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Application of Biolog FF MicroPlate for substrate utilization and metabolite profiling of closely related fungi

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ABSTRACT

Filamentous fungi are known to have unique biochemical pathways to assimilate a vast array of simple and complex nutrients available to them and to produce a variety of metabolites. Morphological and biochemical uniqueness of these organisms are commonly used for their identification, but differentiation of closely related cultures requires extensive phenotypic and genomic investigations. The Biolog FF MicroPlate was recently introduced for rapid identification of common filamentous fungi based on their abilities to utilize 95 discrete substrates. We used the FF MicroPlate for substrate utilization, growth, secondary metabolite and antimicrobial profiles of some fungal cultures important to our microbial drug discovery program. Culture growth was monitored by change in absorbance in each well, and the presence of secondary metabolites and their corresponding bioactivities was detected by LCMS analyses and antimicrobial assays of the extracts of each well, respectively. Fingerprints were created with Spotfire visualization software, and data were analyzed in various ways. The substrate utilization fingerprints were useful in selecting media components for media optimization of secondary metabolite production for the various cultures. In general, a strong correlation was found among substrate utilization, growth, antimicrobial activity and presence of the responsible secondary metabolites. The method was used for dereplication of isolated fungi and in the differentiation of closely related variants within one species.

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1. Introduction

Fungi are ubiquitous in nature and possess unique biochemical pathways to assimilate a vast array of available substrates and produce unique secondary metabolites, some of which are well-known antibiotics and pharmaceuticals (Keller et al., 2005). Screening for useful fungal secondary metabolites has been ongoing for several decades, but a vast majority of fungal cultures in nature are still unexplored. Frequently, a large number of cultures isolated in the labs are left out of the screening process because they appeared too similar to the ones already in the collection or did not grow fast enough to fit into the current screening paradigm. Occasionally, several variants of a lead culture are isolated during the course of production optimization exercises, and it becomes important to understand their characteristics for various reasons. Morphological and biochemical uniqueness of these organisms are commonly used for their identification, but differentiation of closely related cultures requires extensive phenotypic and genomic investigations.

The Biolog FF MicroPlate, based on the company's Phenotype Array Technology, was recently introduced for rapid identification and characterization of filamentous fungi (FF MicroPlate™ Instruction for Use). The FF MicroPlate contains 95 discrete substrates that are utilized differently by different species leading into a distinct substrate utilization and growth fingerprint (Rice and Currah, 2005; Buyer et al., 2001). We used the Biolog FF MicroPlate substrate utilization, growth, secondary metabolite and antimicrobial profiles of some fungal cultures important to our microbial drug discovery program. Since we did not have access to the automated Biolog MicroStation required for rapid identification of fungal cultures, we attempted to use the plate reader available in the lab to monitor the absorbance change in each well and later prepared extracts from these wells for antimicrobial and LCMS analyses. The method was used for distinguishing closely related cultures and media development. A portion of this work was presented at the annual meeting of the Society for Industrial Microbiology held in San Diego during August 10–14, 2008 (Abstract P79).

2. Materials and methods

2.1. Fungal cultures and growth media

Fungal cultures used in the present study were from the Wyeth Microbial Collection of the Natural Products Discovery Group. All

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cultures were stored frozen at −140 °C in 25% glycerol and were revived on a suitable agar medium such as Potato Dextrose Agar (PDA), Bennet's Agar (BA) or 2% Malt Extract Agar (MEA) when needed. Potato Dextrose Broth (PDB, DIFCO Brand) was commonly used for preparing a liquid broth culture, and alternate media were used on an as needed basis.

2.2. FF–IF broth

FF–IF broth was prepared in a clean borosilicate glass bottle by mixing 0.25% Phytagel (P8169 from Sigma Chemical Co., St. Louis, MO) and 0.03% Tween 40 (P1504 from Sigma) in DI water. The solution was autoclaved for 20 min at 121 °C and stored at room temperature until needed.

2.3. FF MicroPlate

Pre-made FF MicroPlates (catalog #1006) containing 95 different carbon and nitrogen sources were purchased from Biolog (21124 Cabot Blvd, Hayward, CA) and stored at 4 °C until needed.

