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An Improved Method for the Screening of Fungal Growth Inhibition

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Summary

There are different ways to determine the antifungal activity of substances. This can be done by means of the disk method, which is a visual method. There are also automatic systems available.

In this paper we describe a method which is based on measuring growth in the form of changes in optical density during the incubation time. The optical density measurements were performed with a Multiskan MCC/340 (Labsystems, Helsinki, Finland). We used 0.375% agar in 20 g/L Malt Extract Broth as end concentration. The wells were filled in total with 225 μL, 75 μL test substance, 75 μL medium with fungal spores, and 75 μL paraffin on top.

If a plate with 96 wells is filled under the above-mentioned conditions, and placed in the Multiskan MCC/340 with stacker, the test can continue with a maximum of 20 plates without any handling for the duration of the experiment. The data were collected and stored in a computer file which was processed using SAS.

The computer software (developed on site) has an option to check the optical density in the form of a graph during the experiment, which allows one to see on-line if a test substance has antifungal activity.

The system can also be used as preliminary screening for mode of action experiments.

Keywords: antifungal substances, plate reader, screening method, fungi

Introduction

Most tests to determine growth rates and germination rates of fungi are performed on agar plates. This is time-consuming and requires a lot of material. Therefore, we evaluated other ways to perform growth (inhibition) tests. Such a system should allow testing on a small scale of a lot of variations in culture conditions in one experiment.

A lot of different methods are known in literature aimed at screening substances for their antimicrobial activity. Especially for filamentous fungi there are plate assays such as the disk method, in which the disk is impregnated with the antifungal substance. Subsequently, the impregnated disk is placed on a petri dish with agar containing mould spores, and the inhibition zone around the disk is measured. The zone can be measured by means of a ruler or automatically with an image analysis system. It is not easy to quantify this method which is based on diffusion of the test substance in the medium.

A method based on the growth of single hypha in the presence of a volatile compound is described in the literature. The antifungal potency is measured by assessing the growth rate of a hypha with and without an antifungal agent, which gives a result in 2–3 hours *(1)*. In short, the method is as follows. The reaction vessel, which contains the mould in an agar layer on the top part of the inside of the vessel, is placed on the stage of the microscope. This is controlled by a microcomputer, and a hypha on the medium is selected and recorded by a video camera. The images are recorded by a video cassette recorder, and also stored in a computer that can analyse the growth rate. The growth is measured until the growth rate is stable. At that point, the test substance is added for a certain period of time. Finally, the test substance is removed and the vessel is flushed with air. A disadvantage of the system is that only one sample can be measured at a time.

Another method from the same group of researchers is based on the method mentioned earlier and is called the Bio-Cell Tracer *(2)*. In this system, 6 combinations can be tested at the same time by using a multi-well plate.

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This system is suitable for non-volatile substances and is also based on the growth of a single hypha. The bottom of the well is coated with poly-L-lysine. A drop of spore suspension is added, followed by 1mL medium, and then the plate is incubated. Following the incubation, the plate is placed on the stage of the microscope, the medium is refreshed, and a horizontally growing hypha is selected. A tracing marker is placed on the tip of the hypha on the monitor screen, and every 30 s the distribution of light intensity around the tip is analysed and the growth rate is calculated. The total measuring time is 20 min. When the growth rate is stable, the medium is replaced with medium containing the test substance. The measurements are continued for 70 minutes. The technical equipment is the same as described earlier for the volatile components.

A quantitative method for measuring the growth (inhibition) is the use of absorbance measurements. Different equipment can be used for this method, like the Bio-Rad Laboratories micro-plate reader, the Easy Reader EAR340, or the Multiskan MCC/340. In the literature, the methods have been compared and gave the same results *(3)*. This assay is based on the use of spores and mycelial fragments. In short, the method is as follows. The test with the Multiskan MCC/340 is performed in a 96-well flat bottom plate (Nunc). Each well contains the test substance, liquid medium, and a mixture of 2000 spores and mycelial fragments. The total volume is 100 μL. The plates are shaken for a few seconds. Subsequently, the spores are allowed to sediment for 30 minutes, and the plate is measured at 595 nm with a micro-plate reader. After appropriate incubation times, the procedure is repeated.

