Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays

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Introduction
Critical to success in microbial natural products discovery is the manipulation of nutritional and environmental factors promoting secondary metabolite biosynthesis. Even minor variations in environment or nutrition have the potential to impact the quantity and diversity of fermentation products. The deliberate elaboration of cultural parameters, to augment the metabolic diversity of a strain, has been called the OSMAC (one strain, many compounds) approach (Höfs et al. 2000; Bode et al. 2002; Christian et al. 2005; Scherlach and Hertweck 2006). Implicit in the OSMAC approach is a strategy that addresses uncertainties of how to grow uncharacterized strains. The likelihood that one or more growth regimes would be adequate for growth and product formation increases as an organism is empirically tested among multiple conditions. Secondary metabolite screening regimes were adapted to grow fungi in nutritional arrays.

Abstract
Aims: We asked to what extent does the application of the OSMAC (one strain, many compounds) approach lead to enhanced detection of antibiotics and secondary metabolites in fungi? Protocols for bacterial microfermentations were adapted to grow fungi in nutritional arrays.

Methods and Results: Protocols for microfermentations of non-sporulating fungi were validated using known antifungal-producing fungi. Detection of antifungal activity was often medium dependent. The effects of medium arrays and numbers of strains on detection of antifungal signals were modelled by interpolation of rarefaction curves derived from matrices of positive and negative extracts. Increasing the number of fermentation media for any given strain increased the probability of detection of growth inhibition of Candida albicans. Increasing biodiversity increased detection of antifungal phenotypes, however, nutritional arrays could partly compensate for lost antibiotic phenotypes when biodiversity was limiting.

Conclusions: Growth and extraction in microtiter plates can enable a discovery strategy emphasizing low-cost medium arrays that can better exploit the metabolic potential of strains.

Significance and Impact of the Study: Increasing fermentation parameters raise the probability of detecting bioactive metabolites from strains. The protocols can be used to pre-select strains and their growth conditions for scale up that will most likely yield antibiotics and secondary metabolites.
sets of strains by lowering costs for each variation in culture parameters. As a first step in microbial screening, nutritional or environmental arrays could be applied to identify organisms and conditions in which they would be more apt to produce antibiotics or secondary metabolites, resulting in screening populations enriched in biological activity. However, filamentous fungi are rarely fermented below the flask or tube scale, and the extent to which secondary metabolite production can be scaled down is generally unknown. Microplate fermentations of filamentous fungi are difficult to manipulate because mycelia often form dense interwoven tissues or thick cell masses that can clog and interfere with pipette tips when transferring inoculum and extracts. Rates and patterns of hyphal growth vary enormously among species. Some species may grow very fast and can overflow and escape from a well, while another species may barely initiate growth within the same time period. Furthermore, aeration of microplate cultures by orbital shaking may be ineffective because mycelia can quickly form solid masses.

Systems for strain transfer and fermentation in microplates are routinely used for screening of bacteria and yeasts for enzymes and metabolic products (Duetz et al. 2001; Dieting et al. 2005; Zimmermann and Rieth 2006). However, techniques for growing filamentous fungi in microplate systems have lagged behind and have been limited to organisms which produce abundant, easily manipulated spores, e.g. Aspergillus (Lee and Magan 1999), Oidiodendron (Rice and Currah 2005) and Trichoderma (Druzhinina et al. 2006) species. In an effort to obtain fungal growth compatible with microplate technologies, the filamentous fungus Chrysosporium lucknowense was mutated for non-filamentous growth and reduced medium viscosity so that it could serve as a eukaryotic host in high throughput DNA library screening (Verdoes et al. 2007).

To transfer and grow filamentous fungi in a 96-well format, new protocols were needed to adapt the microwell fermentations to the distinctive biology of filamentous fungi, especially those fungi that only grow as hyphae in culture. In the context of secondary metabolite discovery, exclusive dependence on sporulating fungi severely limits the diversity of fungi that can be included in screening experiments. Most culturable fungi, especially those obtained by methods that initiate cultures from vegetative mycelium, sporulate with great difficulty or only form vegetative mycelia in vitro (Bills et al. 2004a; Arnold and Lutzoni 2007; Collado et al. 2007). First, a technique was needed where non-sporulating fungi could be efficiently transferred among microwells as hyphal suspensions. We also assessed whether fungal metabolites could be efficiently extracted from microplates and if metabolite production was adequate for detection of biological activity. A simple screening system was established using heterogeneous collections of 80 fungi grown in 96-well format arrayed across multiple fermentation conditions. To what extent would the application of the OSMAC approach to heterogeneous strain collections improve antibiotic and secondary metabolite detection? To answer that question, the effects of numbers and kinds of media on the detection of biologically active metabolites from fungal extracts were modelled using growth inhibition of Candida albicans as biological readout. The model permitted examination of a long-debated question in microbial screening; should more relative effort be applied to screening more biodiversity or more physiological parameters?

