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Real-time monitoring of fungal inhibition and morphological changes

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ABSTRACT

Mold growth constitutes a problem in many food and clinical environments and there is therefore focus on studying antifungal activity. Methods for determining growth inhibition by measuring colony growth or biomass are, however, time-taking and rapid methods for evaluation of antifungal effects are needed. Propionic acid and diacetyl are antifungal compounds produced by a range of dairy-associated bacteria. Their activity against *Penicillium* spp. was monitored real-time using an optical detection system with tilted focus plane to assess growth and morphological changes of *Penicillium* spp. by image recording inside a 96 well microplate. Images were used for generation of growth curves by using a segmentation and extraction of surface areas (SESA) algorithm and for quantifying morphology changes.

Using image analysis growth could be detected within 15 h compared with more than 30 h when using standard optical density measurements. Induced morphological changes of fungi could furthermore be visualized and quantified using morphological descriptors such as circularity, branch points, perimeter and area of spores and growing hyphae. Propionic acid inhibited two out of two *Penicillium* spp. while morphological changes were strain dependent at the concentrations tested. Diacetyl inhibited six out of six *Penicillium* spp. strains and increased spore size and number of germination sites in two out of six of the strains prior to germination.

1. Introduction

Various methods have been used to monitor fungal growth and inhibition due to antifungal activity of chemical compounds. Optical density (OD) has frequently been used to determine the inhibitory activity of antifungal compounds (Lind et al., 2005; Magnusson & Schnürer, 2001; Ndagano, Lamoureux, Dortu, Vandermoten, & Thonart, 2011). The method is efficient for screening a large number of compounds against several fungal strains and only small amount of medium and compounds are needed. When using OD, light passes through the sample and is scattered by the cell suspension. The disadvantage of using OD for filamentous fungi, however, is that growing hyphae are not evenly distributed in the microplate well and this might give uncertainties in estimation of fungal growth. In addition, sporulation in the surface of the wells might give high OD values and thereby overestimation of growth. OD is therefore mainly suitable for initial detection of mold growth or for growth vs. no growth studies.

Alternative methods for mold growth quantification includes measure of fungal biomass (Ström et al., 2005), measuring the diameter of the colony or the inhibitory zone around molds using the disk diffusion method (Delavenne et al., 2013) or recording images of petri dishes with growing molds and quantifying the growth by multispectral image analysis (Ebrahimi et al., 2015). These methods are often time consuming and require large amount of media and, if antifungals are tested, the compound in question. Morphology is not taken into account and therefore a combination of methods is often necessary when growth and morphology are studied simultaneously. In addition, biomass measurements will only give an end point result.

A newly developed optical detection system called an oCelloScope™ has been used for speeding up determination of bacterial inhibition by antibiotics with the same accuracy as standard OD (Fredborg et al., 2013). The oCelloScope™ detection system is an instrument which can scan a liquid sample in a microplate well. The focus plane of the imaging system is tilted 6.55° and a stack of images are recorded inside the microplate well. The growth of microorganisms can be determined from the images using a segmentation and extraction of surface areas (SESA) algorithm (Fredborg et al., 2013).

Here, we report for the first time the use of the oCelloScope™ to monitor fungal growth and inhibition. The method was compared with OD measurements. We furthermore developed morphological descriptors which allowed us to investigate the influence of propionic

Abbreviations: CDIM, chemically defined interaction medium; OD, optical density; MEA, malt extract agar; SESA, segmentation and extraction of surface areas.

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acid and diacetyl, respectively, on morphology and growth of *Penicillium* spp.

2. Materials and methods

2.1. Chemicals and materials

2,3-Butanedione (diacetyl) with purity 97% (Sigma-Aldrich, Schnelldorf, Germany); propionic acid with purity >99% (Merck, Hohenbrunn, Germany), hydrochloric acid (VWR, Rue Carnot, France) and Tween80 (Merck, Hohenbrunn, Germany). All water was freshly prepared Milli-Q quality (Merck Millipore, Billerica, MA, USA).

2.2. Microbial strains, media and growth conditions

The fungal strains (isolated from fermented dairy products) were supplied by DuPont Nutrition Biosciences ApS. The molds, *Penicillium solitum* DCS 302 and *Penicillium* sp. nov. DCS 1541 (tentative name: *Penicillium salamii*, closely related to *Penicillium olsonii*, Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre), *Penicillium glabrum* DCS 305, *Penicillium brevicompactum* DCS 1540, *Penicillium* sp. DCS 1065 and *Penicillium* sp. DCS 1066 were grown individually on malt extract agar (MEA) with 30 g/L malt extract (Becton Dickinson AS, Sparks, MD, USA), 5 g/L peptone (Becton Dickinson AS), 15 g/L agar (Becton Dickinson AS) (Galloway and Burgess, 1952) for 5–7 days at 25 °C and stored in 20% glycerol + water and Tween80 at −80 °C until use.