The system we used for optical density measurements is also the Multiskan MCC/340 (Labsystems, Helsinki, Finland). However, there are a few parameters we have changed, first of all the media. We used a semi-solid medium, because the spores and when they grow out the hypha, are fixed in the medium. The measuring spot is then always in the same place, which prevents fluctuation in the measurements, that can occur when a liquid medium is used. Secondly, we used spores only. In the literature, spores and mycelium fragments are used. We used spores because we wanted to investigate spore germination and outgrowth. Finally, the system we used is fully automatic. It has a stacker that transports the plates through the Multiskan MCC/340. That means that we fill the multi-well plates, store them in the Multiskan MCC/340, and the measurements are done automatically at different time intervals. The Multiskan MCC/340 uses 96-well micro-titre plates with a well volume of 250 μL. With a stacker attached to the Multiskan MCC/340, one can use 20 96-well plates simultaneously measuring different culture combinations fully automatically. In this paper, we describe the optimisation of the experimental conditions.

Material and Methods

Mould strains (optional)

The mould strain we used for the set up of the method was *Penicillium roqueforti*, (equivalent to CBS221.30). For the study of antimicrobial activity *Paecilomyces variotii* (equivalent to CBS338.51) was used. Both strains were isolated in our laboratory and identified by the »Centraal bureau voor schimmel cultures in Baarn« the Netherlands.

Methods

Preparation of the spore suspension

Spore suspensions were prepared as follows: Malt extract agar plates, prepared from malt extract broth (MEB: 20 g/L) (Oxoid, Unipath LTD Basingstoke, Hampshire, England) and agar-agar (20 g/L) (Difco, Difco laboratories, Detroit, Michigan, USA) were inoculated with the mould strain, and incubated at the appropriate temperatures until good sporulation. The spores were harvested by loosening them with a Drigalski spatula in sterile demineralized water. The suspension was filtered through a sterile double layer of tissue and divided in aliquots of 0.6mL using Nunc tubes. The tubes were stored at –80 °C. The CFU's (colony forming units) were determined after at least 3 days of storage.

Test system

Different parameters involving the system were tested. This included different media, filling amounts, and the absence or presence of a paraffin covering (Merck, Darmstadt, Germany). After testing a variety of combinations, of malt extract broth, glucose, and agar or gelatine as solidifier, we settled for the combination of 0.75% agar with 2 × malt extract broth (40 g/L). The medium was diluted 1:1 with the test substance in the well. The mould spores were added to the malt extract agar in a concentration which results in 10–1000 spores per well.

The micro-titre plate wells were filled as followed: 75 μL test substance, 75 μL malt extract agar with spores, and 75 μL paraffin oil. After being filled, the plate was covered with an oxygen permeable seal and placed in the Multiskan MCC/340. The plates were measured automatically. The Multiskan MCC/340 was placed in a temperature-controlled incubator. In the case of filamentous fungi, the culture temperature was usually 20 or 25 °C. It is possible to use the stacker and measure 20 plates at the same time in one test. Every experiment was at least performed in duplicate.

It is important to randomise the conditions because the place of the well on the plate influences the time needed to outgrowth of the moulds.

Multiskan MCC/340

Below, a short description of key characteristics of the Multiskan MCC/340 is given. The Multiskan MCC/340 measures optical density. It is possible to connect a computer to the Multiskan MCC/340, and to design in-house computer software to control the Multiskan MCC/340 and store the data. The Multiskan MCC/340 set-up depends on the experiment. The wavelenght is dependent on the colour of the media. If the absorbance of the medium itself is high, the wavelength at which the measurements are performed needs to be chosen carefully. In the experiments described in this paper, we opted for a wavelength of 620 nm. One can shake the plate before measurement, but this option was not used because we chose a solid medium. The total monitoring

time can be selected in days with a maximum of 90 days. The total monitoring time in our tests was a maximum of 4 weeks with customised reading intervals. The output was recorded and all the data files were concatenated. Finally they were processed in SAS.

Results and Discussion

Agar or gelatine as solidifier

A system with a solid medium is necessary, because the mould spores have to grow in a rigid matrix. If the test is performed in a liquid medium, the germinating mould spores will float through the broth and thus give rise to fluctuations in the optical density readings.