Materials and methods

Equipment

The System Duetz (SD) is a set of tools that was developed at the ETH, Zurich, Switzerland for the parallel preservation and cultivation of sets of bacterial and yeast strains in 96- or 24-well microplates (Duetz et al. 2000, 2001; Minas et al. 2000). A detailed description of its components and protocols for cultivation and preservation of bacteria and yeasts can be found at http://www.enzyscreen.com. Culture aeration, biomass and secondary metabolite production of several bacteria and actinomycete species with the SD have been shown to be comparable with that of shake-flask cultivation (Minas et al. 2000; Duetz and Witholt 2001).

Multichannel pipettes or a Multidrop Combi (Thermo Fisher Scientific, Inc., Waltham, MA, USA) were used to fill sterilized deepwell (2·5 ml well<sup>−1</sup>) 96-well plates. Kühner environmental chambers (ISF-4-V) equipped with inclinable (approximately 75°) tubes racks (Kühner SM317025, 25 mm diameter, 16 tubes per rack) were used to incubate and agitate inoculum tubes. Culture manipulations, master plate preparation and plate replications were carried out in biosafety cabinets. Transfer Tubes (Spectrum Laboratories, Rancho Dominguez, CA, USA) were used to fill individual wells of inoculum master plates with hyphal suspensions. Extraction solvents were added to and removed from individual well cultures with multichannel pipettes or automated liquid handling stations (e.g. Quadra 96; Tomtec, Hamden, CT, USA or Biomek FX; Beckman Coulter, Nyon, Switzerland).

Generation of hyphal suspensions for master plates

Fungi used to prepare hyphal suspensions were grown 2–3 weeks in 60-mm Petri dishes containing YM agar (Fluka or Difco malt extract 10 g, Difco yeast extract 2 g,
agar 20 g, distilled H₂O 1000 ml). Strains were selected based on differences in culture morphology, and in some cases microscopic characteristics, and were judged to represent different species (Bills et al. 2004a; Okuda et al. 2005). The technique of presorting large numbers of isolates after growth under standard conditions and comparison of their morphological features, followed by consolidation of the populations so that selected strains approximate meaningful species, has been corroborated by independent phylogenetic analysis of ribosomal DNA sequences (Arnold and Lutzoni 2007; Collado et al. 2007). Species that formed dry air-borne spores (e.g. Aspergillus spp.) or species with aggressive and fast radial growth (e.g. Trichoderma spp.) were avoided unless noted otherwise. Strains were sequentially numbered and data (e.g. geographic origin, substratum, strain number) were recorded in spread sheets. An adjacent column of the spread sheet indicated the master plate number and well coordinates which linked subsequent manipulations of extracts and assays results to the master plate.

Three to four mycelial discs were cut from each 60-mm plate with a sterile Transfer Tube. Mycelia discs were extruded from the Transfer Tube and crushed in the bottom of tubes (25 × 150 mm) containing 8 ml of SMYA medium (Difco neopeptone 10 g, maltose 40 g, Difco yeast extract 10 g, agar 4 g, distilled H₂O 1000 ml) and two cover glasses (22 mm²). Tubes were agitated on an orbital shaker (200 rev min⁻¹, 5 cm throw), and rotation of the cover glasses continually sheared hyphae and mycelial disc fragments to produce nearly homogenous hyphal suspensions consisting of minute hyphal aggregates and fine mycelial pellets. Tubes were agitated 4–6 days at 22°C, or occasionally longer for slow-growing species. Although growth varied among strains, most exhibited moderate to dense growth to the unaided eye. Microscopic inspection revealed that many tubes that seemed to grow sparsely contained a dense suspension of hyphal fragments and aggregates. After growth, tubes that seemed contaminated, that did not grow, or that were too densely overgrown to pipette with a transfer tube were eliminated. Eighty strains were selected for loading of each master plate.

Master plate preparation

The master plate held the hyphal inoculum in a 10-column × eight-row pattern for replication. The master plate consisted of a sterile 96-square deepwell plate with thick walls and a lid with sealing rings (F378600001, F378600005; Bel-Art, Pequannock, NJ, USA) so that the plate could be frozen to provide a backup culture. The inoculum tubes were arranged to correspond to a numbered spread sheet and the central 80 positions (columns 2–11) in the master plates. Columns one and 12 were reserved for assay controls and media blanks. Approximately 1 ml of hyphal suspension from each tube was transferred to its corresponding well with a Transfer Tube. Simultaneously, 1-ml aliquots of each hyphal suspension were transferred to cyrovials with 20% glycerol and frozen at −80°C for later use. Once loaded, the master plate was covered with its sterile lid until ready for replication.

Preparation and inoculation of media for growth and metabolite production

Media formulations were selected from a proprietary database of microbial fermentation media maintained at Merck Research Laboratories. Media included in nutritional arrays were selected to represent a range of simple and complex carbohydrates or glycerol as carbon sources, and inorganic, amino acid, or complex nitrogen sources. Media also varied with respect to osmolarity, phosphate levels, micronutrient supplements, and the addition of buffers. Solid-state, semi-solid and liquid media were included in arrays, although liquid media were generally preferred to better take advantage of automated liquid dispensing.

