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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.

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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, Schnellendorf, 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 Aunbjerg 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 neighbors. 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

were applied on the same wells in a microtitre plate making the results directly comparable.

The oCelloScope™ measurements revealed growth at a much earlier state than what could be achieved with OD. Based on the OD measurements using a cut-off value of ~0.1, growth of *Penicillium* sp. nov. DCS 1541 and *P. solitum* DCS 302 in CDIM with no propionic acid was observed after between 30 and 38 h (Fig. 1A and B). When analyzing the same samples by the oCelloScope™ detection system, using a cut-off value of ~0.25, growth was observed clearly within 15 h (Fig. 1C and D). Cut-off values were selected based on visual detection of growth on curves. The growth curve and images of *Penicillium* sp. nov. DCS 1541 without propionic acid generated by the oCelloScope™ software can be seen as an example in video 1.

3.1. Growth of fungi in the presence of propionic acid

Addition of 0.5 mg/mL propionic acid almost completely inhibited the growth of the two molds as judged by the OD curves (Fig. 1A and B). The oCelloScope™ curves confirmed that *Penicillium* sp. nov. DCS 1541 was inhibited by propionic acid for about 60 h after which growth was detected (Fig. 1D). When studying the oCelloScope™ images visually, swelling and irregularities of the *Penicillium* sp. nov. DCS 1541 hyphae were observed when grown in the presence of 0.5 mg/mL propionic acid. This was not the case for hyphae formed without the presence of propionic acid (Fig. 1). For *P. solitum* DCS 302, growth in the presence of 0.5 mg/mL propionic acid was detected by the oCelloScope™ after 24 h (Fig. 1C). *P. solitum* DCS 302 was more tolerant to propionic acid with no marked impact on the morphology under the experimental conditions used (Fig. 1).

After 9 h the oCelloScope™ growth curve increased slightly for *Penicillium* sp. nov. DCS 1541 in the presence of propionic acid (Fig. 1D). When studying the oCelloScope™ images visually and by use of the oCelloScope™ software it was seen that there was an increased number of spores after 9 h as compared to the first measurement (results not shown). This could be due to some spores not being in focus at the first measurement.

3.2. Quantification of morphological differences in hyphae development

The morphological difference in hyphae development between molds in the presence and absence of propionic acid was studied visually from the oCelloScope™ images and quantified using morphological descriptors. These descriptors are dependent on segmentable objects so the images have to be recorded before extensive or overlapping hyphae formation has occurred. A range of descriptors are available in the oCelloScope™ software for quantifying morphological differences in hyphae development. One way to discriminate between hyphae fragment with similar area but different shape is to use the 'Perimeter' descriptor which is the length of the hyphae border. Circular hyphae fragments will have a low 'Perimeter' value whereas any other hyphae shape will give larger values. In the presence of 0.5 mg/mL propionic acid *Penicillium* sp. nov. DCS 1541 hyphae were irregular and swollen giving a higher circularity and lower perimeter and skeleton length compared to hyphae formed without the presence of propionic acid. Two hyphae fragments of similar area size formed in the presence and absence of propionic acid are shown as an example in Table 1.

The morphological descriptors can either be expressed as an average of all objects in the oCelloScope™ images or they may be used to follow