After testing a variety of combinations, we decided to use the combination of 0.75% agar with malt extract broth (MEB) covered with paraffin oil. The 0.75% agar is the highest concentration that can be used at 40 °C. The temperature cannot be higher because the mould spores are added to the medium. The final concentration is 0.375% agar.

We also tested gelatine as a solidifier, because this can be handled at temperatures lower than 40 °C. We decided not to use gelatine because moulds can decompose the gelatine and turn it into liquid. If this happens, the solid matrix is lost and the optical density of different time intervals cannot be compared (see Introduction).

Rich or poor media

The conditions of products that are spoiled by moulds usually consist of sub-optimal growth conditions for the mould. For that reason, we wanted to test the system for cells growing at optimal and sub-optimal conditions. We compared a glucose (poor) and malt extract (rich) medium. The general results were that the growth curves with glucose had a longer lag-time (time till growth, absorbance increase), and the total yield was lower. Noticeably, spore formation also took place, and spore formation influences the absorbance. The explanation for spore formation in the presence of a glucose medium can be that a nitrogen limitation occurred, followed by stress-induced spore formation. Such events have been described in the literature for *Aspergillus nidulans (4)*. As indicated earlier, spore formation influences the optical density measurement in a non-reproducible way. Figs. 1 and 2 illustrate typical examples of the difference in growth rate in the presence of malt extract and glucose.

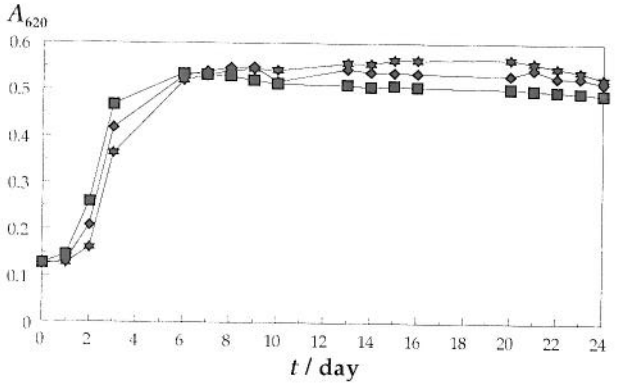

Fig. 1. Growth of fungi in 1 × MEB (malt extract broth) with different inoculation levels, and gelatine as solidifier. The well is covered with paraffin. (1e4 ■; 1e3 ◆; 1e2 ✱)

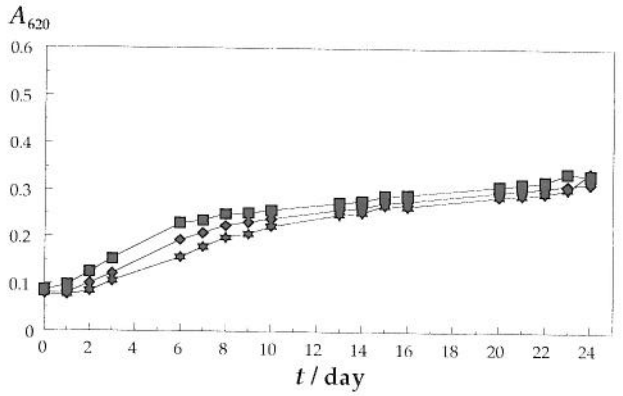

Fig. 2. Growth of fungi in 50 mM glucose with different inoculation levels, and gelatine as solidifier. The well is covered with paraffin. (1e4 ■; 1e3 ◆; 1e2 ✱)