Liquid and semi-solid media were prepared in 100-ml aliquots in Erlenmeyer flasks. Of the aliquot, 80 ml was used to fill the 80 central wells of the growth plates with 1 ml of medium. Media formulations with soluble components were dispensed into plates with a Multidrop Combi. Media with complex insoluble components (e.g. cornmeal, tomato paste) were dispensed into plates with a multichannel pipette with wide-bore tips while the medium was mixed with a magnetic stirrer. Media were usually dispensed into the plates the day before inoculation, and the plates refrigerated upright at 4°C until inoculated.

Seed-based solid media were prepared by filling a microtiter plate with 200-μl wells with seeds (wheat, rice, millet, cracked corn) and levelling the seed depth to the tops of the wells. The open wells of an inverted growth plate were aligned on top of the seed-containing microtiter plate, and two plates were rapidly flipped, so that a measured seed volume fell into each well. A liquid basal medium (700 μl) was added to the seeds; the plates were closed with the SD’s sandwich lids, and the assembled plates were autoclaved.

The SD’s cryoreplicator pin tool was sterilized by immersing the pins in 70% ethanol for 1 min and then evaporating the ethanol on a hotplate. The cooled cryoreplicator pins were lowered several mm into the hyphal suspensions of the master plate and then raised. The master plate was switched for a medium-filled growth plate,
and the inoculum-covered pins were lowered to the bottom of the liquid medium. The growth plate was switched again for the master plate, and the inoculation was repeated three to five times. Repeated inoculation from the master plate ensured that sufficient fungal cells were transferred to initiate growth. The process was repeated again for each different medium plate. Sterilization of the cryoreplicator during multiple transfers from the same master plate was unnecessary because the cryoreplicator press prevented accidental lateral movements, and the inoculum source for each well remained constant. Cyroreplicators were changed and sterilized between consecutive inoculations of master plates.

The clamps included in the SD that pressed the sandwich cover onto growth plates and attach them to shaker platforms were awkward for static incubation, and therefore were not used. Instead, plates were incubated statically at about an angle of 85° (Fig. 1a–c). Inclined incubation increased the surface area of the liquid media and improved aeration. Plates were incubated for 2- to 3-week growth cycles at 22°C. During early experiments, the sandwich covers were held in place on growth plates with heavy rubber bands (Fig. 1c). A second generation closure used modified carpenter’s wood clamps to clamp lids securely to growth plates (Fig. 1b). Finally, stainless steel plate holders that simultaneously clamped plates shut with two lateral wingnuts and supported them at an angle of 85° were custom built (Fig. 1a).

After inoculating the medium plates, Omnitray plates (Nunc; Thermo Fisher Scientific Inc.) filled with YM agar were inoculated from the same master plate (Fig. 2) and incubated at 22°C. A second Omnitray plate filled with Luria broth (Becton Dickinson, Le Pont de Clax, France) agar was inoculated and incubated at 28°C to check for bacterial contamination. Replication of master plates onto agar verified that each well contained viable inoculum and that strains were not contaminated (Fig. 2). After inoculation, 500 µl of sterile glycerol was added to each well of the master plate. Plates were closed, agitated briefly, wrapped in aluminum foil, and frozen at −80°C.

Extract preparation

After growth, plates were opened and inspected for contamination. The sandwich cover’s 96-hole silicone mat was replaced with a solid silicone mat, and the breathable cotton layer was removed. One millilitre of solvent (acetone or isopropanol) was added to each fungal culture. Mycelia adhering to the well walls were gently dislodged, and mycelial masses were crushed and mixed with the solvent with the cryoreplicator tool. Sandwich covers with the solid silicone mats were reassembled and clamped on a shaker board and agitated for 1 h. After about 30 min, the shaker board was reversed to change the direction of agitation. Finally, plates were centrifuged for 5 min to settle mycelia to the bottom of the wells.

A total of 900 µl of the acetone- or isopropanol-medium supernatant from each well was transferred to 2-ml wells of a recipient plate. To minimize metabolite
precipitation during solvent evaporation, 100 µl of DMSO was added to the 900 µl of the culture extract. The culture–solvent–DMSO mixture was reduced to 50% of its original volume under a stream of N₂ from a 96-tip manifold or by vacuum evaporation. After solvent removal, approximately 500 µl of aqueous extract with 20% DMSO remained per well, and extracts were stored at −20°C until assayed. Extract plates were thawed and briefly shaken on a plate mixer prior to assay.

**Antifungal assay**

Antifungal susceptibility was tested with the strains *C. albicans* MY1055 or MY2369 from the Merck Culture Collection. Thawed stock inoculum suspensions from *C. albicans* MY1055 (strain MY2369) and cerulenin and helvolic acid production in *S. oryzae* were separated with a Hypersil BDS-C₁₈ cartridge column (3-µm particles, 4 × 100 mm, Agilent) maintained at 40°C. The column was eluted at 1 ml min⁻¹ of a gradient of 10% v:v acetonitrile in water to 100% acetonitrile over 30 min followed by 5 min at 100% acetonitrile. Both elu-