2.4. Inoculum preparation

Pure cultures from the frozen stocks were streaked onto PDA plates and incubated at 22 °C for 7 to 21 days depending on the culture type. For sporulating cultures, sterile cotton-tipped applicators (Pur-Wraps from Hardwood Products Company, Guilford, ME) were used to collect spores or conida from the agar plates, avoiding carryover nutrients from the agar medium, and spores were suspended into a tube containing 5 ml of FF–IF broth. Absorbance (at 600 nm) was adjusted to approximately 0.20 by adding additional FF–IF broth. For nonsporulating cultures, approximately one-half square centimeter of the fungal mycelia was scrapped from the surface of the agar, avoiding the agar itself, and transferred into a 15 ml sterile centrifuge tube containing 2 ml of FF–IF broth. The mycelia were thoroughly macerated using a spatula or a battery-operated mini-grinder to fragment the mycelia. An additional 5 ml of FF–IF broth was added to the tube, and the contents were gently vortexed and then centrifuged for 5 min at 3000 rpm. Supernatant was discarded, the cells were suspended into 10 ml FF–IF broth, and the tube was placed in a rack to allow settling of bigger mycelial clumps. One to two milliliter from the top of the suspension was used to prepare inoculum with an absorbance of 0.20.

2.5. Collection of absorbance data

A multilabel Victor^{2}V counter (PerkinElmer, 940 Winter Street, Waltham, MA) was used to read the absorbance at 490 nm on a daily or weekly basis depending on the growth rate of the fungal culture under investigation. Data was exported into an Excel spreadsheet for analysis.

2.6. Extraction and sample preparation

At the end of the incubation period, Biolog FF MicroPlates were lyophilized for 24–48 h, and each well was extracted with 100 µl of methanol twice. Methanolic extracts from the six replicate plates of one culture were combined into a deep-well plate, and the contents were chilled to −80 °C and then dried under vacuum using a Speedvac concentrator (Savant Instruments, Holbrook, NY). The dried extracts were then dissolved in 60 µl of DMSO to obtain a 10× solution.

2.7. Biological activities of the extracts

Five microliters of the above extracts were tested for antimicrobial activities by microbroth dilution method as described earlier (Singh, 2006).

2.8. Chemical analysis of the extract

Ten microliters of each extract was analyzed using a Hewlett Packard model HP1100 liquid chromatograph with tandem photodiode array and mass spectral detection with either a Finnigan LCQ ion trap mass spectrometer or LCQ DECA with an ESI probe (ThermoQuest, River Oak Parkway, San Jose, CA).

2.9. Spotfire analyses

Data from the Excel sheet was imported into Spotfire software (Spotfire Inc, Somerville, MA) and then analyzed for growth pattern, substrate utilization, antimicrobial activity and UV peaks for the target secondary metabolites produced. Scatter plots and heat maps were used for easy visualization of the results.

2.10. Medium formulation based on the Spotfire analyses

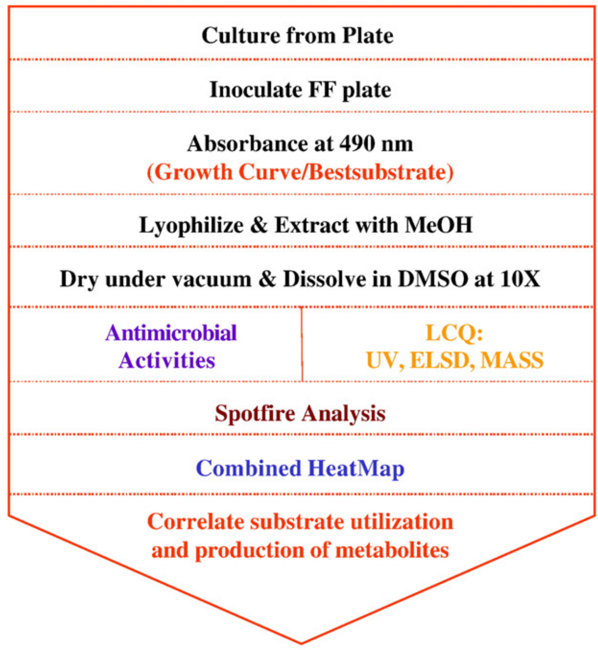
Carbon and nitrogen sources supporting the growth and production of the target compound were used to supplement the basal fungal medium for 100 ml and 1 l fermentations.

