *Aconitum carmichaeli Debx*, and *Strobilanthes cusia* were collected in the Medicinal Botanical Garden of Huaxi, Sichuan University. Samples were immediately placed in plastic bags, stored at 4°C and processed within 2 days of collection.

Isolation of endophytic fungi

The plant materials were washed in running tap water, followed by several rinses in sterile distilled water and then cutting into 3-cm fragments. The plant fragments were surface sterilized by immersion in 75% ethanol for 15 min and washed three times with sterilized distilled water. Leaves and stems were ground in the sterilized mortar with 1 mL sterile distilled water. Samples of the suspensions were submitted to serial dilutions, which were inoculated into 90 mm Petri dishes with selected media (g/L): glucose, 5 g; yeast extract, 1 g; peptone, 3 g; $ZnSO_4$, 0.01g; $NaHCO_3$, 1 g; disodium EDTA, 0.1 g; sodium glutamate, 1 g; $MgSO_4 \cdot 7H_2O$, 0.08 g; ferric citrate, 0.01 g; $CaCl_2 \cdot 2H_2O$, 1 g, K_2HPO_4, 0.6 g; agar 2 g; H_2O 1000 mL; 20 µg/mL Streptomycin at original pH.

The roots were cut into pieces (1 ×1 cm) and then evenly placed on the surface of the selected media. To confirm the sterilization efficiency, the last washing water was inoculated into Petri dishes. The Petri dishes where incubated at 28°C for 15 days and checked daily. A hyphal tip of the endophytic fungus appearing on the plate was removed, made pure, subcultured and kept in Glycerin at -80°C.

Lipid content test

Mycelium of each strain was stained by immersing in Sudan black (Burdon, 1946) for 8 min, immediately rinsed with 70% ethanol for 5 s, and then Observed under the light microscope (Figure 1a). Stain which had dark blue lipid particles in its mycelium was selected for further study of lipid content.

The 3-days-old cultures in the seed medium (5 g/L peptone, 2 g/L yeast extract, 20 g/L glucose, 3 g/L KH_2PO_4, 1.5 g/L $MgSO_4$, and pH 6.2 to 6.6) were transferred into 100 mL nitrogen-limited culture medium (Lin et al., 2010b) (100 g/L glucose, 1.5 g/L yeast extract, 0.5 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4 \cdot 7H_2O$, 7 g/L KH_2PO_4, 2.5 g/L NH_4NO_3, 0.2 g/L $CaCl_2 \cdot 2H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.01 g/L $ZnSO_4 \cdot 7H_2O$, 0.001 g/L $MnSO_4 \cdot 4H_2O$, 0.0005 g/L $CuSO_4 \cdot 5H_2O$) in the 250 mL shake-flasks , and grown at 150 rpm and 28°C. After 7 days of growth, cultures were harvested by centrifugation at 12000 rpm, at 5 min.

The cell wall of the mycelia was broken by modified acid-heating extraction (Schulz et al., 2002). 4 M HCl was added to the biomass in a ratio of 1: 6 (w/v), and the mixture was heated in boiling water for 10 min with periodic vortex homogenization. Subsequently, freezing at -80°C for 20 min was done. This process was repeated for three times, followed by centrifugation at 6000 rpm for 10 min. The sediment sample was dried at 65°C prepared for conventional Soxhlet extraction (Eikani et al., 2007) using ether as media.

Morphological identification of the endophytic fungi

The fungi was cultured on the PDA plates at 28°C for 5 days. The ultrastructure of ML-GEN.1 was studied by the scanning electron microscopy (Kirschner, 2009). The Mycelium of ML-GEN.1 growing on the solid media was cut into 1 × 1 × 1 cm. The sample was processed before observing in the scanning electron microscope through the following steps: primary fixation with 2.5% glutaraldehyde for more than 2 h, post fixation with 4% glutaraldehyde for 4 h, followed by washing with 0.1 Monophosphate buffer; three changes of 15 min each. Sequential dehydration (Kirschner, 2009) was initially done with 30% ethanol for 15 min, followed by 50% ethanol for 15 min, 70% ethanol for 15 min, 80% ethanol for 15 min , 90% ethanol for 15 min, and 95% ethanol for 15 min. Critical point drying was done using liquid CO_2 at its critical point (31.5°C at 1100 PS I. Sputter coating was done using Gold 10 nm think film coating. The sample was observed in Scanning electron microscopy.