The hypothesis that metabolite titers in microplate fermentations were comparable with those in Erlenmeyer flask fermentations was tested by monitoring brefeldin A production by *Eupenicillium brefeldianum* (strain F-146,140) and cerulenin and helvolic acid production in *Sarocladium oryzae* (Bills et al. 2004b) (KF-140). For both fungi, inoculum was grown for 4 days in tubes of SMYA medium with cover glasses as described above. For *E. brefeldianum*, inoculum (0-5 µl aliquots) was added to flasks with 50 ml liquid YES medium (Frisvad and Samson 2004) (sucrose 150 g; yeast extract 20 g; MgSO₄·7H₂O 0-5 g; CuSO₄·5H₂O 0-005 g; ZnSO₄·7H₂O 0-01 g; distilled H₂O 1000 ml). For *S. oryzae*, inoculum (0-5 µl aliquots) was added to flasks with 50 ml liquid GGP medium (Iwai et al. 1973) (glucose 10 g; glycerol 30 g; peptone 5 g; NaCl 5 g; CaCO₃ 3 g; distilled water 1000 ml). Flasks of liquid YES and GGP were agitated at 220 rev min⁻¹. Microplate fermentations for each fungus were prepared by dispensing 1 ml of medium per well in the three central rows of 12 wells. Each well was inoculated by dipping the tips of a multichannel pipette into the liquid inoculum and then submerging the tips in the wells. Fermentations were incubated at 22°C with 12 h fluorescent light per day.

At day 14, 50 ml of isopropanol was added to each of three replicate flasks. The flasks were agitated with solvent for 1 h. A 1-ml aliquot was removed from each flask. Each of the three rows of the microplate was treated as a replicate sample. One millilitre of isopropanol was added to the three replicated central rows of microplates. The solvent and mycelium were mixed and broken apart with the tips of the pin tool and agitated for 1 h. Aliquots of 60 µl of extract were removed from each well, and the aliquots pooled for each row. All extracts were evaporated to dryness in a stream of N₂, and the residue reconstituted in methanol at 1:5× for *E. brefeldianum*, and 3× for *S. oryzae*. Metabolite titers were evaluated by reverse phase HPLC coupled to a diode array detector (Agilent 1100). Reconstituted methanol extracts (10-µl injections) were separated with a Hypersil BDS-C₁₈ cartridge column (3-µm particles, 4 × 100 mm, Agilent) at 40°C. The column was eluted at 1 ml min⁻¹ of a gradient of 10% v:v acetonitrile in water to 100% acetonitrile over 30 min followed by 5 min at 100% acetonitrile. Both elu-

To confirm that antifungal metabolites would be detectable in microplate fermentations, well-characterized antifungal-producing fungi were grown in microplate fermentations and their extracts were tested against *C. albicans*. Eleven fungi that had been previously determined to produce different structural classes of antifungal metabolites were selected. In each case, the antifungal component of their extracts had been previously isolated by bioassay-guided fractionation and their structure determined by high resolution MS and/or NMR experiments in the Department of Natural Products Chemistry, Merck Research Laboratories, Rahway, NJ, USA. The fungi, their strain numbers, and their expected antifungal metabolites were: *Moria nigrosporeoides*, (F-090,354), morini-

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Monocillium sp., (F-210,948) furanocandin; Xylaria sp., (F-212,836); xylarin; Leiotheicum sp., (F-215,757), asperochlorine; Paecilomyces lilacinus, (F-236,792), leucinostatin; Glarea lozoyensis, (F-239,379) pneumocandins; Favolaschia pustulata, (F-242,456), 9-methoxystrobilurin K; Monascus purpureus, (F-242,596), lovastatin; Paecilomyces inflatus, (F-253,279), BE-49385; and Emericella rugulosa, (F-173,113), echinocandin B and sterigmatocystin.

Structures of the principal antifungal metabolites are depicted in Fig. 5 and information on each can be found in the Dictionary of Natural Products (Buckingham 2007), except for sesterterpenoid metabolite BE-49385 (Kushida et al. 1999). The eight rows of a microplate (A–H) were filled with a different medium selected from our database of fermentation media; the fungi were arrayed across 11 columns (2–12) of a microplate so that each fungus was grown in eight media (Fig. 6a,b). The same master plate was also replicated onto plates filled with single media to confirm the results of the eight media by 11 fungi array. Plates were extracted with acetone and 2- and 5-µl aliquots of the water-DMSO extracts were assayed against C. albicans strain MY1055 in alternating columns of Omnity plates. The plates were prepared in triplicate. ZOIs were recorded and measured by image analysis (Fig. 6).

Effects of increased number of media on screening efficiency and detection of antifungal activity

How much effort was needed to profitably test different media against each strain was unclear. In a preliminary simulation, cost-effective numbers of conditions were judged by inoculating fungal sets onto 12 different media weekly over a 6-week period. Twelve media were selected from the database and were held constant during 6 weeks. Each week, 80 new fungi selected from our fungi isolation program were used to prepare a master plate and inoculate the 12 media. Fermentation plates were incubated 2 weeks, extracted with isopropanol, and aqueous extracts in 20% DMSO were prepared. Each week, 1888 extracts were tested against C. albicans MY1055. ZOIs >7 mm were recorded by image analysis. Sample-based accumulation curves were plotted from the positive strain-by-eight media matrix for each week to visualize how the size of the medium array influenced detection of antibiotic signals.

