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Glass bead cultivation of fungi: Combining the best of liquid and agar media

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ABSTRACT

Production of bioactive compounds and enzymes from filamentous fungi is highly dependent on cultivation conditions. Here we present an easy way to cultivate filamentous fungi on glass beads that allow complete control of nutrient supply. Secondary metabolite production in *Fusarium graminearum* and *Fusarium solani* cultivated on agar plates, in shaking liquid culture or on glass beads was compared. Agar plate culture and glass bead cultivation yielded comparable results while liquid culture had lower production of secondary metabolites. RNA extraction from glass beads and liquid cultures was easier than from agar plates and the quality was superior. The system allows simple control of nutrient availability throughout fungal cultivation. This combined with the ease of extraction of nucleic acids and metabolites makes the system highly suitable for the study of gene regulation in response to specific nutrient factors.

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1. Introduction

Fungi have been cultivated under artificial conditions in laboratories for over 100 years (Ainsworth, 1986). They are traditionally cultivated on solid agar in petri dishes or in liquid media. Liquid cultivation requires vessels such as Erlenmeyer flasks, fermentation tanks or other reservoirs. In experimental systems, it is difficult to obtain uniform growth in liquid culture as the fungus may develop mycelium on the sides of flasks or form clumps of mycelium. Alternatively, fungi can be grown on solid media in petri dishes with agar as the solidifying agent. Agar consists of two polysaccharides, agar and agaropectin (Araki, 1937). Agar also contains impurities such as trace elements (Whittaker, 1911), and the quality may vary from one batch to another (Scholten and Pierik, 1998). Some fungi are able to grown on media solely made of water and agar (Midgley et al., 2006), which makes this medium unsuitable for studies of carbon metabolism.

Different solid state fermentations (SSF) have been used for fungal cultivation. Physical conditions during SSF such as aeration influence oxygen access, removal of carbon dioxide and temperature, which are all important factors in fungal growth and metabolism (Krishna, 2005). Natural materials such as whole grains and seeds or inert support materials combined with liquid media have been used to produce specific secondary metabolites and enzymes (Ooijkaas et al., 2000; te Biesebeke et al., 2002; Rangaswamy, 2012). Cultivation on natural material does not allow control of the nutrient composition, and in the case of grains, it is difficult to separate the mycelia from the growth medium. The last is also true when small particles are used as the inert support. Glass beads have been used successfully to culture soil actinomycetes (Bottcher and Conn, 1942) and filamentous Gram-positive bacteria streptomycetes (Nguyen et al., 2005). Glass beads do not influence nutrient availability and provides the solid support, which has proven to be an advantage for induction of secondary metabolite production.

The increasing number of available fungal genome sequences and technological advances has provided a platform for whole transcriptome analyses. *Fusarium* genomes contain a high number genes predicted to be involved in the production of as yet unidentified secondary metabolites (Hansen et al., 2012). Many genes are silent under standard laboratory cultivation conditions (Lysøe et al., 2011), and it would be an advantage with a system that allows rapid change of nutrients to identify inductive factors. Methods such as quantitative RT-PCR, RNA sequencing and SAGE are available for determination of transcript level that may be correlated to, e.g., secondary metabolite production. This requires high-quality RNA from fungi grown under reproducible conditions. Isolation of RNA from total fungal mycelia grown on agar is impaired by saturation of the columns used in the RNA extraction kits. Liquid cultivation of fungi is preferable for RNA extraction but does not give optimal production of secondary metabolites. A reproducible cultivation system that allows efficient extraction of nucleic acids, proteins and metabolites would aid in the discovery of nutrient factors that control biosynthesis of the different secondary metabolites. Here we test a glass bead cultivation system for two species of *Fusarium* and show that RNA extraction and purification

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is highly efficient and that secondary metabolite production is comparable to that on agar plates.

2. Materials and methods

2.1. Fungal strains

Fusarium graminearum strain PH1 (NRRL31084) was obtained from the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA, whereas *Fusarium solani* f. sp. *pisi* (FGSC 9596) was obtained from the Fungal Genetics Stock Center University of Missouri, Kansas City, Missouri, USA. Strains were grown on Czapek–Dox (CZ) agar (Difco, 233818), and the conidia used for inoculation of different media were produced on Spezieller Nährstoffarmer Agar (SNA) (Nirenberg, 1976) with alternate 16 h light–8 h dark cycles.

2.2. Cultivation

Fusarium were grown on yeast extract sucrose (YES) medium (Samson et al., 2010) for secondary metabolite and RNA extraction and on YPG for DNA extraction. The fungi were cultivated under three different conditions: (1) solid agar media prepared in 90 mm petri dishes, (2) 50 mL liquid media in 250 mL baffled bottles at 100 rpm and (3) in petri dishes with 2 layers of acid washed 3 mm glass beads and 15 mL liquid medium. Agar and glass bead plates were inoculated with 10 μl spore suspension (1×10^6 spores/mL) placed in the middle of the petri dishes, whereas the liquid cultures were inoculated with 10 μl spore suspension (1×10^6 spores/mL) directly into the bottles. The growth on the three different conditions was initially observed on YES medium by cultivating the two *Fusarium* species at 25 °C in the dark.