2.3. Growth medium and culture conditions

A chemically defined interaction medium (CDIM) was used for mold inhibition studies. The medium was prepared as described by Aunsbjerg et al. (2015b). CDIM at pH 6.5 and CDIM acidified to pH 4.0 with HCl were mixed with 0.075 mg/mL of diacetyl or 0.5 mg/mL of propionic acid, respectively. All batches were made in triplicates.

The microplate was left at room temperature for about 1 h after inoculation to allow the spores to position in the well. This was important in order to keep the right focus on the growth development when measuring with the oCelloScope™ during the incubation period.

2.4. Determination of inhibitory activity and morphological changes of fungi

The antifungal activity of propionic acid was tested against the molds, *P. solitum* DCS 302 and *Penicillium* sp. nov. DCS 1541. The antifungal activity of diacetyl was tested against all six *Penicillium* spp. strains. Inhibition of fungi in the microplates was assessed by measuring $OD_{600\ nm}$ using a Varioskan™ Flash (Thermo Fisher Scientific Oy, Finland). In parallel time-lapse imaging scanning through a fluid sample was conducted thereby generating series of 40 images in each well. The images covering an area of 1755 × 1200 μm, were measured using an oCelloScope™ detection system (objective, 4×) (Philips BioCell A/S, Denmark) as described in detail by Fredborg et al. (2013). The image distance was 5.13 μm and the illumination exposure time was 2 ms. Growth curves were generated from images by using the segmentation and extraction of surface areas (SESA) algorithm. The images were in addition used to compare morphological differences of fungi in batches without and with added propionic acid or diacetyl. The plates were measured for up to 118 h and stored at 25 °C between measurements. Both methods were used on the same microplates.

2.5. Morphological descriptors

Some of the parameters of interest for characterization of *Penicillium* spp. morphology are 1) time of germination, 2) the increase in size of spores and hyphae fragments pr. time unit, and 3) morphological differences between formed hyphae in the presence and absence of propionic acid and diacetyl. From the features provided by the oCelloScope™ software, the following descriptors were chosen to estimate the parameters in question:

2.5.1. Area

The 'Area' descriptor measures the total number of pixels covered by an object. The 'Area' value is not affected by object shape; e.g. objects with identical 'Area' values may have different shapes. 'Area' can be used to monitor changes in object size over time and is also useful as a classifier to discriminate between different types of objects or to exclude irrelevant objects from the analysis based on their size.

2.5.2. Perimeter

The 'Perimeter' descriptor measures the object perimeter and can be used to discriminate between objects with equivalent areas, but with different shapes. 'Perimeter' values are computed based on the length of the object border. Large objects will result in a higher 'Perimeter' value than small objects. The shape will affect the 'Perimeter' value in cases of objects possessing identical areas; e.g. a circular object has a lower value than objects with any other shape.

2.5.3. Circularity

The 'Circularity' descriptor measures how similar the object shape is to a circle independently of the object size; e.g. objects with identical shape but different in size will have the same 'Circularity' value. The circularity is calculated as the ratio between the perimeter of a circle with the same area as the object, and the perimeter of the object. Circular objects have an area of ≥1 and any other shape will be <1. Due to technical irregularities in the software calculations the value of a circular object can be marginally larger than 1.

2.5.4. Branch points

'BranchPoints' values are computed based on thinned images obtained by continuously removing pixels from the object boundary (without breaking the object apart) until only a one pixel thick line or point remains. Using the thinned image, the 'BranchPoints' algorithm measures the number of branch points, i.e. object pixels with more than two adjacent object pixel neighbor's. The 'BranchPoints' descriptor can be used to quantify the complexity of the shape of an object. A complex shaped object will have a high number of branch points.

2.5.5. Skeleton length

'SkeletonLength' values are computed based on thinned images (see previous section). The 'SkeletonLength' algorithm measures the length of individual objects as the sum of skeleton pixels. The 'SkeletonLength' can be used for determining the length (in pixels) of an object including protrusions. This can be useful e.g. when separating objects growing in a budding pattern like yeast or in rod or chain-like pattern like some bacteria. In this context the 'SkeletonLength' can be used for characterization of the length of the hyphae protruding from the spore. The 'SkeletonLength' can like the 'Circularity' be used for finding the time of the germination.

Unwanted object in images such as dirt or non-germinated spores can easily be removed from the calculations using the oCelloScope™ software. E.g. non-germinated spores can be removed by choosing objects with a specific circularity or by deselecting the unwanted objects manually.

3. Results

Here we studied the effect of two known antifungal compounds, propionic acid and diacetyl, on growth and morphology of *Penicillium* spp. Mold growth was determined by use of either OD measurements or by recording images of the growing mold in a liquid sample using the oCelloScope™ detection system. The two measuring methods