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Combining microdilution with MicroResp™: Microbial substrate utilization, antimicrobial susceptibility and respiration

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ABSTRACT

Pharmacological studies focus on susceptibility of pathogenic microbes against specific drugs or combinations of them, ecological studies on substrate utilization efficiency of variable microbial communities. The MicroResp™ system was especially developed to study soil microbial communities. It was slightly modified to facilitate exploring of microbial growth efficiency in a concentration-dependent fashion (microdilutions of carbohydrate mixtures or specific toxic chemicals). After turbidimetric growth assessment, colorimetric indicator plates (cresol red agar) were mounted to the assay plates. The substrate utilisation design is illustrated by glucose and a plant carbohydrate mixture, the antimicrobial susceptibility design by the naphthoquinone juglone. Dose–response effects are explored by curve fitting of nonlinear models that especially have been developed to detect hormetic effects that are characterized by stimulation at lower followed by inhibition at higher dosages (U- and inverse U-shaped effects). Multivariate analyses are presented utilizing metavariables that were obtained in the curve fitting process of the measured parameters growth and respiration and the factor growth efficiency.

1. Introduction

The exploration of microbial substrate utilisation and susceptibility by and against various substances or substance mixtures has received a lot of attention in medical and environmental science. Depending on the specific research question, studies differ in their focus.

Clinical studies mainly target single specific pathogens, either by identifying the strain based on its specific utilization of a substrate set, or determining its susceptibility against established antimicrobial drugs; besides, the potential of specific synthetic compounds, natural products or antibiotics as "chemical weapons" against other microbes are explored in on-going screening efforts to identify potential candidates for drug development, especially when facing multidrug resistance of microbial pathogens (Coates et al., 2002). Environmental studies, by contrast, are aimed more at exploring the extent of changes caused by specific factors on the structure of microbial communities that is revealed by community level physiological profiling by assessing utilisation pattern of a standardized substrate set (Campbell et al., 2003).

Broth microdilution methods are established as standard in determining MIC values (minimal inhibitory concentration) of antimicrobial substances (Pfaller et al., 2011; Wiegand et al., 2008). Today, turbidimetric assays in 96-well microplates are prevalent in academic and clinical studies (De La Fuente et al., 2006; Engelmeier and Hadacek, 2006).

Environmental studies are aimed at determining community level physiological profiles (CLPP) by assessing microbial substrate utilization of a wide range of carbon sources, but only at a single concentration (Garland, 1997; Lehman et al., 1995). Again, the most recommended scoring method is turbidimetry. Kits such as Ecoplate™ (Biolog Inc, Hayward CA, USA) assess the growth on 32 substrates using optical density (turbidimetry) at 590 nm (Garland and Mills, 1991). In 2003, researchers from the Macaulay Land Use Research Institute in Aberdeen (Scotland, UK), introduced the MicroResp™ kit, in which substrate induced respiration (SIR) was measured colorimetrically on a second indicator microplate that is clamped to a deep well plate containing the soil samples (Campbell et al., 2003). Both plates are connected by an air-tight seal that facilitates the movement of the evolving carbon dioxide to the indicator plate that contains agar augmented with cresol red and $NaHCO_3$. The following chemical reaction occurs:

$$CO_2 + H_2O + HCO_3^- \rightarrow 2CO_3^- + 3H^+$$

The cresol red changes from pink to yellow colour with decreasing pH. The advantage of the MicroResp™ method is that it relies on a

Abbreviations: CFU, colony forming units; G, growth; GE, growth efficiency; MS, Murashige–Skoog; GC–MS, gas chromatography–mass spectroscopy; LB, Luria-Bertani; MDS, multidimensional scaling; MEA, malt extract agar; R, respiration; TSA, tryptic soy agar.

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colour change of a pH sensitive dye that is not in direct contact with the medium and tested microbes. In solutions, water soluble tetrazolium salts are often used as redox-sensitive indicators of microbial growth. The risk of a potential reduction of the dye by other unattended strong chemical reducers that also may be present in the medium does not apply to MicroResp™ (Bhupathiraju et al., 1999).

Here we want to introduce a modified MicroResp™ method that explores substrate utilization and susceptibility of specific compounds or compound mixtures that are added to liquid culture media in dilution series. This assay provides both growth and respiration as quantitative data, allowing to calculate a growth efficiency factor (Del Giorgio et al., 1997) that interrelates changes in growth and respiration rates of treatments compared to untreated controls (cultures on non-supplemented media). It represents a highly sensitive assay not only to study substrate utilisation but also susceptibility against toxic carbohydrate compounds, such as natural products, drugs or xenobiotics.

Media for carbon utilization and susceptibility differed in their initial carbon content; the former had a low carbon content to force microbes utilizing the offered carbohydrate. Conversely, higher carbon content facilitates more vigorous growth that helps detecting inhibitory effects. To illustrate the applicability of this assay, we chose the carbohydrate glucose, a substrate that is used to study microbial carbon-use-efficiency (Keiblinger et al., 2010), a complex carbohydrate mixture as it occurs in plant tissues, and a secondary metabolite, the naphthoquinone juglone that is well known for its antimicrobial activity (Hadacek and Greger, 2000). To explore dose–response relations we used several non-linear growth models that were especially developed to study hormetic effects, stimulation at lower dosages that is followed by inhibition in higher. This effect is not limited to pharmacological but also occurs in biological models (Calabrese and Blain, 2009).

The fact that the pepper bark tree, *Warburgia ugandensis*, of the family Canellaceae, is used as source of plant carbohydrates and bacterial and fungal isolates (endophytes), is due to a current research project, from which data have been chosen in attempts to illustrate specific aspects of the introduced assay.