The ability to utilize agar as carbon source was examined by growing *F. graminearum* on homemade CZ medium without carbon source (1 L: 1 g K_2HPO_4; 0.5 g KCl; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.01 g $FeSO_4 \cdot 7H_2O$, 1 mL trace solution (1 g $ZnSO_4 \cdot 7H_2O$ and 0.5 g $CuSO_4 \cdot 5H_2O$ in 100 mL H_2O). Agar (20 g/L; Prolabo, VWR) was added only to the solid medium. The strain was cultivated for 28 days at 25 °C in the dark and the growth was observed visually.

Table 1
Yield and quality of RNA from mycelia incubated in liquid, liquid with glass beads, agar plugs and aerial mycelia from agar YES media. Concentrations are measured by spectrophotometry with nanodrop. RIN[e] value and S28/S18 ratios are measured in Tapestaion (Agilent) using R6K reagent with 2200 TapeStation software. Values are presented as mean ± SEM from three independent replicates.

	Concentration (ng/μL)	Rin[e]	S28/S18
Liquid	262 ± 65	9.37 ± 0.38	1.08 ± 0.14
Glass beads	57 ± 48	9.20 ± 0.4	1.29 ± 0.15
Agar plugs	34 ± 16	8.57 ± 0.12	1.02 ± 0.09
Aerial mycelia from agar	72 ± 14	9.13 ± 0.21	1.03 ± 0.18

2.3. Production of secondary metabolites

The two *Fusarium* species were cultivated under the three different cultivation methods in YES medium at 25 °C in the dark in order to compare the secondary metabolite production. Extraction from solid agar plates were carried out after 10 days as described previously (Smedsgaard, 1997). In brief, 9 plugs were transferred to a 14-mL culture tube and extracted in an Bransonic 2510 UltraSonic bath (Branson) for 45 min with 2 mL ethyl acetate–dichloromethane–methanol (3:2:1) buffered with 1% formic acid. The extracts were transferred to a new tube and lyophilized. The samples were then re-dissolved ultrasonically for 10 min with 500 μL methanol and transferred to 2 mL HPLC vials. Whole mycelia from cultures grown on glass beads for 10 days were lifted with forceps into 14 mL glass tubes to a volume of 3 cm^3 and extraction carried out as described for the agar plugs. Metabolites were extracted from liquid cultures after 7 days by transferring 25 mL fungal suspension to 100 mL glass bottles containing 25 mL 3:2:1 extraction solution and then shaken at 180 rpm for 2 h. The organic phase was transferred to 50 mL flask and lyophilized. The extracts were re-suspended in 2 mL 100% methanol and transferred to HPLC vials. Extractions were performed as independent triplicates from individual cultures.

The samples were analyzed on an Agilent 1260 LC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector. A 5-μL extract was injected and separated on a 100 × 2.1-mm kinetex 2.6 μm XB-C18 (Phenomenex, Torrance, CA, USA) using a flow of 0.4 mL/min with a linear water–acetonitrile gradient, where both

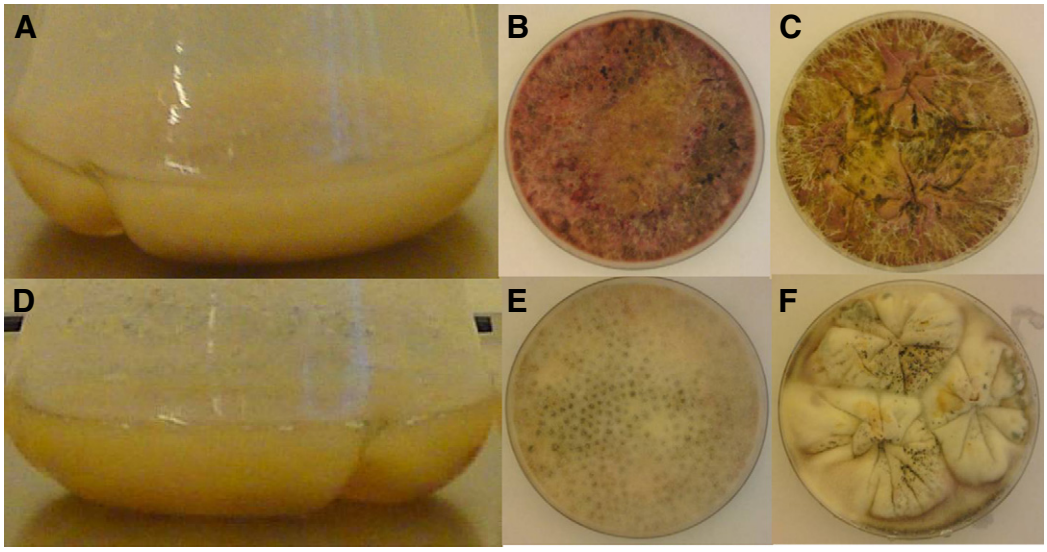

Fig. 1. *Fusarium graminearum* and *Fusarium solani* mycelial growth on different YES media at 25 °C. (A) *F. graminearum* cultivated in liquid media in 50 mL Erlenmeyer flask grown for 7 days with 100 rpm rotation. (B) *F. graminearum* cultivated on glass beads with liquid media in petri dish for 14 days. (C) *F. graminearum* cultivated on agar for 14 days. (D) *F. solani* cultivated in liquid media in 50 mL Erlenmeyer flask grown for 7 days with 100 rpm rotation. (E) *F. solani* cultivated on glass beads with liquid media in petri dish for 14 days. (F) *F. solani* cultivated on agar for 14 days.

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