The mean number of active strains for one to eight media derived from 11 weeks of screening and a theoretical percentage of expected active strains for one to eight media were calculated. The theoretical percentages were used to predict the relative contributions of screening more strains or more media towards the yield of antibiotic signals within a finite number of screening experiments.

Results

Inoculation and growth in 1-ml cultures

We tested heavily sporulating and predominantly non-sporulating fungi with the protocols suggested for unicellular organisms (Duetz et al. 2000, 2001). Dense conidial suspensions of two sets of 90 sporulating fungi, mostly Penicillium, Aspergillus, and Fusarium species, were prepared in 10% glycerol, and 1-ml aliquots were frozen in individual wells of a master plate at −80°C to provide stock cultures for replication. Plates frozen for 9 weeks were sampled with the cryoreplicator, and the replicator tips were pressed onto YM agar plates for one to five times. Efficient replication from spores was achieved with 92–100% of strains successfully transferred to the agar. Frozen hyphal suspensions, however, replicated poorly with only 30–45% of the strains transferable to agar surfaces. When unfrozen fresh hyphal suspensions were loaded in the master plate and replicated three to five times directly to agar, transfer efficiencies of 95–100% of the strains were achieved (Fig. 2). Therefore, fresh unfrozen hyphal suspensions were used for replication of sets for fungi onto multiple fermentation media.

Microplate cultures were not agitated, because many fungi completely filled wells in a few days making agitation ineffective. Plates were therefore incubated statically.
Gas exchange was potentially growth limiting in static cultures; therefore, the plates were inclined at a near vertical (85°) angle to improve aeration (Fig. 1). Inclination of plates caused fungi in individual wells to grow as wedge-shaped mycelial masses. Often, and depending on the medium composition, culture surfaces exhibited elaborate morphological differentiation comparable with that of agar cultures or solid state fermentations on seed-based or vermiculite-based media. Differentiation was manifested in natural and sometimes intense pigmentation, pigmented exudations and production of complex reproductive structures, e.g. stromata, pycnidia, and ascomata (Fig. 3).

Cross contamination of strains between adjacent wells occurred rarely, if at all. In nearly all cases when contamination occurred, its source was from the inoculum tubes. Usually, such contamination could be detected by replicating the master plate onto agar media that promoted fungal or bacterial growth (e.g. Fig. 2).

**Scale down of antifungal-producing strains in microculture wells**

*Comparison of antibiotics between microplates and flasks*

Production of brefeldin A was assessed in agitated Erlenmeyer flasks with liquid media and in 1-ml microcultures of *Eupenicillium brefeldianum* at day 14 (Fig. 4a). Inspection of HPLC chromatographs indicated brefeldin A production was greater in liquid culture, but qualitatively, the metabolite profiles from microwell fermentations resembled those from agitated liquid cultures (Fig. 4a). In *Sarocladium oryzae*, titers of cerulenin and helvolic acid between agitated liquid cultures and those in static 1-ml fermentations were similar (Fig. 4b). However, extracts from 1-ml fermentations exhibited a more complex metabolite profiles because of a series of peaks eluting between 20 and 30 min.

**Scale down of antifungal-producing strains in microplates**

All of the 11 characterized producers of antifungal antibiotics produced ZOIs on at least one medium at one of the two concentrations tested. We could not discount the possibility that some antifungal activity in some of the conditions may have been due to metabolites other than the principal fungal metabolites. Some of fungi, e.g. *Moriaria pestalozzioides* (Fig. 6a, column 2), *Glarea lozoyensis* (Fig. 6a, column 8) and *Emericella rugulosa* (Fig. 6a, column 12) produced antifungal metabolites on most of the media set. Extracts of *Monascus purpureus* (Fig. 6a, column 10) were only weakly active, and hazy ZOIs were evident on most media. Other strains, e.g. *Arthrinium arundinis* (Fig. 6b, column 3), *Monocillium sp.* (Fig. 6a, column 4), *Xylaria sp.*, (Fig. 6b, column 5), *Leiothecium sp.* (Fig. 6a, column 6), *Paecilomyces lilacinus* (Fig. 6b, column 7), *Favolaschia pustulata* (Fig. 6b, column 9) and *Paecilomyces inflatus* (Fig. 6b, column 11) showed medium-specific production of antifungal activity. When the same set was replicated onto plates of a single media, ZOIs of inhibition were usually consistent among the eight wells of a single column (data not shown). The occasional well-to-well inconsistencies of ZOIs could be attributed to variable inoculation of wells with very slow-growing species, e.g. the basidiomycete, *Favolaschia pustulata*, or a sporadic failure of extracts to be released from pipette tips onto assay plates.

**Effects of increased number of media on antibiotic detection**

During small- and large-scale simulations of a microbial screening program, expanding the medium array for any given strain increased the probability of detection of ZOIs against *C. albicans* (Figs 7, 8). In the preliminary 6-week screening, the extracts of 80 of 480 fungi grown on 12 media (16.7%) exhibited growth inhibition of *C. albicans*. Interpolating from the mean of the rarefaction curves, only 44 (9.2%) of the same strains would have yielded an antifungal signal in a three-medium screening regime (Fig. 7). The mean number antifungal signals from an 80-fungus set increased from 7-4 with three media to 13-1 with 12 (89% increase). Inspection of the slopes indicated that the rate of detection of new antifungal signals tended to decrease past six to eight media in some sets of fungi. Therefore, to reconcile concerns...
about excessive media manipulation relative to input of strain diversity, panels of eight media were applied to larger screenings of fungi.