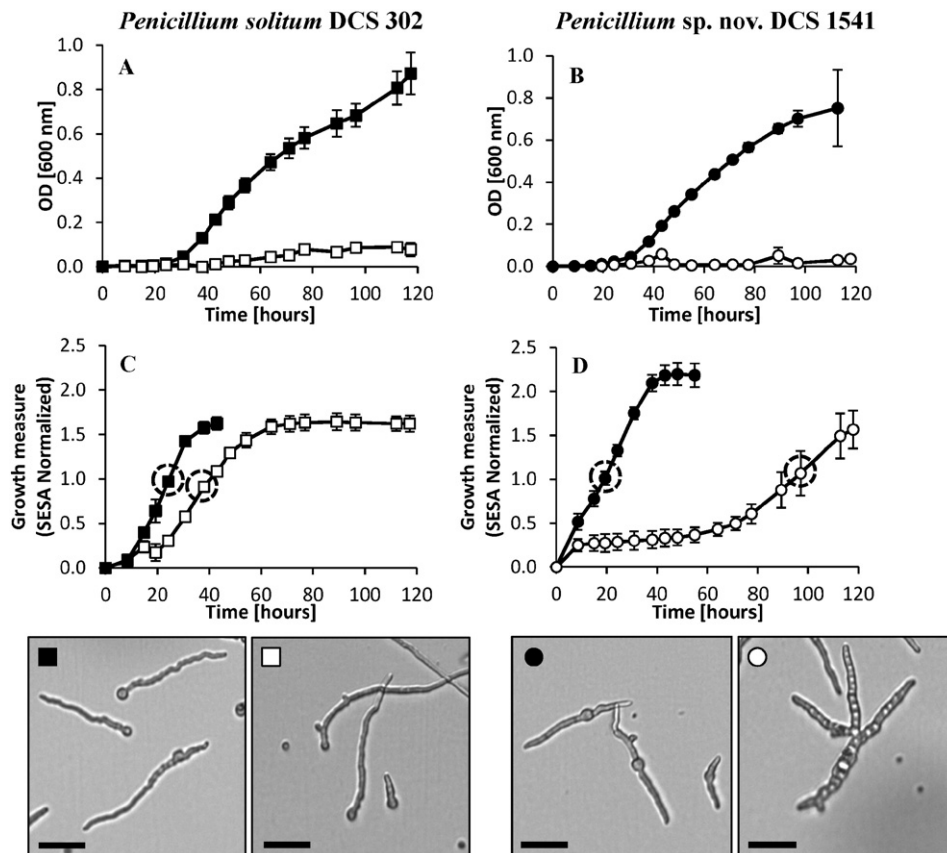
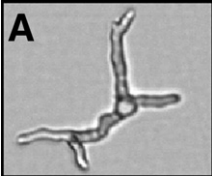
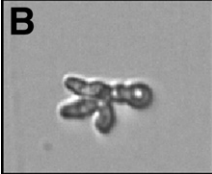


Fig. 1. Inhibition of *Penicillium* species by propionic acid. Growth of *Penicillium solitum* DCS 302 (■, □) and *Penicillium* sp. nov. DCS 1541 (●, ○) measured as OD₆₀₀ using Varioskan™ Flash (A, B) and as Normalized SESA units using oCelloScope™ and the SESAs algorithm (C, D) in CDIM with 0 (●, ■) or 0.5 (○, □) mg/mL of propionic acid. Measurements are averages of three replicates and error bars show the standard deviation. Below graphs: oCelloScope™ images at growth measure close to 1. Measurements corresponding to images are marked on graphs by dotted circles. Black bar: 40 μm.

Table 1

Comparison of *Penicillium* sp. nov. DCS 1541 hyphae in the presence and absence of propionic acid. Hyphae grown to an area of similar size in the absence (A) and presence (B) of 0.5 mg/mL propionic acid (after 24 and 89 h, respectively), by use of morphological descriptors.

	Area [μm^2]	Perimeter [μm]	Circularity	Skeleton length [μm]	Branch points
	426.0	453.3	0.1748	217.3	4
	423.5	147.9	0.5089	62.7	3

the development of a single spore. It is possible to remove and insert the plate at the same position in the oCelloScope™ instrument allowing tracking of the individual structures (Table 2).

If the average of all objects is used, it should be taken into account that spores not germinating will also influence the results. Here, we defined germinated spores as objects having circularity below 0.95. This was done in order to remove spores from the dataset thereby allowing comparison of the DCS 1541 hyphae formed in the presence and absence of 0.5 mg/mL propionic acid. The hyphae fragments vary markedly in size and therefore the averages and perimeter without standard deviations are shown in the Fig. 2A. An example of the variation in area and perimeter between hyphae fragments is shown at two time points in Fig. 2B. Besides delaying onset of growth (Fig. 1), fewer spores germinated in the presence of propionic acid as seen by 89% fewer hyphae fragments compared to hyphae grown without propionic acid (Fig. 2B). The DCS 1541 hyphae formed in the presence of 0.5 mg/mL propionic acid had a smaller perimeter than hyphae

with the same area formed in CDIM without the presence of propionic acid (Fig 2).