DNA extraction and amplification

Endophytic fungus ML-GEN.1 was identified on the basis of microscopic morphology and nucleotide sequence of ribosomal RNA. Genomic DNA was extracted from fungal mycelia using the Plant Genomic DNA Kit (TIANGEN, China) according to the manufacturer's instructions. PCR primers ITS86 (GTGAATCATCGAATCTTTGAAC) and ITS4 (TCCTCCGCTTATTGATATGC) were used to amplify the ITS2 sequence from the fungal chromosomal DNA (Life Technologies, Barcelona, Spain). Final concentrations for 25 µL PCR reactions were as follows: 5 min at 94°C followed by 30 cycles of 50 s at 94°C, 50 s at 51°C, and 2 min at 72°C with a final extension period of 10 min at 72°C. The amplified DNA was purified using the Universal DNA Purification Kit (TIANGEN, China) according to the manufacturer's protocol. The purified PCR products were cloned into pMD19-T vector (Takara, Japan) in accordance with the manual, and transformed into *Escherichia coli* JM109 competent cells (Sambrook and Russell, 2001). The recombinant plasmid DNA was purified and sequenced. For tentative identification, ITS rDNA sequences were compared with NCBI (http://www.ncbi.nlm.nih.gov) database.

Phylogenetic analysis

MEGA 5.0 was used in this study (Tamura et al., 2007). Fungal rDNA – ITS sequence of ML–GEN.1 and the matched sequences from GenBank were edited and aligned .

Neighbour-joining (NJ) trees were created using Kimura two-parameter distances. The quality of the branching patterns for NJ was assessed by bootstrap resampling of the data sets with 1,000 replications.

Fatty acid methyl ester (FAME) analysis

The crude lipids which were obtained from the fungus cells was solved in hexane and transesterified to biodiesel by base catalysis with 2 N KOH dissolved in methanol, followed by 5 mL water and placed in 30°C for 15 min to stratify. The layer of hexane were introduced directly into the GC MS-QP2010 Gas chromatograph mass spectrometer equipped (Shimadzu Corporation) with Rtz-Wax column (30 m ×0.25 mm x 0.25 m). Helium was used as the carrier gas. Temperature was 160 – 230°C; 10°C/min and the sample volume was 0.4 µL; split 1 : 80(v/v).

Waste molasses fermentation

Cane molasses was obtained from Lutang Sugar refinery (Guangxi, China). Molasses media was as follows: 20% molasses solution, 1.5 g/L yeast extract, 0.5 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4 \cdot 7H_2O$, 7 g/L KH_2PO_4, 2.5 g/L NH_4NO_3, 0.2 g/L $CaCl_2 \cdot 2H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.01 g/L $ZnSO_4 \cdot 7H_2O$, 0.001 g/L $MnSO_4 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$ 0.0005 g/L, and pH 6.5. The crude molasses was pretreated with H_2SO_4 according to the reported method (Yu et al., 2008). Un-pretreated molasses was added to the fermentation media as a comparison.

