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DETERMINATION OF THE MINIMAL INHIBITORY CONCENTRATION OF THYME ESSENTIAL OIL ON THE GROWTH OF *PENICILLIUM PANEUM* WITH THE DILUTION METHOD

Abstract

The aim of this study was to develop a fast, reproducible and accurate micro-dilution turbidimetric screening method for the antifungal activity of essential oils (EOs). The influences of water activity (aw 0.88, 0.93, 0.95 and 0.97), pH (4.8, 5.0, 5.5 and 6.0) and room temperature (22 and 30°C) were discussed, and were correlated with results of the macro-dilution method. As a result, the minimal inhibitory concentration (MIC) of thyme EO (*Thymus zygis*) in a semi-solid medium on the growth of *Penicillium paneum* was determined. This study shows that results of the optimized method are comparable with results of the macro-dilution method regarding the influence of aw, pH and temperature and thyme EO. Growth at aw 0.88 was significantly reduced compared to aw 0.93, 0.95 and 0.97. The modelled MIC values ranged between 0.89 and 3.52 µL/mL. This study also showed the growth behavioral effect of *P. paneum* at pH 6. The micro-dilution method proved to be a rapid, accurate and reproducible method which can be used for the screening of antifungal activity of essential oils. Furthermore, the method can be applied for antifungal activity screening of other potential water or oil soluble extracts.

Goals

- Optimization of an antifungal screening method for essential oils and plant extracts
- Gaining insight in the growth behaviour of *Penicillium paneum*
- Validating the results of the optimized screening method with the well-described macro-dilution screening method

PhD research goals

- Gaining insight in the different bread spoilage micro-organisms
- *In vitro* screening of biopreservatives through different microbial assays
- Investigating the preservation potential of biopreservatives in (par-baked) bread
- Investigating the impact of production on the quality of par-baked bread
- Assessing the final bread quality: microbiological, technological & sensorial

Experimental design

Dilution method

The dilution method is performed in either a solid / semi-solid medium.

- Solid (malt extract agar, MEA - cfr. macro-dilution – reference method)
  - Data was modelled according to Baranyi & Roberts (1994).

  OR
- semi-solid (yeast extract sucrose - cfr. micro-dilution – optimized method)
  - Data was modelled according to Lambert-Pearson (2000).

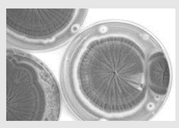

Fig 1 Radial growth

Fig 2 Spectrophotometer

modelling

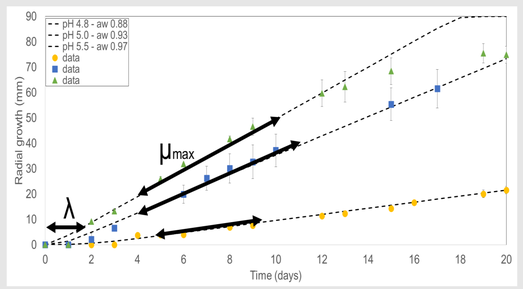

Fig 3 Macro-dilution (µmax & λ)
Maximal growth rate (mm/day) and lag phase time (days)

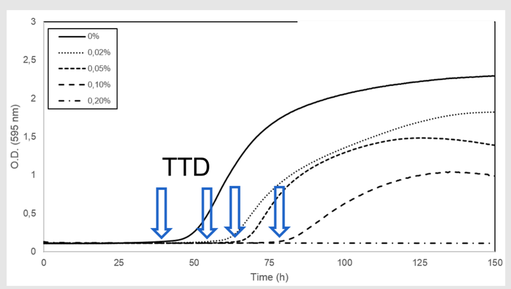

Fig 4 Micro-dilution **(TTD)**
Time to detect (h)

$$RTD = \frac{1}{TTD}$$

$$RTD = P_0 \cdot e^{-\left(\frac{x}{P_1}\right)\cdot P_2}$$

$$MIC = P_1 \cdot e^{\frac{1}{P_2}}$$

Equations to determine MIC with the Lambert-Pearson model
Rate to detection (RTD, 1/h)
Minimal inhibitory concentration (MIC, µL/mL)

