Encapsulation of thyme and oregano CO_2 extracts in Ca-alginate hydrogels for antimicrobial applications

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INTRODUCTION AND OBJECTIVE

Because of the increasing resistance to drugs by some pathogenic microorganisms there is a renewed interest in the use of natural products like plant extracts as antimicrobial agents. The focus of this research is to develop formulation methods that stabilize the active substances, control their release and provide an appropriate handling.

For our encapsulation research we chose *Corynebacterium jeikeium* as a model for a multidrug-resistant microorganism. *C. jeikeium* is a "lipophilic" and multidrug-resistant bacterial species of the human skin flora. It is the most frequently recovered medically significant corynebacterial species at intensive care facilities and has been recognized with increasing frequency as a serious nosocomial pathogen.

MATERIALS AND METHODS

Plant compounds CO_2 plant extracts were kindly provided by FLAVEX Naturextrakte GmbH, Germany. Individual components of plant extracts were purchased with appropriate purity from usual chemical companies.

Preparation of emulsions Oil-in-water emulsions of various lipophilic CO_2 plant extracts or lipophilic individual components of plant extracts were prepared as follows. The components were heated up to 60 °C to lower the viscosity. First, one part lipophilic substance was mixed with one part emulsifier (vortex mixer for µL scale; ULTRA-TURRAX® for mL scale). Then, water was added to a resulting concentration of at most 250 mg/mL for both. The concentrated emulsions can be diluted with water as required.

Encapsulation of CO_2 plant extracts Lipophilic CO_2 plant extracts were encapsulated with a concentration of 250 mg/mL in Ca-alginate hydrogel beads without any emulsifier or with 10 mg/mL Tween® 80.

Cultivation of C. jeikeium *C. jeikeium* K411 originally isolated from the human axilla (Kerry-Williams and Noble, 1984) was grown at 37 °C in BYT complex medium, consisting of 37 g/L brain-heart broth, 10 g/L yeast extract, 1% (v/v) Tween® 80 (Tauch et al., 2004) and optionally 15 g/L select agar.

Agar diffusion test In a first screening it was tested if different free or encapsulated CO_2 plant extracts show any antibacterial potential against *C. jeikeium* in agar diffusion tests.

MIC assay Minimum inhibitory concentrations for emulsions (MIC_E) of different CO_2 plant extracts and for lipophilic individual components of plant extracts were determined with a broth dilution method. Therefore, a *C. jeikeium* culture (exponential growth phase) was diluted to a concentration of $2*10^4$ cells/mL (OD_{600} = 0.0002). The broth was incubated at 37 °C on a microtitre plate in wells containing progressively lower concentrations of the testing substance (100 µL total volume per well). After 24 h 30 µL 0.01 % Resazurin, a visual indicator, which changes its colour from blue to pink in the presence of viable cells, was added and after another 24 h, the MIC, which corresponds to the concentration in the blue well with the highest dilution, was determined.

Determination of release kinetics To investigate the release kinetics of the active ingredients encapsulated plant extracts (with 10 mg/mL Tween® 80) were placed into dH_2O and incubated at 37 °C. The accumulation of active ingredients was tested by making use of the MIC test (Fig. 1). At certain points in time, samples were taken, mixed with Tween® 80 to a concentration of 10 % (v/v) to stabilize the emulsion and diluted, if necessary, with water to obtain sample concentrations of 90 %, 80 % and 60 % (v/v). The samples were serially diluted on a microtitre plate and handled according to the MIC assay. The accumulation is expressed via the dilution which is needed to obtain a minimum inhibitory concentration for emulsions of release samples (MIC_R). This so called accumulation factor can be defined as the theoretically concentration (c_t) divided by MIC_R.

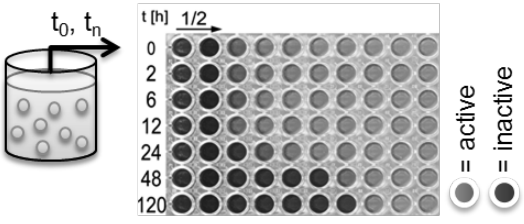

Figure 1: Release of thyme se extract in dH_2O and quantification using a MIC assay

RESULTS AND DISCUSSION

Agar diffusion tests For oregano leaf extract, thyme leaf extract and sage leaf extract agar diffusion tests with 1% (v/v) Tween® 80 showed considerable inhibitory effects for the pure extracts on filter discs and the corresponding encapsulated CO_2 extracts (Fig. 2). After three days the pure extracts showed greater inhibition zones than the corresponding beads. That is probably due to a slowed release of the active substances from the beads.