3. Results and discussion

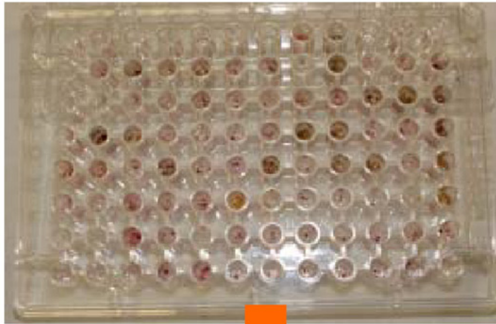
An outline of the steps involved in the profiling of a fungal culture using the Biolog FF MicroPlate is shown in Scheme 1. Freshly grown culture was scraped from an agar plate and macerated to obtain a suspension of fragmented mycelia. A standardized inoculum (A_{590} adjusted to about 0.20) was then inoculated into a set of six FF MicroPlates, and plates were incubated stationary at 22 °C. Growth in each well was observed visually on a daily basis, and absorbance of each well was read daily for fast-growing cultures and less frequently for slow-growing cultures. Visual observation of color and mycelial growth in each well was used to quickly differentiate between cultures and their natural variants. Commonly used 96-well plate readers (Perkin-Elmer or Molecular Devices) provided the data needed to compare and de-replicate closely related fungi of interest. At the end of the incubation period plates, were lyophilized and extracted with 3×200 µl of methanol. Extracts were pooled, dried under vacuum, and reconstituted into 60 µl of DMSO. The resulting extract of each well was tested for antimicrobial activities, and the presence of the target secondary metabolite was confirmed by LC-MS analysis of the extracts (Scheme 1).

The Biolog FF MicroPlate was found to be suitable and convenient for substrate utilization studies of closely related fungi. Two sets of closely related natural variants of cultures 1087B and 309 were compared with three additional cultures in the same genus (Fig. 1). Variants A and B arising from the original cultures of 1087B and 309 were identical by genomic analyses; however, they produced different levels of secondary metabolites of our interest. FF MicroPlate profiling revealed a significant difference between the culture 1087B-A and culture 1087B-B (1st two columns in Fig. 1); however, only a small difference was revealed between culture 309A and 309B. Taxonomically distant cultures in the same genus group showed significant differences in their substrate utilization profiles as depicted in the heat map (Fig. 1).

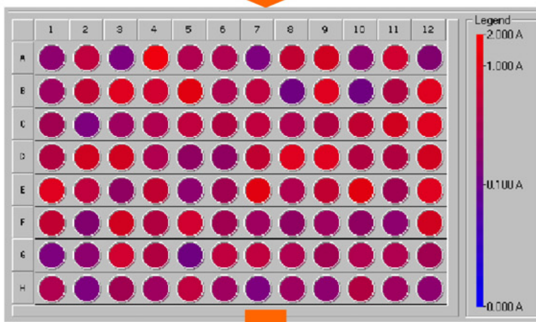
Spotfire analyses of data enabled an easy visualization of various characteristics of the cultures tested. The absorbance data collected



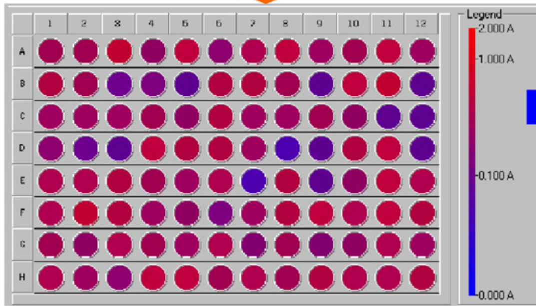
FF Microplate
after 5 wk at 22°C

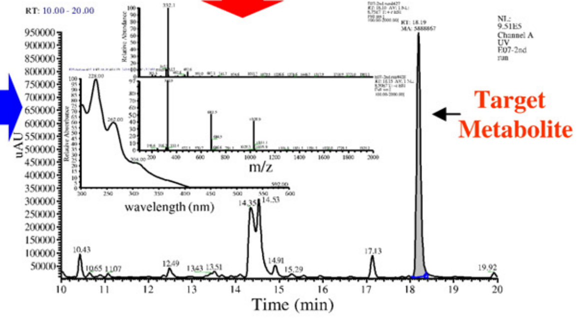


FF Microplate
read on Victor



Inhibition of
C. albicans





Scheme 1. Steps involved in the profiling of fungal cultures using FF MicroPlate.