Application of eight media to a large screening population (2586 strains) similarly enhanced the detection of antibiotic signals against *C. albicans* (Fig. 8). A total of 515 strains (19.9%) produced at least one extract with anti-*C. albicans* activity, of which 218 strains (8.4%) were active only in one medium. In contrast, only five strains (0.2%) were active across all of their respective sets of
eight media. The mean number antifungal signals from a 236-fungus set increased from 19 ± 6 strains with three media to 32 ± 9 with 12 (84% increase). Inspection of the slopes indicated that the rate of detection of new antifungal signals would continue to increase with more than eight media for most weekly sets of fungi.

Figure 5 Structures of known antifungal compounds produced by characterized fungi used to validate 1-ml fermentations (see Fig. 6). Note that some fungi may produce more than one other compound from the same family or other antifungals. (a) Moriniafungin from Morinia pestalozzioides (b) Arundifungin from Arthrinium arundinis. (c) Furanocandin from Monocillium sp. (d) Xylarin from Xylaria sp. (e) Aspirochlorine from Leiothecium sp. (f) Leucinostatin A from Paecilomyces lilacinus. (g) Pneumocandin A0 from Glarea lozoyensis. (h) 9-methoxy-strobilurin K from Favolaschia pustulosa. (i) Lovastatin from Monascus purpureus. (k, l) Sterigmatocystin and echinocandin B from Emericella rugulosa. (j) BE-49385 from Paecilomyces infilatus.
Detection of new antifungal signals not only depended on the number of media tested but also on the species composition of the fungal screening sets as evidenced by differences in the slopes among the six 80-strain sets tested in the preliminary screening and the 11-week screening of 236-strain sets. In some of the preliminary sets (Fig. 7), the discovery curves reached their asymptote within six media (e.g. week 22-March-2005), while in other weeks (e.g. 29-March-2005), detection of new antifungal signals continued increasing up to 12 media. Differences among weeks in the large-scale screening were more pronounced. Not surprisingly, the two sets that were enriched based on previous observations of *C. albicans* or *Aspergillus fumigatus* activity (Fig. 8, weeks 200622 and 200623) exhibited the highest rates of antibiotic phenotypes. Up to 49% (115) of strains grown on eight media (Fig. 8, week 200622) exhibited antibiotic activity towards *C. albicans*. At the other extreme, only 19 strains gave antibiotic signals among eight media during week 200611.

Interpolation of data from the mean of the rarefaction curves of the 11-week period supported the idea that both phylogenetic diversity of strains and multiplicity of growth conditions impacted discovery rates (Table 1). For example, assuming a capacity to produce and screen 3200 extracts, 400 strains could be grown on eight media with a predicted yield of 55Æ8 antifungal signals, while 800 strains grown on only four media would yield 78Æ4 antifungal signals (Table 1). However, doubling the number of new fermentation conditions with the SD involves

![Figure 6](image_url)

**Figure 6** Detection of antifungal activity from known antifungal-producing fungi grown in 1-ml volumes in microplates. Omnitray assay plates were seeded with *Candida albicans* MY1055. Amphotericin B is in position 1b and kanamycin in 1g. Five-microlitre aliquots from extracts applied to assay plate. To prevent fusing of ZOIs, extracts from the single growth plate were applied in alternating columns on two different assay plates. (a) Extracts applied to even-numbered columns. Organisms and their products are listed in legend. (b) Extracts applied to odd-numbered columns. Organisms and their products are listed in legend. Each of rows a–h is a different fermentation medium. Codes for each medium are listed in the legend.

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negligible effort compared to the work needed to collect, isolate and establish double the number of new fungal strains for screening.

Discussion

Fungi regulate their metabolite synthesis, probably to ensure that the energy and precursors used in their synthesis are expended only in the environments where the metabolites are advantageous. In other cases, synthesis may be regulated globally or tightly coordinated with growth and developmental stages (Hoffmeister and Keller 2007). It is also evident among all fungal genomes within the Pezizomycotina studied to date that the number of presumed secondary metabolites gene clusters exceeds the number of metabolites detectable in a given species (Scherlach and Hertweck 2006). In the absence of information on gene regulation among the seemingly infinite numbers of filamentous fungi, empirical manipulations are the most direct option for inducing changes in secondary metabolite expression during primary screening (Gross 2007; Schroeder et al. 2007). Exhaustive manipulation of medium and growth conditions to better interrogate the biosynthetic capacities of micro-organisms has been proposed as an alternative to traditional microbial screening paradigms that ferment many strains in limited numbers of flask or tube fermentations (Bode et al. 2002; Tormo et al. 2003).

