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Production of hydrocarbon compounds by endophytic fungi *Gliocladium* species grown on cellulose

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ABSTRACT

Endophytic fungi belonging to the genus *Gliocladium* are able to degrade plant cellulose and synthesize complex hydrocarbons under microaerophilic conditions. These fungi could thus be used to produce biofuels from cellulosics without the need for hydrolytic pretreatments. Gas chromatography–mass spectrometry–solid-phase micro-extraction (GC–MS–SPME) of head space gases from *Gliocladium* cultures demonstrated the production of C_6–C_{19} hydrocarbons including hexane, benzene, heptane, 3,4-dimethyl hexane, 1-octene, m-xylene, 3-methyl nonane, dodecane, tridecane, hexadecane and nonadecane directly from the cellulosic biomass. Hydrocarbon production was 100-fold higher in co-cultures of *Gliocladium* and *Escherichia coli* than in pure *Gliocladium* cultures. The dry mycelia weight is stable at stationary period in co-culture condition which may lead to synthesize more hydrocarbons. These fungi could potentially be developed into cost-effective biocatalysts for production of biofuels.

1. Introduction

Microbes synthesizing extracellular long-chain hydrocarbons are of interest since they could be exploited for biofuel production. Some fungal species have been shown to synthesize such hydrocarbons depending on environmental conditions and growth media (Sunesson et al., 1995; Wackett, 2008). For example, *Gliocladium roseum* (NRRL 50072) (now designated as *Ascocoryne sarcoides* (Stadler and Schulz, 2009; Strobel et al., 2010; Griffin et al., 2010)), which lives inside the Ulmo trees in the rain forests of Patagonia (Argentina and Chile) was able to directly degrade plant cellulose and synthesize a broad array of hydrocarbon compounds which are remarkably similar to diesel fuel (Strobel et al., 2008). In this fungus, hydrocarbon production appears to be a defense mechanism since the production of gases rich in hydrocarbons and hydrocarbon was stimulated by exposure to antibiotics (Strobel et al., 2008). The current study was undertaken to determine if co-cultivation with bacterium would be a means to increase hydrocarbon production by three *Gliocladium* species. *Escherichia coli* strain Nissle 1917 (DSM 6601) was chosen as the test bacterium because it is non-pathogenic, metabolically versatile and able to grow under the microaerophilic conditions required by the fungus.

2. Methods

2.1. Fungal and bacterial test strains

Gliocladium 62724, *G. roseum* 1165 and *G. roseum* 62726 were obtained from DSMZ (German collection of microorganisms and cell cultures, Inhoffenstrabe, Germany), ATCC (American type culture collection, Manassas, VA, USA) and from a private Danish collection at Copenhagen Institute of Technology at Aalborg University respectively. *E. coli* strain Nissle 1917 (DSM 6601) was obtained from ATCC. The freeze dried fungal spores were suspended in sterile 15% (v/v) glycerol, plated on potato dextrose agar (PDA, Difco Laboratories) and incubate at 23 °C for 18 days until good sporulation occurred. The spores were harvested by washing the petri plates with sterile water containing 1% Triton X-100 (Fisher Scientific, Orangeburg, NJ, USA).

2.2. Culture media

Cellulose pre-culture medium (Ahamed and Vermette, 2008) consisted of: 10 g L^{-1} avicel (Sigma–Aldrich, St. Louis, MO, USA), 5 g L^{-1} corn steep liquor (Sigma–Aldrich, St. Louis, MO, USA), 1.5 g L^{-1} proteose peptone (Fischer Biotech, Fair Lawn, NJ, USA), 0.5 g L^{-1} yeast extract (Fischer Biotech, Fair Lawn, NJ, USA), 1.7 g L^{-1} $(NH_4)_2SO_4$, 2 g L^{-1} KH_2PO_4, 0.3 g L^{-1} $CaCl_2{\cdot}2H_2O$, 0.3 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.005 g L^{-1} $FeSO_4{\cdot}7H_2O$, 0.0037 g L^{-1} $CoCl_2{\cdot}6H_2O$, 0.0016 g L^{-1} $MnSO_4{\cdot}H_2O$, 0.0014 g L^{-1} $ZnSO_4{\cdot}7H_2O$. The composition of production medium was the same as that

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of the cellulose pre-culture medium, except that it contained 35 g L^{-1} avicel, 10 g L^{-1} corn steep liquor, 3 g L^{-1} proteose peptone, 2 g L^{-1} yeast extract, 2 g L^{-1} $(NH_4)_2SO_4$, 4 g L^{-1} KH_2PO_4 and 0.2 ml L^{-1} tween 80. The production medium was sterilized at 121 °C for 45 min.

2.3. Inoculum and shake flask culture

Concentrated aqueous spore inocula (10^6 mL^{-1}) of the *Gliocladium* strains preserved in 2-mL vials at −80 °C in 15% (v/v) glycerol were inoculated into modified 1000-mL graduated Erlenmeyer flasks containing 300 mL of cellulose pre-culture medium and incubated at 23 °C on a rotary shaking incubator with an agitation speed of 125 rpm. The initial pH was 5.7 and was not controlled during pre-culture. The flasks were sealed with a rubber stopper with two holes through which stainless steel tubes were inserted to allow collection of liquid and air samples. After 72 h of cultivation, the *Gliocladium* mycelium suspension from the pre-culture medium corresponding to 10% (v/v) of the total production medium volume was aseptically inoculated into two 500-ml flasks containing 250 mL of production medium. One of the flasks was also inoculate with 1.89×10^6 CFU mL^{-1} of *E. coli* Nissle 1917. Microearophilic conditions were maintained in both flasks through hose cock clamp fixed on rubber tube with sterile air filter, turning these clamps on and off two times a day facilitate the microaerophilic conditions and the flasks were incubated at 23 °C for 20 days on a rotary shaker (125 rpm). Control flasks containing autoclaved cultures were also incubated. The shake flask culture experiments were carried out in duplicate.