3.3. Influence of diacetyl on growth of fungi

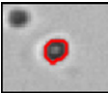
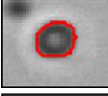



We recently showed that diacetyl was inhibitory towards *Penicillium* spp. in the concentrations produced (0.075 mg/mL) by an antifungal *Lb. paracasei* strain in a solidified chemically defined interaction medium (CDIM) (Aunbjerg et al., 2015a). Here we showed that 0.075 mg/mL diacetyl was also inhibitory in liquid culture towards *Penicillium* spp. (Fig. 3A). Similar results were observed for five other *Penicillium* strains (results not shown).

3.4. Growth of fungi in the presence of diacetyl

Circularity can be used as an indicator for time of germination. A smooth, circular formed spore will give a circularity value around 1.

Table 2

Growth development and morphological descriptors used on a single *Penicillium* sp. nov. DCS 1541 spore grown in the presence of 0.5 mg/mL propionic acid.

	Growth time [hours]	Area [μm^2]	Perimeter [μm]	Circularity	Skeleton length [μm]	Branch points
	8.3	15	12.16	1.13	5.5	0
	31	41	21.37	1.06	10.5	0
	55	100	42.72	0.86	18.7	0
	89	516	179.25	0.45	98.5	3
	112	1291	529.09	0.25	277.2	7

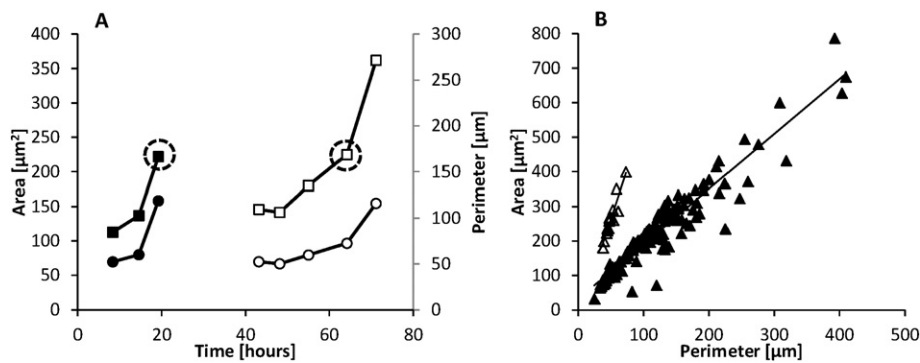


Fig. 2. A) Average area (■, □) and perimeter (●, ○) of hyphae formed in the absence (black markers) and presence (open markers) of 0.5 mg/mL propionic acid. B) Area and perimeter of all hyphae formed in the absence of propionic acid (▲) after 19 h and hyphae formed in the presence of propionic acid (△) after 64 h. Averages corresponding to hyphae fragments in B) is marked with dotted circles in A).

When the spores start germinating circularity will decrease below 1. For two strains, *Penicillium* sp. DCS 1065 and DCS 1066, the spore size increased markedly before germination when grown in the presence of diacetyl (Fig. 3B). When using the circularity to determine time of germination and the area for detecting growth it was seen that the average area of the spores before germination was 2.9 times larger for samples with added diacetyl compared with control samples. *Penicillium* sp. DCS 1066 is shown as an example (Fig. 3B). This tendency for increased spore size in the presence of diacetyl was not seen for the four other strains of *Penicillium* sp. (results not shown). Diacetyl furthermore increased the number of germ tubes from the spores of *Penicillium* sp. DCS 1065 and 1066 (Fig. 4) compared to control samples. This was not the case for the four other *Penicillium* strains.

4. Discussion

Here, we studied for the first time, induced changes in growth and morphology of *Penicillium* strains using a segmentation and extraction of surface areas (SESA) algorithm on images recorded inside a microtitre plate by an oCelloScope™ detection system. For comparison OD was chosen, since it is considered useful for early detection of growth, many samples can be measured simultaneously and small amounts of liquid and test chemicals can be used. The inhibitory activity of antifungal compounds was also shown by optical density measurements but results were obtained much faster by the real-time image recording.