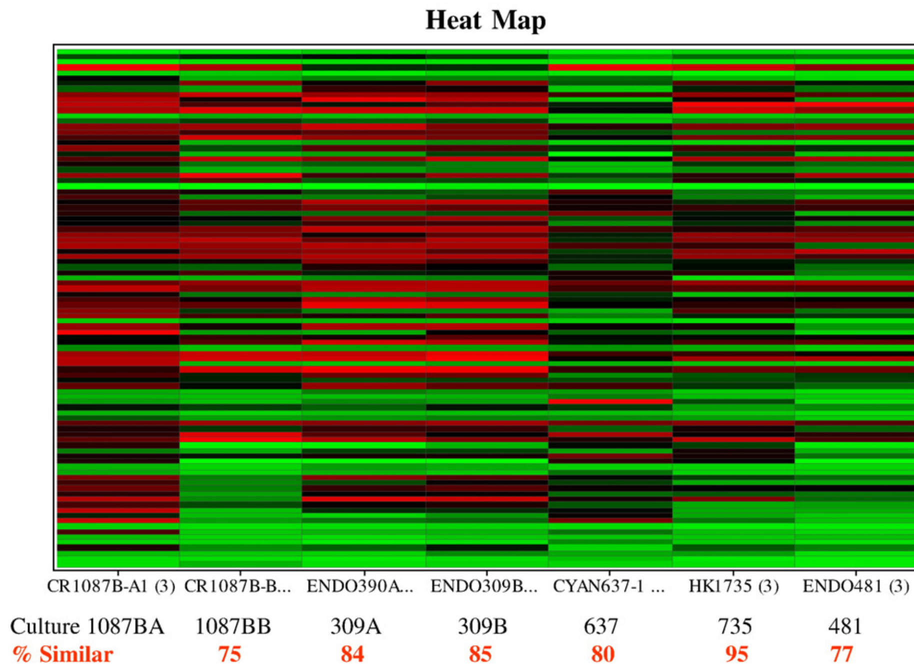


Fig. 1. Comparative heat map of related *Phomopsis* spp. Grown in FF Microtiter Plates. Green to dark red colors are the indication of poor to high growth and substrate utilization by the cultures tested. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

during the course of this study permitted the creation and visualization of substrate utilization profiles and growth kinetics on these substrates (Fig. 2). The culture shown here was slow-growing, and an extended incubation and monitoring was required to visualize its growth kinetics. Further Spotfire analysis of the data set was used to visualize the substrates that supported growth of the culture (Fig 3A), production of antimicrobial activity (Fig. 3B), and the target secondary metabolite (Fig. 3C). Strong correlation was observed between growth, substrate utilization, antifungal activity and the target secondary metabolite produced by