Our experimental model illustrated the impact of nutritional manipulation on fungal antibiosis; however, it is easy to imagine the empirical manipulation of other culture parameters. Varying fungal medium by addition of complex plant–host-derived components (Aldred et al. 1999; Overy et al. 2006), using solid supports to promote cell differentiation (Barrios-Gonzales and Mejia 1996; Nielsen et al. 2004; Bigelis et al. 2006), and addition of metabolic inhibitors (Christian et al. 2005; Hanlon 2006) can further stimulate product diversity. Likewise, temporal changes in metabolite profiles and appearance of ephemeral metabolites can be exploited by replicating sets and extracting them at different times. A consequence of this approach is that often the majority of strains will be active in only one among several media (Yarbrough et al. 1993; Wildman 1997; Tormo et al. 2003; Scherlach and Hertweck 2006; Schroeder et al. 2007). That same pattern, where strains were more often active on one or a few media, was observed in both the preliminary and large-scale screening simulations in this study, and illustrates the importance of microbial nutrition to the outcomes of antibiotic screening.

Instead using analytical methods to discriminate among extracts derived from differential fermentations (Tormo et al. 2003; Scherlach and Hertweck 2006; Schroeder et al. 2007), eukaryotic cell perturbation with C. albicans was used as an indirect measurement of bioactive metabolite production. Although binary ZOI data lacks the information content of analyses of HPLC-UV spectra, HPLC-MS spectra or NMR-spectroscopic data which could guide selection of new metabolites, the ZOI data focuses attention on the biologically active components. A ZOI assay with whole microbial cells, e.g. C. albicans, not only has the potential to detect antifungal metabolites from multiple structural classes, but also cell-penetrable molecules that directly inhibit intracellular enzymes or modulate intracellular signal induction or transcription (Yim et al. 2008).
2006; Xu et al. 2007). Multiple metabolites and antibiotics are often produced in the same fermentation, and the effects of toxic or reactive metabolites may mask detection of unique antibiotics. Such masking effects can be especially confounding when screening with whole-cells assays, e.g. ZOI assays with unfractionated extracts, and when the only criterion for selection of actives is potency. Therefore, a potential positive outcome of the OSMAC approach is that a growth condition may be found that represses toxin formation relative to production of a unique bioactive metabolite, thus allowing its detection in a target-specific assay able to discriminate among multiple bioactive molecules. Growth on multiple media from a single inoculation source also builds in a degree of reproducibility that aids in interpretation of screening results (Fig. 6). Multiple active extracts originating from the same culture are likely real and repeatable actives. On the other hand, actives resulting from a single growth on a single medium may point to potentially unique metabolites or exceptional medium-organism combinations (Scherlach and Hertweck 2006; Schroeder et al. 2007).

We recognized that OSMAC and the application of multiple fermentation conditions were general methods that could enhance the probability of successful discovery from a given strain. The results of this study are the first to demonstrate that OSMAC can be carried out on large screening populations of fungi; in our model, OSMAC increased the probability of detecting antifungal phenotypes. However, the screening paradigm described in our study may only be suitable for metabolite screening in specific situations. An obvious application would be to generate large numbers of extracts of organisms under varied fermentation parameters for preliminary assays for antibiosis or cytotoxicity. Those organism and medium combinations yielding extracts with a minimum potency and activity spectrum could be scaled up to provide larger fermentations suitable for further processing for an extract library or for chemical fractionation. Such an enriched extract library would be especially appropriate for screening paradigms seeking cell-penetrable molecules with whole-cell bioassays. In our experience thus far, scale-up from 1-ml to 100-ml or even to the liter scale has been relatively straightforward, and to date, we have successfully scaled up sufficient quantities for isolation and structure determination of known, as well as new, fungal metabolites. A single organism can be repeated in 96 wells filled with the same growth medium to yield nearly 100 ml of culture. In an effort to move a fermentation process to a more conventional vessel, the fermentation can be repeated in statically incubated Erlenmeyer or Fernbach flasks. When multiple media from an organism yielded activity, then multiple starting points were available for scale-up.

Parallel microfermentations of bacteria and yeasts have gained widespread acceptance, but few precedents suggested that growth and product formation from filamentous fungi in microplates would be effective. Most of the available protocols have employed fresh conidial suspensions to inoculate plates for profiling substrate utilization or screening for enzymatic activity. The best known microplate systems for fungi are the Biolog plates (http://www.biolog.com) for determining patterns of carbon and nitrogen utilization. For example, growth characteristics of Hypocrea jecorina were comparable among agar, shake flask and the Biolog Phenotype Microarray system (Druzhina et al. 2006). A microplate growth system has been applied to screening soil fungi for organic acid production, but protocols were not available (Magnuson et al. 2003). Mycotoxin and secondary metabolite production are routinely evaluated from agar growth in Petri plates (Smidsgaard 1997; Frisvad and Samson 2004). The successful application of agar plug microextraction techniques to fungi suggested that secondary metabolite production in small scale static fermentations would be feasible.