Propionic acid induced swollen hyphae and irregular growth of *Penicillium* sp. nov. DCS 1541 (Fig. 1). The swollen hyphae resulted in a smaller perimeter than hyphae with the same area formed without the presence of propionic acid. By calculating area and perimeter of formed hyphae in the presence and absence of propionic acid using the oCelloScope software, we were able to quantify the morphological differences. These differences could not be detected using OD measurements alone. Propionic acid has previously been shown to induce swelling of *Aspergillus flavus* hyphae (Al-Hilli and Smith, 1992) and *Neurospora crassa* (Parton et al., 1997).

The antifungal activity of weak organic acids such as propionic acid have been shown to increase with decreasing pH (Lind et al., 2005). At low pH the uncharged undissociated form of the weak organic acids is favored which allows the acid to easily enter the fungal cell. At pH 4.0, 88% of the compound is undissociated. The higher pH inside the fungal cell results in dissociation of the acid causing a release of anions and protons. The anions accumulate inside the fungal cell and the protons acidifies the cytoplasm thereby inhibiting many metabolic processes (Brul and Coote, 1999; Mollapour and Piper, 2008). One study suggested that the antifungal mechanism of propionic acid involved a conversion of the propionic acid to propionyl Coenzyme A, which possibly inhibited enzymes important for the glucose metabolism (Brock and Buckel, 2004). This could explain the decreased growth of fungi observed in the present study.

The antifungal activity of diacetyl in concentrations up to 0.075 mg/mL was fungistatic rather than fungicidal, as observed by

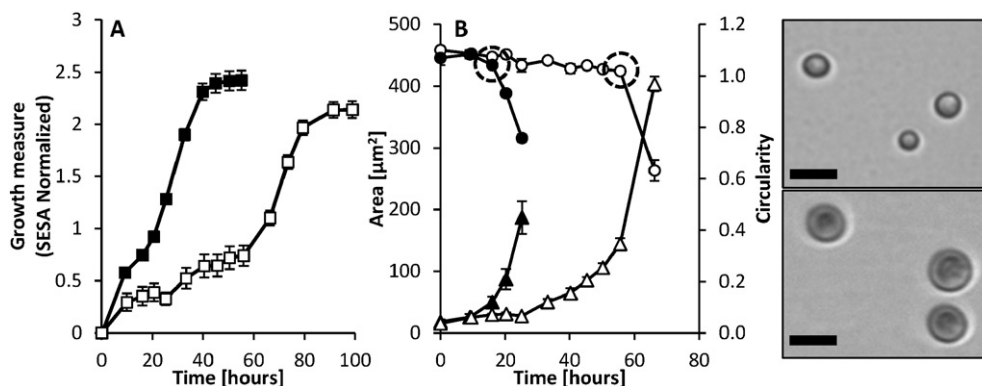


Fig. 3. A) Influence of diacetyl on growth and spore size of *Penicillium* sp. DCS 1066. Growth of *Penicillium* sp. DCS 1066 in the presence of 0 (■) or 0.075 (□) mg/mL diacetyl measured by the oCelloScope™. B) Average spore area (▲, △) and average spore circularity (●, ○) of *Penicillium* sp. DCS 1066 grown in CDIM at 25 °C with 0 (black) and 0.075 (white) mg/mL diacetyl. Measurements are averages of three replicates (▲: only two replicates) and error bars show the standard deviation. Images next to graph show spores just before germination. Measurements corresponding to images are marked on graphs by dotted circles. Black bar: 20 μm .

