Development of a biological tick control agent based on an innovative Attract–and–Kill strategy

DISSERTATION

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Content

List of abbreviations

a_w	Water activity
AG	Amyloglucosidase
CFU	Colony forming unit
CMC	Microcrystalline cellulose
CO_2	Carbon dioxide
EPF	Entomopathogenic fungi
H_2S	Hydrogen sulfide
HCl	Hydrogen chloride
$MgSO_4$	Magnesium sulfate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_3	Ammonia
PEG	Polyethylene glycol
±sd	Standard deviations
±se	Standard errors
Tris	Tris(hydroxymethyl)aminomethane

1 Summary

As vectors of a large number of pathogens, ticks pose a considerable problem not only in Germany, but also in most parts of the world. Most bites in Germany are caused by *Ixodes ricinus* for which there is no specific scientifically proven control agent available. An extensive application of synthetic chemical acaricides has lethal effects on non-target organisms and can further cause resistances in the target organism. However, repellents reduce the contact probability between host and tick, but do not regulate the further distribution of diseases. Although biological control options like entomopathogenic fungi are known to have high potential, there is a considerable lack of studies on formulation and applicability. The disadvantages, such as a low persistence as well as a not consistent efficacy of entomopathogenic fungi (EPF) in the field, still predominate over explicit advantages of biological control agents like a high target specificity, resulting in less toxicity for non-target organisms and a decreased probability of causing resistances in targets. Encapsulation of biological control agents in a biodegradable polymer matrix with supplemented nutrients can improve their stability, virulence as well as applicability for the above ground control of pests, such as ticks. Therefore, the overall aim of this work was to develop a biological tick control agent for the application in the field. To reduce the required dosage of a control agent, a biological attractant was encapsulated. *S. cerevisiae* releases CO_2, attractive to ticks, by metabolizing supplemented nutrients. A co-encapsulated kill component, such as EPF biomass, germinates inside the bead and produces aerial conidia, the infection unit of EPF, on the bead surface. The attraction due to the attractant enables direct contact of the tick to the kill component, ideally resulting in high mortality rates.

This work is subdivided into three chapters, focusing on different aspects of the development of an Attract-and-Kill formulation:

Attract

An Attract formulation was developed aiming at a slow CO_2 release for an application above ground in order to attract ticks. Biodegradable calcium alginate beads containing *S. cerevisiae* cells, granular corn starch and 0.1 $U \cdot g^{-1}$ amyloglucosidase fulfilled the required release of 0.2 $mL \cdot (g \cdot h)^{-1}$ CO_2 at a temperature range between 10 °C and 30 °C for both moist as well as dried and rehydrated beads. By supplementing urea as nitrogen source, the CO_2 release of moist beads was further increased, at least for a short time.

Dried and rehydrated Attract beads were demonstrated to have a significant attractive effect on *I. ricinus* nymphs in a dual-choice experiment.

Kill

In this chapter, three highly virulent pre-selected *Metarhizium* spp. isolates were cultivated under different conditions on solid medium and in submerged culture, in order to screen for the most promising isolate under biotechnological aspects. The highest blastospore concentrations were obtained with the isolate *M. brunneum* Ca8II both for conidiation on solid medium and in submerged cultures. However, *M. pemphigi* X1c produced the highest total dry weight in submerged cultures. Since submerged cultivation provides several advantages over solid cultivation of EPF, blastospores of all three isolates produced by submerged cultivation were encapsulated in calcium alginate with supplemented nutrients. These microfermenter beads allowed a growth of fungal biomass and ensured formation of aerial conidia on the bead surface with the highest concentration for moist beads containing starch and *M. brunneum* Cb16III blastospores. By replacing starch with chitin, a significant increase in conidiation by more than two-fold for all isolates was attained. With chitin as sole nutrient, *M. pemphigi* X1c showed the highest conidiation on both moist and dry and rehydrated calcium alginate beads. The supplemented nutrients enabled a significant increase in drying survival of the sensitive blastospores with a maximum survival of 14.7%.