2. Material and methods

2.1. General

Tryptic soy broth (TSB), juglone, KH_2PO_4, KCl, $NaHCO_3$ and Amberlite XAD-1180 were purchased from Fluka (Buchs, Switzerland); methanol (p.a.), MS medium, and cresol red (indicator grade) were obtained from Sigma Aldrich (Schnelldorf, Germany), D-(+)-Glucose, peptone and ethanol absolute from Merck (Darmstadt, Germany), LB broth and sucrose from Roth (Karlsruhe, Germany). NaOH (50% aqueous solution) was purchased from J.T. Baker (Deventer, Netherlands).

DNA polymerase was obtained from Solis BioDyne, (Tartu, Estonia), endonucleases *Hae*III and *Alu*I and Go Taq® Green Master kit from Promega GmbH (Mannheim, Germany). Water had Milli-Q quality.

2.2. Plant carbohydrates

The plant carbohydrates were obtained by extraction of 3 g dried leaves of *W. ugandensis* Sprague (Canellaceae) at ambient temperature (80 ml methanol for 24 h). The extract was filtered (MN 615; Macherey-Nagel, Düren, Germany) and concentrated to 10 ml under vacuum. Two hundred milligrams of the crude extract were fractioned over Amberlite XAD-1180. Glass columns (15 mm diameter) were filled with 20 g resin and prepared according to manufacturer's guidelines. Two 50 ml fractions were eluted, one with water, one with absolute ethanol. The concentrated eluates were stored at −20 °C until further use.

For GC–MS measurements, 100 µg of the dried aqueous eluate were dissolved in 100 µl *N*-methyl-*N*-TMS-trifluoroacetamide (MSTFA, Thermo Scientific, Waltham, MS) for derivatisation into trimethylsilyl ethers and esters. One microliter of this solution was injected into an AutoSystem XL gas chromatograph (Perkin Elmer, Waltham, MS) in splitless mode, the injector temperature was 250 °C. The column was a Zebron 5 ms column (18 m × 0.18 mm, 0.18 µm film thickness; Phenomenex, Torrance, CA), the helium follow rate 0.8 ml/min. The temperature gradient started at 70 °C and, after 3 min, rose to 300 °C at a rate of 3 °C/min. The gas chromatograph was linked to a TurboMass™ quadrupole mass analyzer (Perkin Elmer, Waltham, MS); the transfer line temperature was set to 280 °C, the ion source to 200 °C, the filament current to 70 eV. The mass spectrometer was run in the TIC mode from 40 to 620 amu. The obtained chromatograms were integrated with TurboMass 4.1.1 (Perkin Elmer, Waltham, MS) and the peak areas were expressed as relative amounts of the total peak area (100%). Mass spectra were identified tentatively with the Golm Metabolome Database (Potsdam, Germany; http://gmd.mpimp-golm.mpg.de; Kopka et al., 2005).

2.3. Microorganisms

2.3.1. Strain isolation and identification from Warburgia ugandensis

2.3.1.1. Bacteria. For isolation of endophytic bacteria, leaves and roots embedded in MS agar were carefully pulled out of the agar and remaining agar was removed with a sterile scalpel. The plant material was washed in sterile, distilled water, rinsed with ethanol, and finally flamed. Roots and leaves were tested for sterility on tryptic soy agar plates incubated for 8 days at room temperature. No growth was observed. All plant material was cut into small pieces, macerated by grinding in sterile mortars, and suspended in 5 ml of 0.9% NaCl solution. Portions of 50 µl of the supernatant and of a 10^{-1} dilution were spread on 10% TSA and R2A plates, respectively. Plates were incubated for 2–8 days and room temperature. Ninety-six colonies in total were randomly picked and further analyzed. For isolation of genomic DNA, bacteria were grown overnight in 5 ml of tryptic soy broth in a rotatory shaker at 27 °C. DNA was isolated as described (Reiter and Sessitsch, 2006). 16S rDNA PCR was carried out by using primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) (Edwards et al., 1989) and 1520r (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards et al., 1989) and primers pHr (5′-TGCGGCTGGATCACCTCCTT-3′) (Massol-Deya et al., 1995) and P23SR01 (5′-GGCTGCTTCTAAGCCAAC-3′) (Massol-Deya et al., 1995) were used for amplification of the 16S–23S rRNA IGS. PCRs were performed in a total volume of 50 µl and contained 1 µl of extracted DNA, 1× PCR reaction buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.15 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate, and 2.5 U FIREPOL DNA polymerase. Cycler conditions were as following: 5 min denaturation at 95 °C, 30 cycles consisting of denaturation for 30 s at 95 °C, primer annealing for 1 min at 53 °C, polymerization for 2 min at 72 °C, and final extension for 10 min at 72 °C. Aliquots of the PCR products containing 200 ng of amplified DNA were digested with 5 U of endonucleases *Hae*III and *Alu*I individually for 3 h at 37 °C. The resulting DNA fragments were analyzed by gel electrophoresis in 2.5% agarose gels. One isolate of each ribotype was identified by 16S rDNA sequencing with the primer 8f making use of the sequencing service of the company AGOWA (Berlin, Germany). Retrieved sequences were visualized with sequence alignment editor package of BioEdit (Ibis Biosciences, Carlsbad, CA) and identified by BLAST analysis.

2.3.1.2. Fungi. For isolation of fungi MS agar-embedded plant tissue was incubated at room temperature for two weeks. Growing fungal hyphae were carefully examined with the naked eye and with an Olympus SZH10 research stereo microscope (×140) to distinguish endophytic fungi that grew directly out of plant material from