Results

Raw data

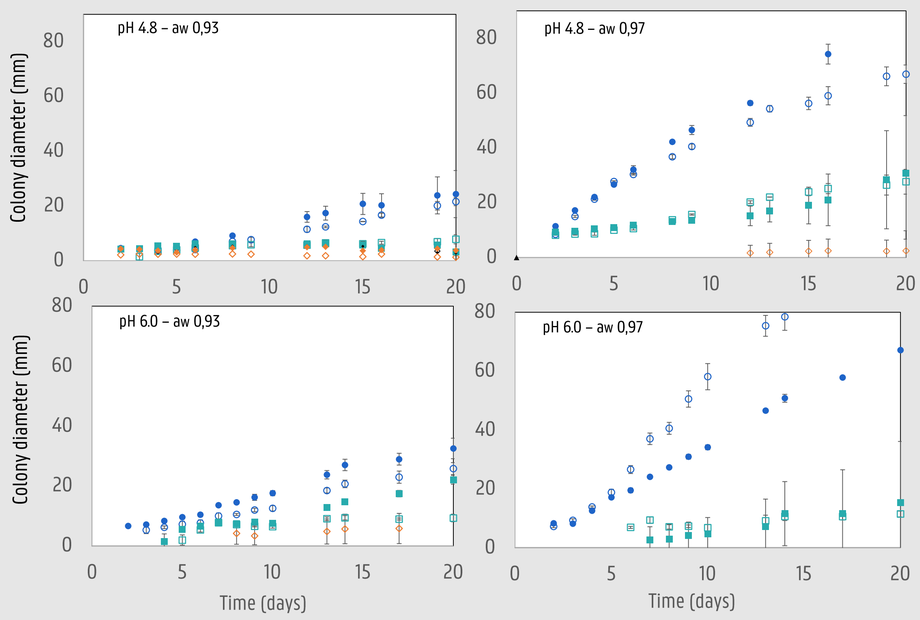

Fig 5 Macro-dilution – Radial growth curves of *Penicillium paneum* obtained at various combinations of pH, a_w & thyme essential oil: 0 µL/mL (○), 0.2 µL/mL (□), 0.5 µL/mL (◊) & 1.0 µL/mL (△). Incubated at 22°C (empty symbols) or 30°C (full symbols) (n = 3).

Macro-dilution

Advantages

- Well-described method
- Easy modelling
- Well-known model
- Microscopic imaging is possible

Disadvantages

- Time-consuming
- Labor intensive
- More material use
- Surface treatment only
- Less data points
- Not automated

Micro-dilution

Advantages

- Less-labor intensive
- Low material need
- Start of growth is accurately recorded
- Easy MIC determination
- Suitable for exploring effects of pH, aw, T, conc

Disadvantages

- Medium must be clear
- Modelling is not suitable for low aw
- Initial cost of filters and spectrophotometer

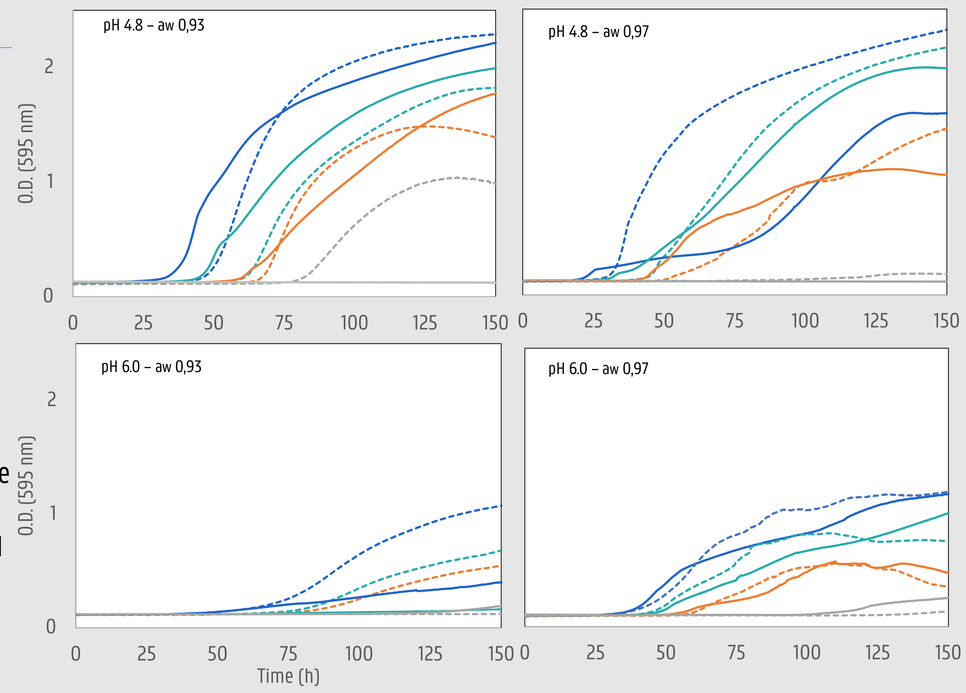

Fig 6 Micro-dilution – Average growth curves (8 individual curves) showing growth of *Penicillium paneum* obtained at various combinations of pH, a_w & thyme essential oil: 0 µL/mL (blue), 0.2 µL/mL (green), 0.5 µL/mL (orange) & 1.0 µL/mL (grey). Incubated at 22 (striped lines) and 30°C (full line)