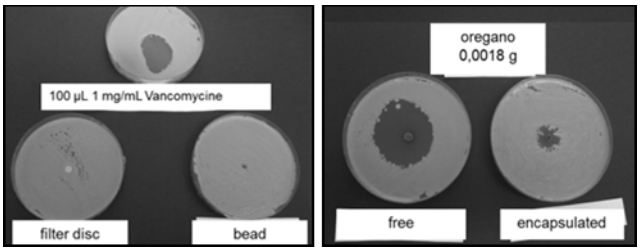

Figure 2: Agar diffusion test with a positive (vancomycine) and a negative control (filter disc and bead without active ingredients) and with oregano extract.

MICs Different emulsifiers were tested and Tween® 80 has proved to be appropriate for the stabilization of emulsions. Affected by Tween® 80, the MIC_E of thyme and oregano CO_2 extracts is significantly lower than that of the principle components (p-cymene, carvacrol, thymol), even in combination (Tab. 1).

Table 1: MIC_Es for emulsions of different CO_2 plant extracts and for lipophilic individual components affected by Tween® 80

	MIC [µg/mL]
Oregano to CO_2 extract	50
Thyme se CO_2 extract	39
Thyme to CO_2 extract	62,5
p-Cymene	>2500
Carvacrol	1000
Thymol	1000
Thymol + Carvacrol (1:1)	1000
Thymol + p-Cymene (1:1)	>1250
Carvacrol + p-Cymene (1:1)	>1250
Thymol + p-Cymene + Carvacrol (1:1:1)	>1250
Rifampicine (control, without Tween® 80)	12,5

Broth dilution methods are widely used to determine MICs but they cannot so easily be used for hydrophobic compounds. The assays are often fitted by using agents like emulsifiers which on the one hand stabilize the test medium but on the other hand incorporate the active substances within the micelles whereby the antimicrobial activity may be influenced. Tween® 80 probably causes further changes in the physicochemical properties of the test system, because it is used as substrate by *C. jeikeium*.

Release kinetics The active substances of e.g. thyme se CO_2-extract accumulate in the medium (Fig 3).

Because the MIC test system is probably influenced by the concentration of Tween® 80 MIC_E and MIC_R should not be equated why the accumulation factor was introduced. Nevertheless and despite the limitations mentioned above the presented method is sufficient to describe release kinetics. Moreover, the test system is a suitable method to display the release of all active substances collectively in contrast to methods that detect only individual components. As we still can't link the high activity of CO_2 plant extracts (thyme, oregano) to one or more principle components (p-cymene, carvacrol, thymol) it is more suitable to observe the efficacy in total than the concentration of single substances.

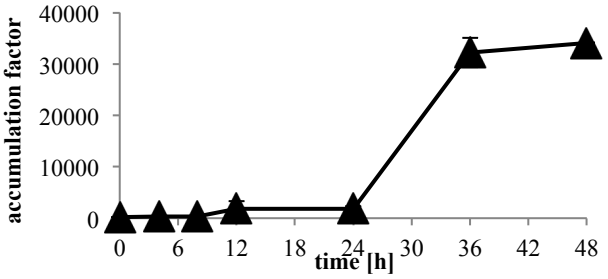

Figure 3: Release of the active compounds of encapsulated thyme se CO_2-extract

CONCLUSIONS

Hydrogel encapsulation is principally suitable for slow release of some CO_2 plant extracts. The presented test system for the detection of MICs and for the characterization of release kinetics will be optimized and still more fitted to release experiments with hydrophobic compounds. Further investigations will deal with characterization of encapsulation materials, bead morphology and emulsifiers as well as their influence on the release kinetics.
Moreover, other individual components of thyme and oregano CO_2 extracts will be tested solitary and in combination.

REFERENCES

- Tauch, A., Bischoff, N., Pühler, A., Kalinowski, J., 2004. *Comparative genomics identified two conserved DNA modules in a corynebacterial plasmid family present in clinical isolates of the opportunistic human pathogen Corynebacterium jeikeium*. Plasmid 52, 102–118.

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