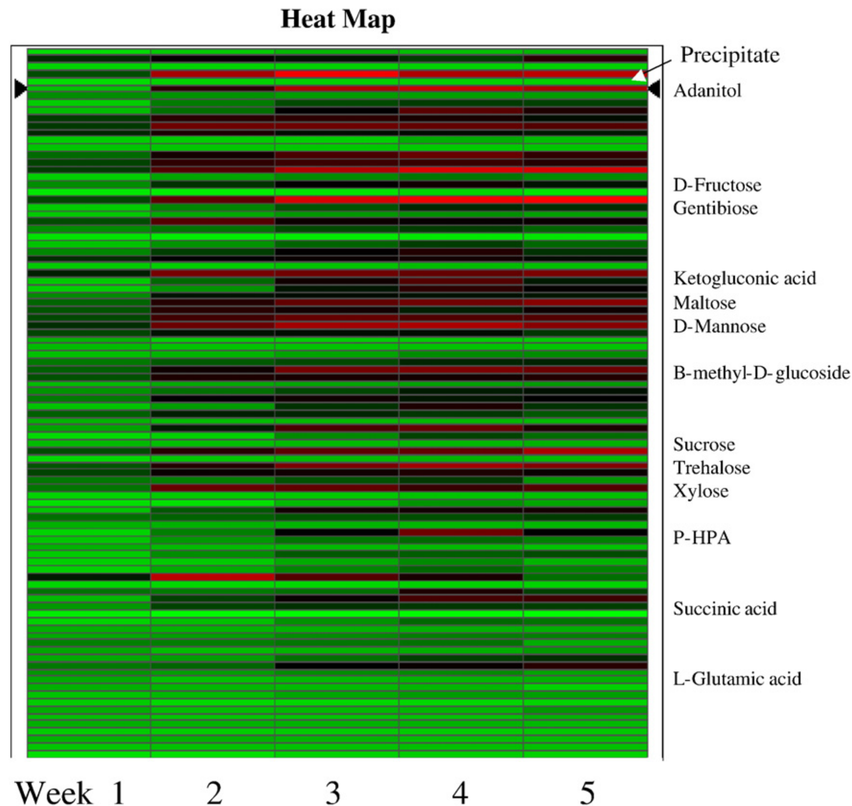


Fig. 2. Growth kinetics of a very slow-growing culture LV-2841 on various substrates.

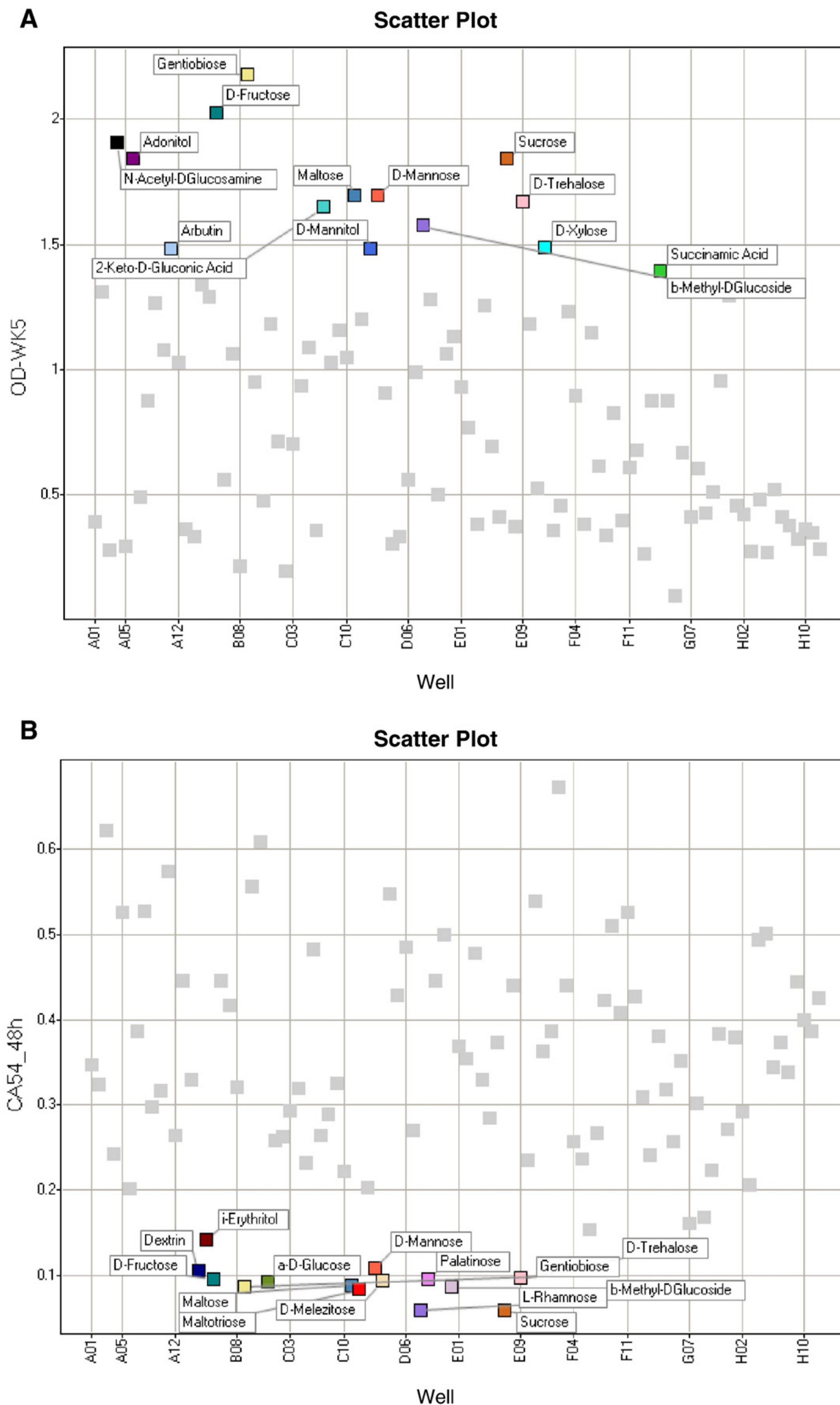


Fig. 3. Spotfire analyses of data obtained for substrate utilization (A), antifungal activity (B) and production of the target compound (C) produced due to various substrates utilized by the fungal culture.