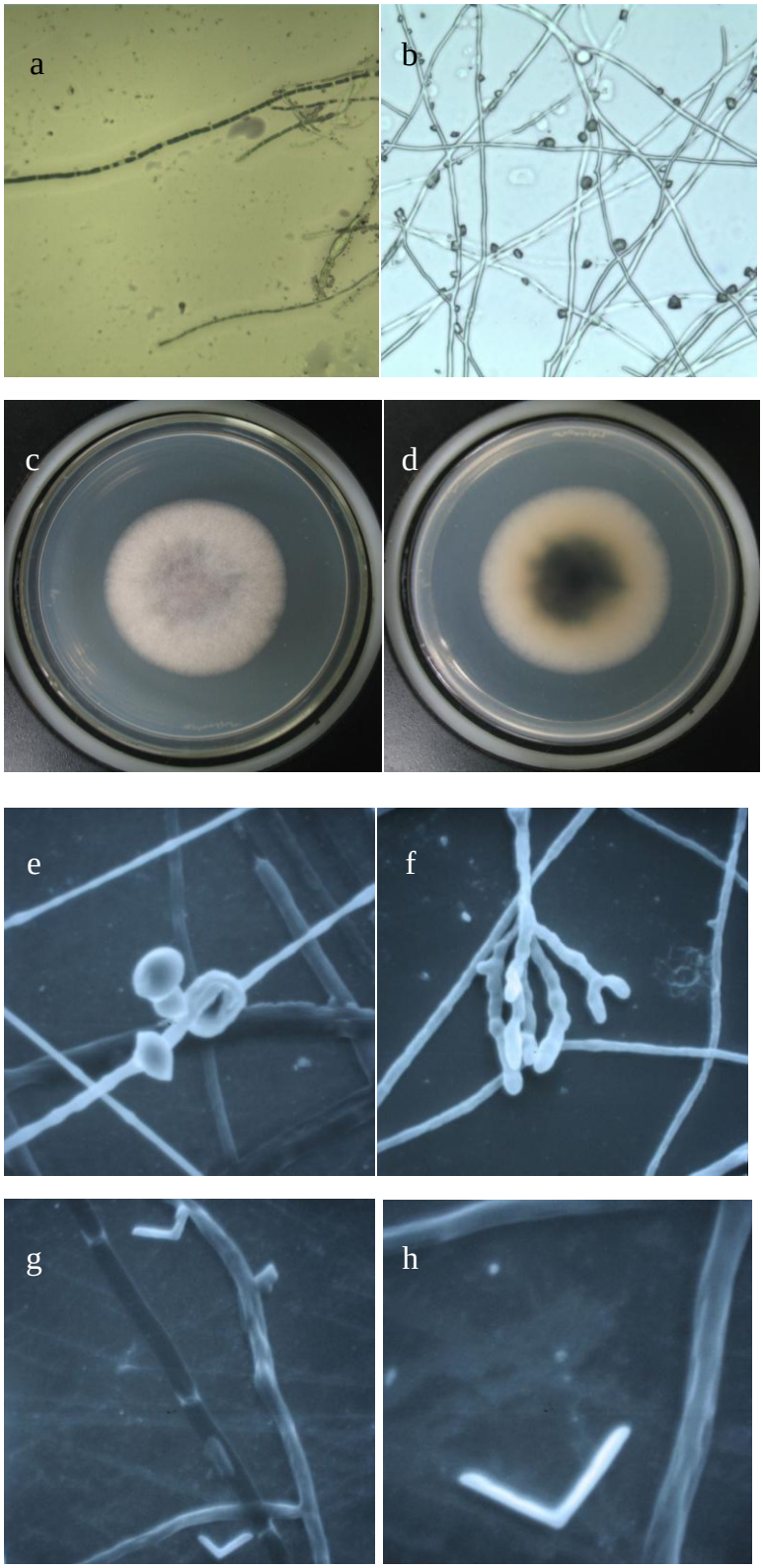

Figure 1. Morphological examination of strain ML-GEN.1. **a, b,** Light micrograph(×40 magnification) of a Sudan black staining hyphae (a) and chlamydoconidia (b) originated from hyphae. **c, d,** The obverse (c) and reverse (d) of colonies of ML-GEN.1 growing on a PDA plate for 5 days. **e-h** Scanning electron micrograph of chlamydoconidia (e), conidiophores (f) and conidia (g, h).

Table 1. Results of waste molasses fermentation.

Characteristic	Un-pretreated waste molasse	Pretreated waste molasse	Nitrogen-limited culture medium
Lipid content (%)	31	29	59
Biomass (g/L)	15.5	22.8	12.7
Lipid Production (g/L)	4.085	6.612	7.493