2.4. Determination of residual cellulose and fungal dry biomass

Cellulose content of *Gliocladium* fungal cultures were determined by a simplified version of the method of Updegraff (1969). Ten milliliter of the culture broth was centrifuged (3000*g*, for 20 min), the supernatant was removed, the pellet was suspended in acetic acid nitric acid reagent (3 mL: 150 mL of 80% acetic acid with 15 mL of pure nitric acid) and boiled for 30 min in a water bath. After cooling and centrifugation (3000*g*, for 20 min), the pellet was washed with distilled water (10 mL), and the residual cellulose was dried at 40 °C under reduced pressure until constant weight. Six replicate samples were measured and the average cellulose content was calculated.

The dry fungal biomass was determined using the method developed by Ahamed and Vermette (2009). The mycelial weight was calculated as the difference between the total dry weight of the solids (comprising mycelium and residual cellulose) and that of the residual cellulose.

2.5. Dimensionless "Henry's law constant" application

Henry's Law Constant (HLC) was applied to determine the concentration of volatiles in liquid and head space gases at equilibrium. The equation is as follows:

$$H' = \frac{H}{RT}$$

H′, Dimensionless Henry's law constant; H, Henry's law constant [atm – m^3/mol]; R, Universal gas constant [8.20575×10^{-5} atm-m^3/mol K]; T, Temperature in kelvin.

Equilibrium of air–water partitioning:

$$(CL)(VL) = (Cle)(VL) + (Cge)(V_g)$$

CL, Concentration in liquid (mg/L); VL, Volume of liquid (L); Cle, Concentration in liquid at equilibrium; Cge, Concentration in head space gas at equilibrium; V_g, Volume of gas.

Liquid phase partitioning:

$$\frac{(CL) \times (VL)}{(VL) + (H' \times V_g)} \cdot \frac{CL}{(1 + H')}$$

Gas phase portioning:

$$(H') \times (Cle) \cdot \frac{(H') \times (CL)}{(1 + H')}$$

2.6. Headspace gases analysis

Ten milliliter of the culture was centrifuged at 10,000*g* for 20 min, and the supernatant was carefully removed with a sterilized glass pipette and used for the measurements of volatile hydrocarbons through GC/MS (7890A GC-system with 5975C inert XL E1/C1 MSD model # G3174A, Agilent Technologies, Wilmington, DE, USA) containing DB-5MS, non-polar, high temperature limit (450 °C), 30 m × 0.250 μm × 0.25 μm column. The CTC analysis CombiPAL robotic arm (G6500-CTC-LHS2.PAL system-CH001210757, CTC Analytics AG, Zwingen, Switzerland) was connected to GC/MS for auto sampling system.

All the samples were sequenced and programmed for incubation at 50 °C in 20 mL head space screw top clear glass vials (VWR 5188-2753) with an agitation speed of 500 rpm and the total extraction time was 2 min. The volatiles from head space gases were adsorbed to sterilized Divinylbenzene/Carboxen/Polymethylsiloxane stableflex silica fiber (Gray, Supelco Cat. 57284-U, Bellefonte, PA, USA) present in solid phase micro extraction (SPME) syringe (Supelco, Cat. 57315, Sigma–Aldrich) connected to CTC-PAL robotic arm for automatic insertion. SPME allows extraction and concentration to be performed in a single step and direct analyte transfer to GC by thermal desorption inside a heated injection port for 1 min and swept into the column by ultra pure helium as carrier gas and the average velocity was maintained at 42.651 cm/s, the analytes were trapped and focused at the column inlet and the stable flex silica fiber of SPME was retracted back into the fiber holder assembly and removed automatically from the GC injection port. The chromatographic separation was performed in the normal manner, where the column temperature program was 30 °C for min^{-1} and ramped at 10–220 °C min^{-1} and the total run time was 21 min. The injector temperature was maintained at 240 °C throughout the entire chromatographic separation. The mass spectrometer was operated in the full scan mode between 50 and 550 amu, the ion source temperature was at 230 °C and the actual electron multiplier voltage was 1047 V.

Initial identification of the unknowns produced by *Gliocladium* was made through library comparison using the NIST database. All chemical names in this study follow the nomenclature of this database. In all cases, kill control flasks were also analyzed and the compounds found therein were subtracted from those appearing in the pure *Gliocladium* or the co-cultures. Tentative identification of the fungal products was based on observed mass spectral data as compared to those in the NIST database. Final confirmatory identification was made for many of the compounds by comparing GC/MS data of authentic standards with the GC/MS data of the fungal products. The retention time of an authentic compound versus the fungal volatile was also measured. The experiments were repeated at least twice with comparable results.

2.7. Identification and quantitative analysis of hydrocarbons

Sixteen commercial hydrocarbon standards were prepared at 10, 20, 30 and 40 μg/mL (ppm) concentrations respectively in