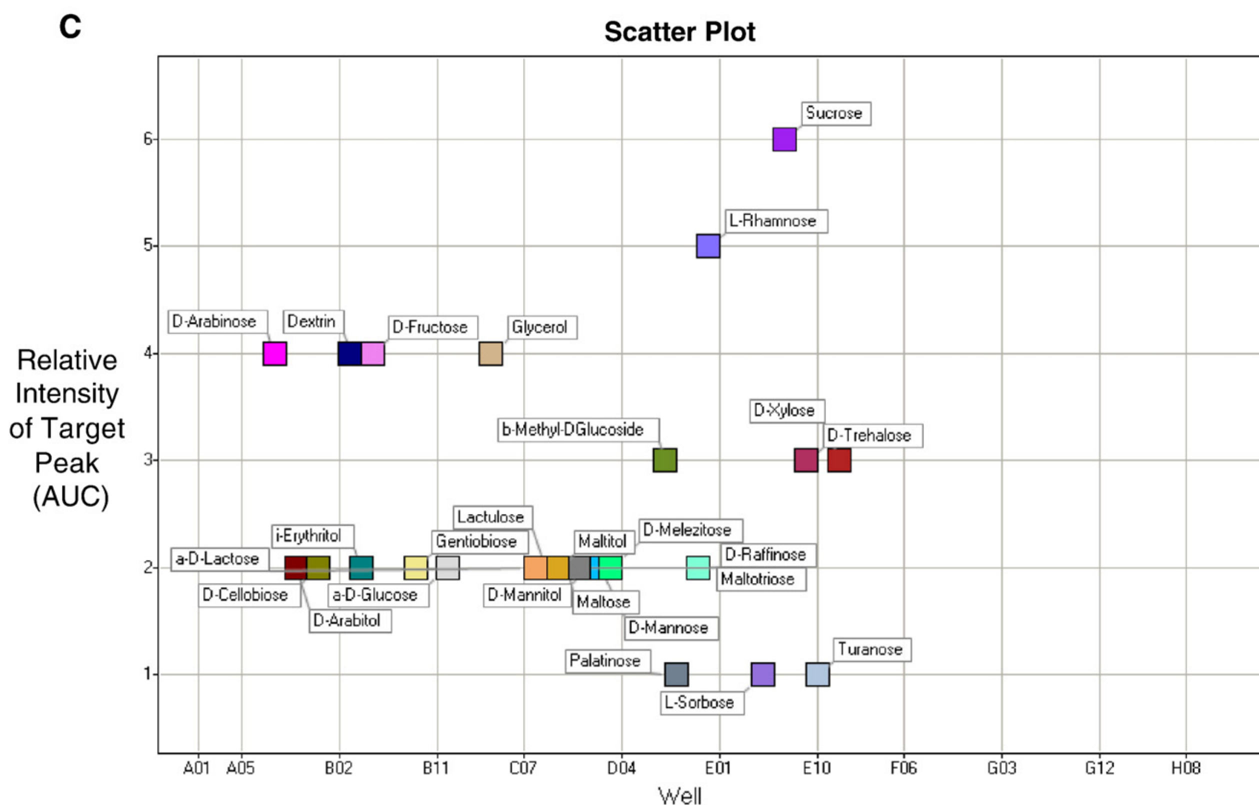


Fig. 3 (*continued*).

the culture (Fig. 4 and Table 1). Some of the substrates tested did appear to support growth but not the production of the antimicrobial metabolite.