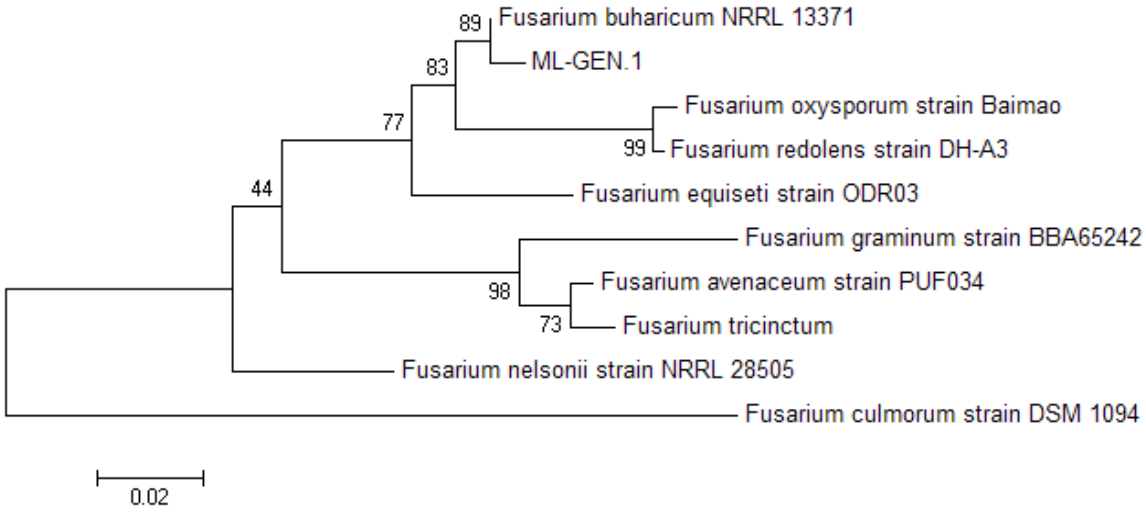

Figure 2. Neighbor-joining tree of ML-GEN.1 based on 5.8S-ITS2-28S rDNA sequences. Confidence values above 50% obtained from a 1000-replicate bootstrap analysis are shown at the branch nodes. Bootstrap values from the neighbor-joining method were determined.

RESULTS AND DISCUSSION

Isolation of endophytic fungi

A total of 76 strains of oil-producing fungi were isolated from plant samples, and each was dyed by Sudan Black and tested for its capability to produce lipid. The isolate ML-GEN.1 produced the highest level of oil with its lipid content of 59% and the biomass of 12.7 g/L. Figure 1a shows there were large amounts of lipid in mycelia cells, which indicated ML-GEN.1 was possibly with high lipid content.

Morphological identification of ML-GEN.1

Colonies of strain ML-GEN.1 grew rapidly; 5 cm in 5 days on a PDA plate. The colony had a cottony aerial mycelium, with a color of white to blue (Figure 1c, d).

The results show the light and scanning electron microscopic studies of strain ML-GEN.1 (Figure 1b, e-h). The hyphae had rough surface and were always branching. Chlamydospores were terminal, hyaline, smooth, and borne in pairs on short lateral hyphal branches. Conidiophores were short, single and multi-branched. The conidia were sickle-shaped, which were the typical formation for the genus *Fusarium*. According to these characteristics described above, strain ML-GEN.1 was identified as a definite species of *Fusarium* sp. It was hard to locate this strain into a named species just from the morphological information. Thus, the molecular identification was employed to identify it.

Molecular identification and phylogenetic analysis of ML-GEN.1

The isolate ML-GEN.1 that produced the highest level of lipid was taxonomically identified according to the ITS sequence. The ITS sequence was deposited with the National center for Biotechnology Information (NCBI) GenBank under the accession number JN248715. The sequence was aligned with other most identical sequences available in the GenBank and compared with closely related sequences retrieved from the GenBank. It displayed the highest degree of homology with those of *Fusarium* when it was compared by BLAST analysis to the sequences in the GenBank. The results of phylogenetic analysis suggestthat strain ML-GEN.1 may represent a novel species within the genus *Fusarium*.