<table>
<thead>
<tr>
<th>Number of media screened</th>
<th>Strains screened</th>
<th>Mean actives from 11 weeks</th>
<th>Percent active (%)</th>
<th>Actives from 400 strains</th>
<th>Actives from 800 strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>236</td>
<td>9:3</td>
<td>4</td>
<td>158</td>
<td>31:6</td>
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<td>25:8</td>
<td>51:5</td>
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<td>19:6</td>
<td>8</td>
<td>33:2</td>
<td>66:4</td>
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<td>236</td>
<td>23:1</td>
<td>10</td>
<td>39:2</td>
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<td>236</td>
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<td>11</td>
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<td>32:9</td>
<td>14</td>
<td>55:8</td>
<td>111:5</td>
</tr>
</tbody>
</table>

1 Mean was derived from the number of predicted antifungal zones from the 11 weekly sample-based rarefaction curves.
2 Predicted numbers of antifungal zones projected from screening larger heterogeneous collections.
Use of the SD with filamentous fungi required significant changes to the protocols recommended for bacteria and actinomycetes. Our results with conidial and hyphal suspensions in frozen master plates indicated the SD’s cryo-replication tool could be used with heavily sporulating fungi, but transfer of non-sporulating fungi was unreliable and required a different approach. Inoculation efficiency from the frozen hyphal suspensions was poor because fungal cell density may be very low compared with bacterial or fungal-spore inoculum. To compensate for lack of unicellular propagules, growing hyphae were sheared while agitating in tubes. Suspensions were loaded in a master plate and replicated while fresh. Dilute agar added to the inoculum medium increased viscosity and cell adherence to the cryoreplicator tips. Media were usually inoculated from the master plate three to five times to ensure enough cells were transferred. Glycerol was added and master plates were frozen after inoculation and replication, or inoculum was frozen separately in vials. When microfermentations needed scale-up, frozen inoculum from individual vials and preparation of new inoculum was much more reliable than resampling frozen wells. Unlike microscopic and optical density calibration of unicellular organism density, estimation and density calibration of large numbers of irregular and often highly pigmented hyphal aggregates was impractical. However, uncertain inoculum density is a generally accepted compromise in microbial screening programs where inocula are usually transferred on a volume-to-volume basis.

Growth plates were incubated statically because forces in agitated microwells were insufficient to shear or, in many cases, move dense hyphal tissues. Plates and the liquid media surfaces were inclined to increase area for gas exchange. Because growth was static, culture volume could be increased from 0.5 to 1 ml; the increased volume yielded more culture extract for assay and follow up. Extraction directly from fungi grown in a 96-well footprint facilitated downstream processing and screening steps. Solvents and extracts could be transferred, reconstituted, and aliquotted by 96-well pipetting stations. Solvent removal could be carried out with 96-well N$_2$ manifolds or vacuum evaporation chambers. Assay of extracts in the same footprint visualized relationships and patterns of media effects on expression of activity (Fig. 6).

The microplate fermentations have other physical limitations. If more than 0.5 ml of extract is needed for primary screening, the 96-well format is too small. A microplate is a series of fused fermentation vessels so accidental loss or contamination of a plate results in loss of many data points. Contaminated cultures cannot be removed from the set. The plates are opaque, and because they must remain closed during growth, they can only be assessed at the end of the fermentation. Manual manipulation of one or a few wells was difficult because of the small interwell distances and the potential for cross contamination. Individual wells cannot be subsampled during the fermentation; therefore all strains within a plate must be grown for the same length of time.

Interwell invasion and contamination between strains was infrequent. Admittedly, avoidance of dry-spored conidial fungi, e.g. *Penicillium* species, and aggressive mycoparasites, e.g. *Trichoderma* species, combined with static incubation reduced the possibilities. In our early trials, occasional cross-well invasions were evident when lids were fastened with rubber bands, but screw-tightened steel clamps (Fig. 1a,b) nearly eliminated this problem. Cross-well contamination of extracts may be a concern in downstream processing of extracted wells if pipette drip and splash are not carefully controlled. Addition of control organisms to the screening population, i.e. a species that produced a known potent antibiotic on many media, e.g. echinocandin B from *E. rugulosa* (Fig. 6a), can prevent plate disorientation and act as a quality control for media preparation.

A general protocol that enables microplate transfer and fermentation for nearly the entire spectrum of cultivable filamentous fungi opens many useful directions for future research. Admittedly, our protocol is more labour-intensive than simple well-to-well transfers with unicellular micro-organisms. However, those techniques work poorly with filamentous fungi, and we believe our modified protocol can significantly advance experimentation with these organisms. Small laboratories working with fungi can use the SD in manual mode with manual downstream processing to expand their experimental capacity. The SD makes it possible to carry out numerous preliminary parallel medium preference and product optimization experiments on a small scale, although once promising parameters are identified, they will require further optimization upon scale-up. Finally, the protocols described herein open up new possibilities for growing and screening filamentous fungi in a fully automated mode compatible with liquid-transfer and plate handling systems.

Acknowledgements

We are grateful to Wouter Duetz, Enzyscreen BV, for advice and encouragement. The steadfast and expert technical support of the CIBE staff is greatly appreciated. Some of the fungal strains used in this study were obtained in collaboration with Landcare Research, Auckland, New Zealand, the University of Turabo, Turabo, Puerto Rico, the New York Botanical Garden, Bronx, New York and the Universitat Rovira i Virgili, Reus, Spain.
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