In summary, the Biolog FF MicroPlate was found to be suitable and convenient for substrate utilization studies of closely related fungi, and it can be used to dereplicate and differentiate cultures by

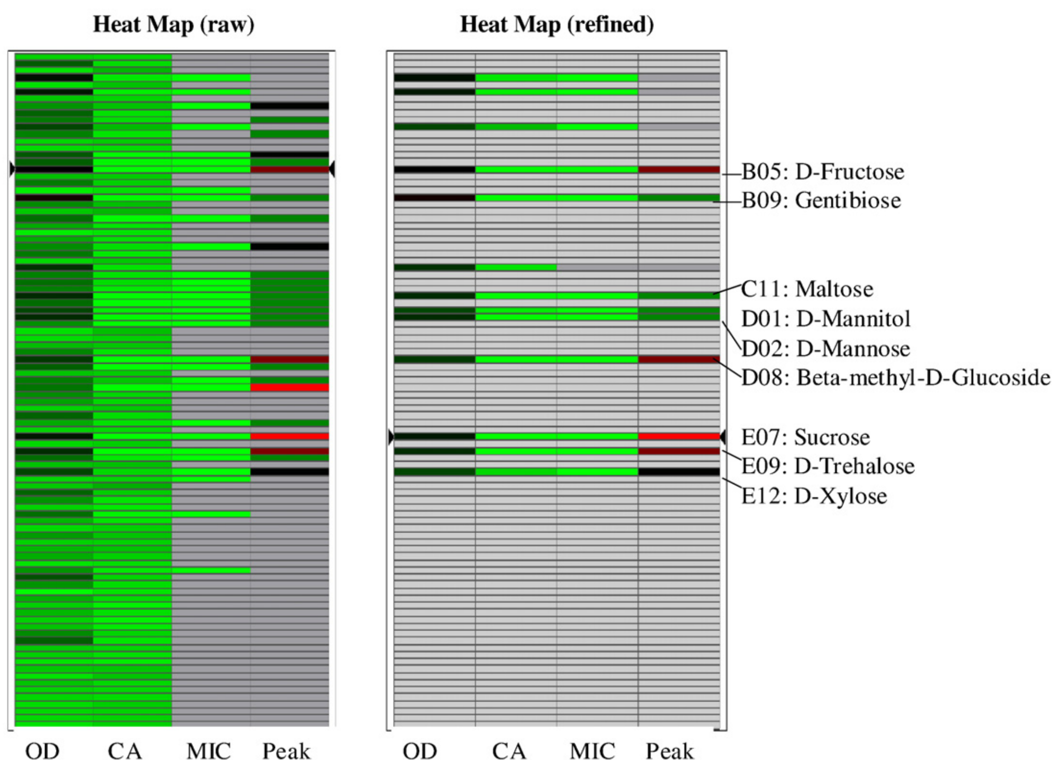


Fig. 4. Combined heat map of all data (OD, absorbance after 5 weeks; CA, absorbance in the bioactivity plate of *C. albicans*; MIC, inhibition of the test organism CA; Peak, UV and mass peak for the target secondary metabolite in the extract).

Table 1
Correlation of substrate utilization, growth, inhibition of yeast, and the presence of target peak in the LC-MS spectra

Well	Substrate	Growth Od_5wk	*C. albicans* inhibition	Visual MIC	Relative intensity of target peak	AUC for Target on DECA
A01	Water	0.394	0.348	No	0	None
E07	Sucrose	1.845	0.059	Yes	4	5,888,867
D12	L-Rhamnose	1.131	0.086	Yes	4	3,287,321
E09	D-Trehalose	1.674	0.097	Yes	3	2,937,421
D08	β-Methyl-d-Glucoside	1.578	0.060	Yes	3	2,853,206
B05	D-Fructose	2.026	0.096	Yes	3	2,845,821
B03	Dextrin	1.341	0.106	Yes	2	1,958,210
C04	Glycerol	0.937	0.320	Yes	2	1,81,611
A08	D-Arabinose	0.876	0.527	Yes	2	1,641,597
E12	D-Xylose	1.488	0.363	Yes	2	1,018,805
C11	Maltose	1.697	0.089	Yes	1	<1 million
C12	Maltotriose	1.201	0.083	Yes	1	<1 million
D01	D-Mannitol	1.482	0.203	Yes	1	<1 million
D02	D-Mannose	1.696	0.108	Yes	1	<1 million
D03	D-Melezitose	0.907	0.094	Yes	1	<1 million
D09	Palatinose	1.282	0.096	Yes	1	<1 million

simple visual observation or by monitoring the development of color and turbidity in the well. Additional biological testing and chemical analysis of extracts prepared from each well were useful in understanding the nature of the culture. Substrate utilization profiles linked to the production of a desired secondary metabolite with a specific biological activity permitted the formulation of fermentation media suitable for higher titers and feeding studies.

Acknowledgments

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