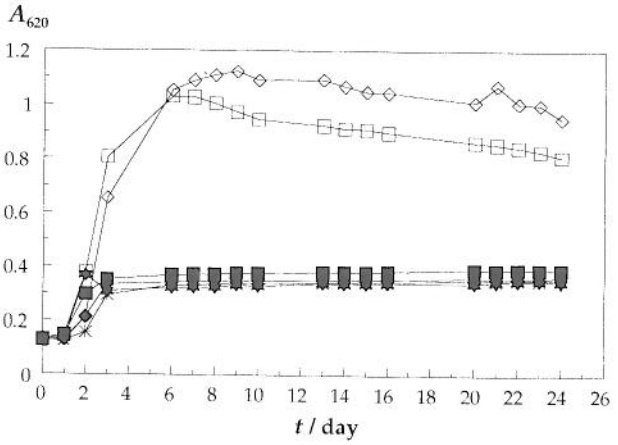

Fig. 3. Growth of fungi in 1 × MEB with varying inocula levels and varying positions. The well is covered with paraffin. [1e4 (well B4) ■; 1e3 (well C4) ◆; 1e2 (well D4) ✱; 1e4 (well F4) □; 1e3 (well G4) ◊; 1e2 (well B4) ×]

Randomisation

The set-up of the experiment must be randomised. The need for randomisation was shown in experiments where every well was filled with similar test solutions. While culture conditions in each well were identical, different absorbance readings were obtained. Thus we concluded that the location of the well on the plate is important, and therefore randomisation is a must. Fig. 3 shows the difference in well position. Different inocula levels were used in this test.

Inoculation level

Different inoculation levels were tested: 1×10^2, 1×10^3, and 1×10^4 per well. Fig. 4 shows the average of multiple wells of different inoculation levels in 1 × MEB with

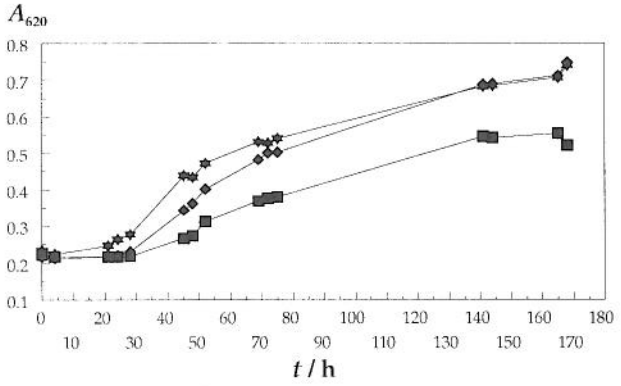

Fig. 4. Growth of fungi at varying inocula levels in 1 × MEB with 0.4% agar as solidifier. The well is covered with paraffin. (1e2 ■; 1e3 ◆; 1e4 ✶)

0.4% agar as solidifier. The apparent lag-times of 1×10^2 and 1×10^3 spores per well were the same, whereas 1×10^4 inoculation level took a slightly shorter time to grow. The total yields of 1×10^3 and 1×10^4 spores per well were the same. As growth rates were similar, we recommend inoculation levels of 1×10^2 and 1×10^3 spores per well in typical antimicrobial screening experiments.

Covering the wells

Paraffin was used to cover the surface of the media to prevent the variation in absorbance measurements due to the spore formation. In the case of malt extract agar, the variation is due to surface growth. Fig. 5 gives an example of aberrant absorbance readings due to spore formation. Note that if one uses the system for the screening of antifungal compounds their partition coefficient into paraffin oil should be determined.

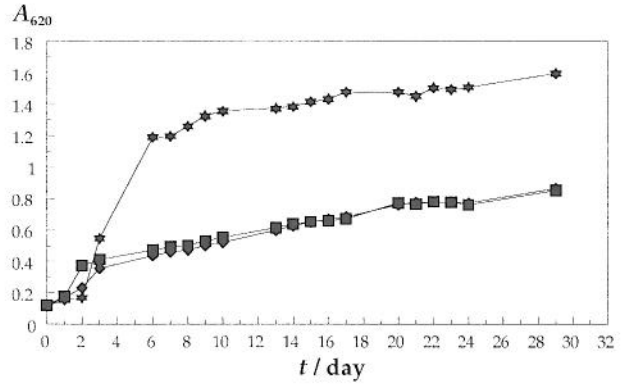

Fig. 5. Growth of fungi at varying inocula levels in 1 × MEB and gelatine as solidifier. The well is not covered with paraffin. (1e4 ■; 1e3 ◆; 1e2 ✶)

An extra cover over the micro-titre plate is a plastic seal which is oxygen permeable (non-toxic from Merlin Diagnostic system). The seal is used to keep the plate sterile and prevent cross-contamination from neighbouring wells. Because the seal is oxygen permeable, it will not significantly inhibit the mould growth. Figs. 1 and 2 are examples in which the wells are covered with paraffin, and Figs. 5 and 6 are the same combinations without the paraffin covering.