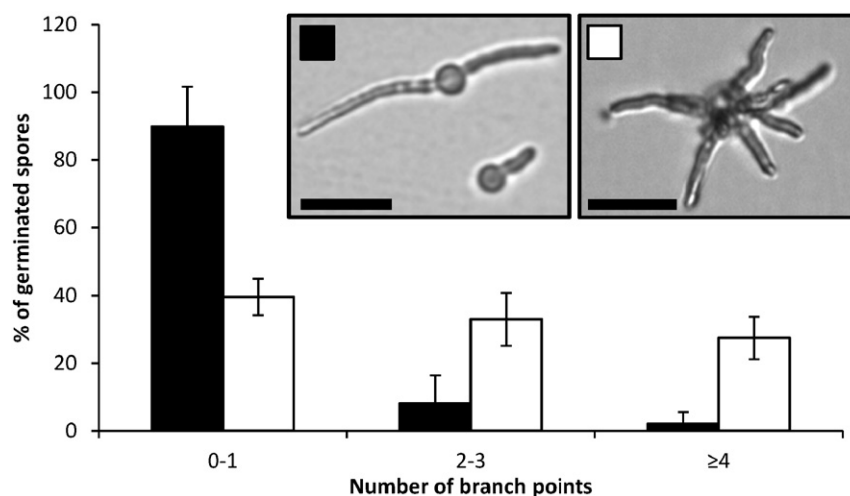


Fig. 4. Number of branch points in germinated *Penicillium* sp. DCS 1066 spores. Mold germination in the presence of 0 (■) or 0.075 (□) mg/mL diacetyl recorded after 25 and 66 h, respectively, by the oCelloScope™, corresponding to the times where germination of most spores had initiated. Measurements are averages of three replicates and error bars show the standard deviation.

increased lag phase (Fig. 3). A possible explanation for the observed growth of fungi in the presence of diacetyl could be that diacetyl was degraded or bound in the medium.

The *Penicillium* spores increased in size prior to germination (Table 2). Most mold spores undergo isotropic expansion prior to germination due to water uptake (Harris, 2006). In two of the six strains the increase of the spores were much more pronounced in the diacetyl exposed culture. It is known that loss of polarity can lead to isotropic growth with increase in spore size (Harris, 2006) and it is possible that diacetyl causes a loss of polarity although this needs further examination.

Diacetyl markedly delayed germination of all six *Penicillium* strains. For many mold spores germination usually starts at one site with a second germ tube emerging at the opposite site of the spore (Wendland, 2001). This was also seen for the *Penicillium* sp. DCS 1066 without the presence of diacetyl whereas an increased number of germ tubes was seen when diacetyl was present (Fig. 4). The bigger spore size and increased number of germ tubes was only observed for two out of six *Penicillium* strains indicating a strain specific mechanism of diacetyl.

The exact mechanism behind antifungal activity of diacetyl has not yet been elucidated. Increased antimicrobial activity of diacetyl compared to related compounds has been suggested to be due to diacetyl being an α -dicarbonyl (Jay et al., 1983) and it has been further observed that a complex can be formed between the α -dicarbonyl of diacetyl and the guanido group of arginine (Gloede et al., 2011; Mathews et al., 2010).

OD is a frequently used method for determination of minimal inhibitory concentration (MIC) of antifungal compounds and MIC has been defined as the lowest concentration of a compound where no growth could be observed in the microplate well (Lind et al., 2005). When using OD for growth assessment of *Penicillium* nov. sp. DCS 1541 in the presence of 0.5 mg/mL propionic acid we saw complete inhibition for 118 h indicating that the MIC value of propionic acid was below 0.5 mg/mL. However, the oCelloScope™ growth curves showed that both molds grew in the presence of propionic acid (Fig. 1), although the number of germinating spores did decrease markedly when propionic acid was present (Fig. 2B). The use of images and the SESA algorithm thus increased the sensitivity as well as the speed of growth detection. The limitations of the method is that it is particular suitable for the initial growth detection before a three dimensional hyphae network has been formed and furthermore, as with all image analysis, it requires more extensive data handling. OD is not optimal for quantification of mold growth due to the unevenly distributed hyphae in the

wells. This may explain the low ODs measured for *Penicillium* spp. in the presence of propionic acid.

5. Conclusions

The oCelloScope™ detection system shows potential for real-time measurements which will allow for fast determination of inhibitory activity of antifungals. Initial growth of tested fungi was obtained in about half the time or less with the oCelloScope™ as compared to the standard OD measurements. The method was particular suitable for the initial growth detection. The use of a microtiter plate made it possible to screen many samples as well as replicates. Notably, the image recording inside the microplate wells made it possible to study the growing hyphae and morphology throughout the experiment in undisturbed samples. Additionally, the development of a single spore could be followed. Information about morphological changes during growth such as irregular growth and variations in hyphae branches can be important when studying the mechanism of antifungal compounds. A faster determination of growth and inhibition is valuable also from a medical point of view when determining fungal resistance and the potential of the method should be explored with medically relevant strains.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.mimet.2015.10.024>.

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