In the neighbor-joining (NJ) tree (Figure 2), strain ML-GEN.1 and *Fusarium buharicum* NRRL 13371 formed a

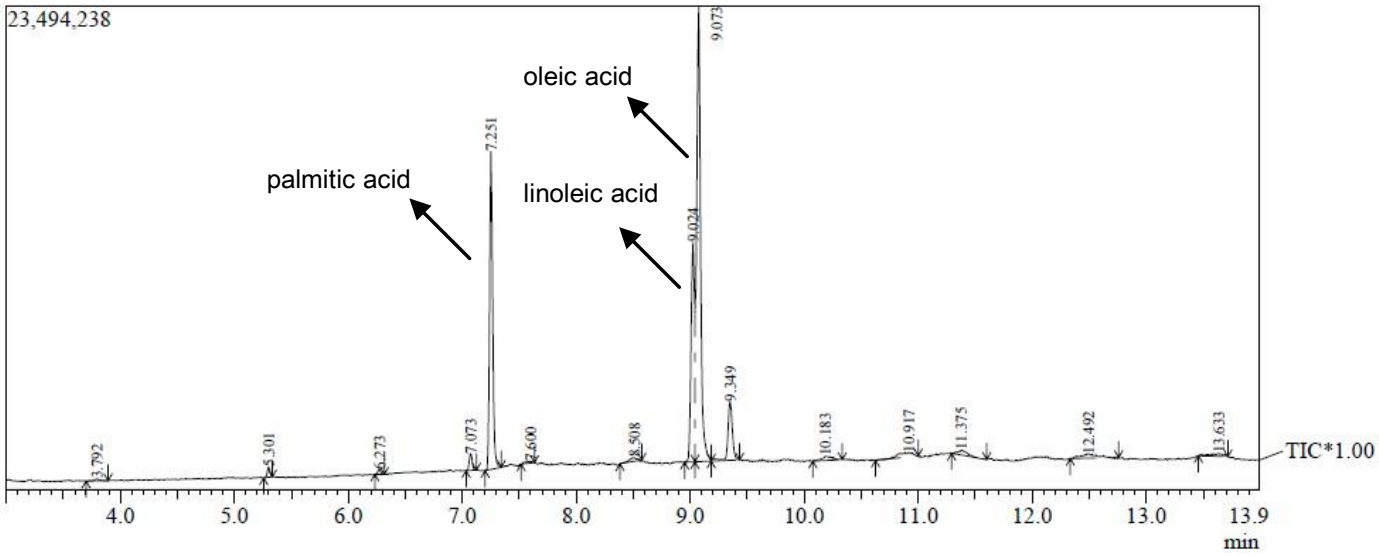

Figure 3. Chromatogram of FAMEs from the lipid of ML-GEN.1.

clade with 89% bootstrap support. The results of similarity comparisons of the 5.8S-ITS2-28S region sequence revealed that strain ML-GEN.1 had the highest nucleotide similarities with *F. buharicum* NRRL 13371. Referring to the results of the morphological and molecular identification, it could be confirmed that ML-GEN.1 belongs to the genus of *Fusarium*.

Analysis of grease composition

GC analysis showed that the lipid extracted from *Fusarium* sp.ML-GEN.1 mainly contained oleic acid (41.66%), palmitic acid (23.26%), linoleic acid (19.18%), the unsaturated fatty acids amounted to about 61%, and it was rich in oleic acid and linoleic acid (Figure 3). The fatty acids profile obtained in this study is similar to that of vegetable oils (Ha et al., 2011).

Waste Molasses Fermentation

Results of waste molasses fermentation are shown in Table 1. In pretreated waste molasses, a biomass of 22.8 g/L and a lipid content of 29% could be achieved after culture for 7 days. In contrast, a biomass of 15.5 g/L and a lipid content of 31% were got in un-pretreated waste molasses. The lipid content in pretreated waste molasses was similar to un-pretreated waste molasses. However, the biomass in pretreated waste molasses was almost 1.5-fold that of un-pretreated waste molasses, leading to the lipid production of 1.6-fold. It is obvious that the lipid content obtained with waste molasses was much lower than that with nitrogen-limited culture medium. The relatively low C/N molar ratio (about 12.5) was thought to be one of the reasons for this (Zhu et al., 2008).

Conclusions

We isolated an oleaginous endophytic fungi ML-GEN.1, which was identified as a novel species within the genus *Fusarium*, which was able to convert the waste molasses into lipid mainly composed of oleic acid, palmitic acid and linoleic acid. The feasibility of efficient and low-cost microbial lipid production by *Fusarium* sp.ML-GEN.1 from waste molasses was subsequently established. *Fusarium* sp.ML-GEN.1 could yield lipid as high as 6.612 g/L under the un-optimized conditions.

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