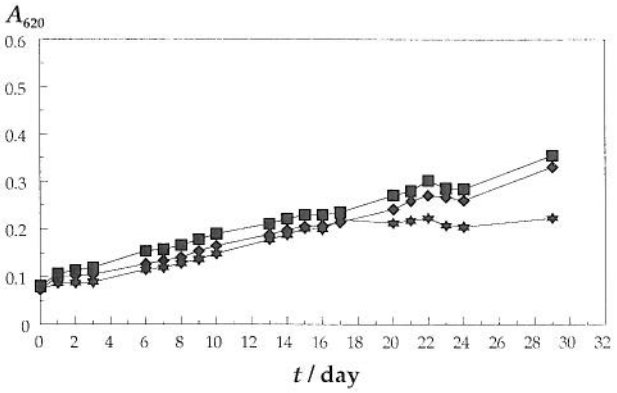

Fig. 6. Growth of fungi at varying inocula levels in 50 mM glucose and gelatine as solidifier. The well is not covered with paraffin. (1e4 ■; 1e3 ◆; 1e2 ✶)

Comparison with a conventional growth assay

Fig. 7 shows the growth measured as the increase of the colony diameter with time. The experiment was done on a malt extract agar plate. The plate was inoculated with 1–3 μL spore suspension containing approximately 1000 spores. The plate was incubated at 20 °C and measured daily with a ruler. In both systems the linear range of the test runs until at least 6 days. Linearity depends on the scale of the assay: the larger the wells or plates, the longer the linear growth phase.

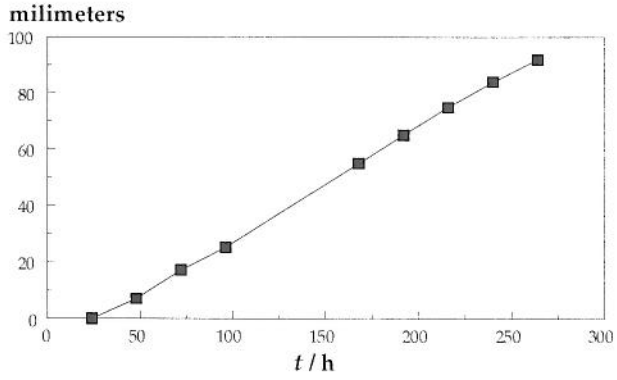

Fig. 7. Growth of fungi on malt extract agar. The growth is measured in time in milimeters

Study of antimicrobial activity

Fig. 8 shows the results of the antimicrobial activity of antifungal substances obtained from Mogen International (the Netherlands). The extracts were purified, chitinase and an antifungal peptide from plant origin *(5)* was added. The proteins were tested as described in Material and Methods. AP24 and chitinase individually had no growth inhibitory effect. After about 100 hours a growth stimulation occurred probably due to adaption of the mould to the proteins. The combination of both proteins did lead to a clear growth inhibition.

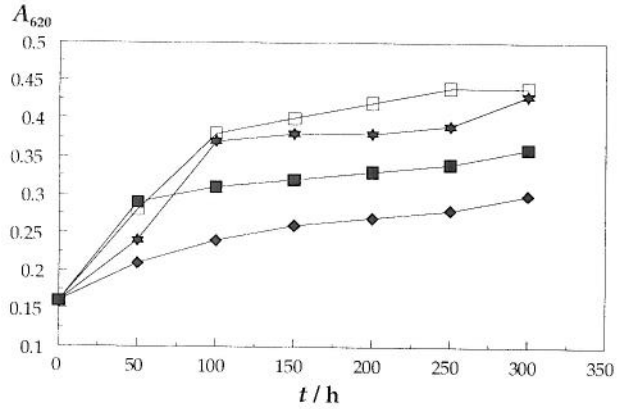

Fig. 8. Growth of fungi in 1 × MEB with agar as solidifier in the presence of an antifungal substance. (blank ■; AP24/Chi ◆; AP24 2.5 ✶; Chi 2.5 □). Concentrations of both compounds are in μg/mL.