Processed data

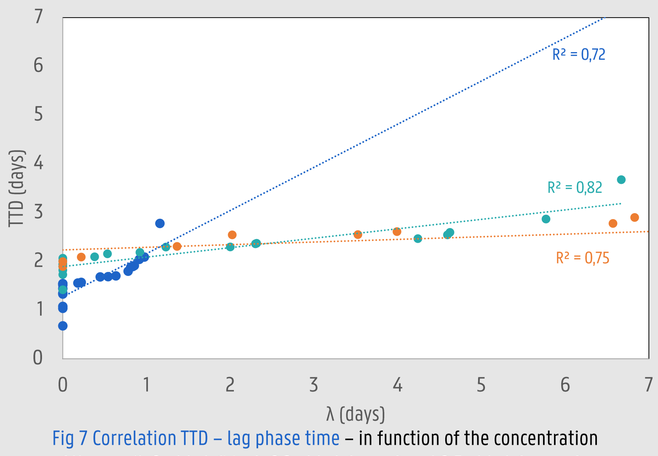

Fig 7 Correlation TTD – lag phase time – in function of the concentration of thyme oil: 0 µL/mL (blue), 0.2 µL/mL (green) and 0.5 µL/mL (orange)

Table 1 MIC - MIC thyme oil concentration values (µL/mL) with their 95% confidence limits (LL: lower limit; UL: upper limit) on growth of *P. paneum* in function of pH (4.8, 5.0, 5.5 & 6.0), a_w (0.93, 0.95 & 0.97) and temperature (22 & 30°C) (* : no model value)

pH		a_w 0.93	LL	UL	a_w 0.95	LL	UL	a_w 0.97	LL	UL
4.8	22°C	2.25	1.74	2.82	2.96	2.10	4.44	1.14	1.04	1.26
	30°C	1.16	1.03	1.32	2.40	2.08	2.80	1.31	1.14	1.53
5.0	22°C	1.12	1.01	1.24	2.74	2.06	3.76	1.70	1.51	1.93
	30°C	1.39	1.13	1.72	2.43	2.01	2.99	2.85	1.91	4.54
5.5	22°C	2.59	2.14	3.12	3.30	2.37	4.88	1.81	1.58	2.10
	30°C	0.89	0.75	1.09	2.65	2.14	3.37	1.61	1.24	2.10
6.0	22°C	3.52	2.78	4.43	2.03	1.69	3.43	2.77	2.26	3.45
	30°C	*	/	/	2.40	2.03	2.89	2.64	2.00	3.64

Results summary

- Growth at a_w 0.88 was significantly reduced compared to a_w 0.93, 0.95 and 0.97.
- Growth at pH 6 and a_w 0.93 was postponed. The inhibitory effect was most present at the highest temperature of 30 °C. These findings indicate that there are mechanisms active that prevent germination of the mould *P. paneum* at pH 6.0 (cfr. "crowding effect").
- The antifungal activity of thyme essential oil was validated with the micro-dilution assay by the extension of the TTD.
- An incubation time of 7 days was suitable to enable a good modelling of the MIC.
- When comparing MIC-values of different authors, it is important to known that there is a difference in conidial germination (micro-dilution) and mycelial growth (macro-dilution). This will definitely impact the end result.

Conclusion

It can be stated that the optimized micro-dilution method is a good alternative for the time-consuming, labor and material-intensive macro-dilution assay based on the follow-up of radial growth of fungal colonies. The micro-dilution method is suitable for all kinds of water or oil solutions, provided that these solutions are clear. To screen the antifungal activity of essential oils as an ingredient of a model matrix or an actual food matrix, it is important to use a dilution test instead of a diffusion test where with the latter the essential oil antifungal activity is tested in the volatile phase only (e.g. application fields: food packaging). In addition, MIC values determined with diffusion assays will be lower compared to dilution assays, as incorporation of the essential oils in a matrix results in a slower release of antifungal active volatile components. The micro-dilution method overcomes issues of phase separation in case of oil testing and provides a rapid screening of multiple substances, at different concentrations, and under various incubation conditions (pH, a_w, temperature). In addition, the easy and reproducible modelling of the MIC is the perfect step-up for *in vivo* trials which are necessary to overcome matrix effects and to fully understand the mode of behavior of essential oil in a food matrix and as well as to include influences of the essential oil on the structural and sensorial properties of food products.

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