The results are in agreement with conventional growth inhibition assays such as the outgrowth of spores on culture plates (results not shown and reference *5*).

Conclusions

The Multiskan MCC/340 is a system that is suitable for screening antifungal substances. The systems described in the literature are usually not suitable for large experiments and are based on a single hypha study. The number of samples that can be tested at the same time is limited. There is one paper that describes the Multiskan MCC/340 *(3)*. That system, however, does not have a stacker, which means that the Multiskan MCC/ 340 is operated by hand. Each plate is shaken for a few seconds, and fungal biomass is sedimented before absorbance measurements are performed. We use a full automatic system with a stacker which is placed in a temperature-controlled incubator. In this way, a lot of parameters can be tested at the same time. Moreover, we use a semi-solid medium so that the spores are fixed in a matrix, and the optical density is measured with every reading on the same spot in the well. This greatly improves the reproducibility of the measurements at different time intervals. The malt extract medium we use is an optimal medium for moulds and yeasts. There is no starvation which can cause the formation of spores formed by stress response. Test conditions that mimic suboptimal growth conditions, which are often observed in practical situations, unfortunately result in spore formation and hence cannot be used reproducibly. The system can also be used for monitoring of yeast growth. However, care should be taken to avoid colony formation of yeast.

In summary, the advantage of the system we use over the systems described in the literature is that we are looking at germination and outgrowth of fungal spores. In the literature, in case of the Multiskan MCC/340 *(3)*, mixtures of spores and mycelium fragments are used, which means that germination and growth inhibition are studied. Another advantage of our system is that it operates fully automatically. This means that plates are filled once, after which the system measures for the time period required without any handling. This system is less time-consuming than a plate assay such as used for the comparison.

Further Use in High Throughput Screening

Finally, the Multiskan MCC/340 system can be used as a screening tool to test substances for their antimicrobial/antifungal activity. Upon identification of antifungal activity, one can study the mode of action with other tools such as fluorescent probes. One can compare the treated and non treated cells with respect to their metabolic activity. The tools that can be used are confocal scanning laser microscopy and flow cytometry. Suitable fluorescent probes are Propidium iodide which gives cells with a permeable membrane a red colour and Calcofluor White which stains chitin in fungal walls as a counter stain *(6)*. There are also probes which can measure changes in Ca^{2+} and Mg^{2+} concentrations. These divalent cations are amongst others important for plasma membrane ATP-ase activity and cellular signal transduction *(6,7)*. Moreover, one can investigate the resistance of mould spores using flow cytometry. Flow cytometry is a technique which can be used to determine physiological characteristics on a single-cell basis. It is possible to sort single cells/spores that are resistant to an antifungal treatment and cultivate them on an agar plate or collect them in a 96-wells plate suitable for growth tests with the Multiskan MCC/340.

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Poboljšani postupak za utvrđivanje inhibicije fungalnog rasta

Sažetak

Postoje različiti načini da se odredi antifungalno djelovanje neke supstancije. To se može postići primjenom disk-metode koja je vizualni postupak. Postoje i automatski sustavi.

U ovom je radu opisan postupak koji se zasniva na mjerenju rasta što se odražava promjenom apsorbancije tijekom inkubacije. Mjerenje apsorbancije provedeno je aparatom Multiskan MCC/340 (Labsystems, Helsinki, Finska). Upotrijebljen je 0,375%-tni agar u 20 g/L ekstrakta slada konačne koncentracije. Jažice su punjene s ukupno 225 μL, od toga 75 μL ispitivanog spoja, 75 μL podloge s fungalnim sporama i 75 μL parafina na površini.

Ako se ploča s 96 jažica napuni pod navedenim uvjetima i smjesti u Multiskan MCC/340 s adapterom, tada se test automatski provodi s maksimalno 20 ploča. Podaci su bili skupljeni i pohranjeni u računalu i obrađeni koristeći SAS. Softwareom se može utvrditi apsorbancija u obliku dijagrama tijekom pokusa, što omogućuje da se vidi ima li ispitivana supstancija antifungalnu aktivnost. Sustav se može koristiti i za preliminarno odabiranje načina provedbe pokusa.