The incorporation of hematin on the bead surface, as aggregating pheromone to extend the contact time between tick and bead, did not affect the conidiation on the beads. A qualitative aggregating effect was observed, however not accompanied with higher mortality rates as shown in a virulence test. The highest mortality of *I. ricinus* nymphs under laboratory conditions was achieved with sporulating beads containing *M. pemphigi* X1c blastospores.

Attract-and-Kill

First steps towards Attract-and-Kill formulation containing both *S. cerevisiae* cells and *M. pemphigi* X1c biomass, supplemented with nutrients were made. At least, a low CO_2 release combined with a moderate conidiation was demonstrated. The influence of the Crabtree effect on conidiation due to high glucose concentrations inside the beads was investigated. High ethanol concentrations inside the beads led to an inactivation of co-encapsulated EPF biomass.

Altogether, this work demonstrates the high potential of the combination of encapsulated attractants and Kill components as biological tick control agents. The attractive effect of a CO_2-releasing formulation on *I. ricinus* nymphs was proven. Furthermore, a *Metarhizium* spp. isolate was identified that fulfills all the required needs, such as a high virulence and the capability to be easily produced with a high yield in submerged cultivation. The possibility of encapsulation and drying of blastospores was demonstrated. By co-encapsulation of *S. cerevisiae* cells with *Metarhizium* spp. blastospores, this work provides a promising approach for the development of an applicable biological tick control agent.

2 Introduction

Ticks are vectors for a multitude of diseases worldwide, posing a risk not only to domestic and farm animals but also to humans [1]. Especially in the agricultural sector, tick bites cause massive economic losses. The transmission of pathogens cause infections in the host animal, frequently leading to losses in productivity as well as increasing morbidity and a higher mortality [2-5]. Tick bites also pose a great danger to humans. Infestation by an infected tick can cause incurable diseases in humans, such as Lyme borreliosis, Tick-borne encephalitis or Babesiosis [6-9]. In Europe and Asia alone, up to 10,000 reinfections with TBE are reported every year [10].

In Germany, the risk of human infections is the most serious concern, whereas agricultural sectors have not been affected so far. Especially the castor bean tick *I. ricinus*, as the most important vector of diseases, causing 90 to 95% of all tick bites, is an important vector [11]. Other species as *Rhipicephalus* spp. or *Dermacentor* spp. are more rarely infesting humans. However, their occurrence in Germany and Europe is increasing due to migratory birds, increased imports of international goods and increased percentage of people travelling.

Even though ticks and tick-borne diseases have gained more public attention, no specific control agent for ticks is available so far. The most widespread method worldwide is the direct application of chemical acaricides on host animals [12; 13]. However, it is reported that around 90% of all pesticides do not reach the target [14], can cause lethal effects on non-target organisms and lead to resistances in the target [15], which makes a long-term application questionable. In Germany, the application of acaricides, such as permethrin, is predominantly restricted to veterinary medicine as spot-on or collar, while the use of such biocides on human skin as well as in larger areas is not carried out for health, ecological as well as economic reasons. Besides acaricides, repellent substances, such as essential oils, can reduce the probability of a direct contact between ticks and hosts [16-18]. However, an application of high dosages can lead to injuries, such as allergic contact dermatitis [19]. Since repellents do not reduce the basic problem and people worldwide get a raising awareness of protecting the environment and are less willing to use harmful chemical acaricides [20; 21], biological control agents have moved into the spotlight within the last decade [8; 22; 23].

Besides entomopathogenic nematodes and parasitoids, entomopathogenic fungi (EPF) have been proven to be suitable candidates for biological tick control. EPF are mainly soil-borne

fungi and one of the natural predators of acari such as ticks [6; 24-27]. Compared to chemical insecticides, EPF present many advantages such as high specificity for the target pest with low environmental pollution and minimal risk for vertebrates [28-30]. The most promising genera are *Beauveria* and *Metarhizium*, which have already proven to be virulent against several tick species [22; 31; 32]. Especially fungi of the genus *Metarhizium* have shown potential to be effective as control agent for *I. ricinus* [23; 33].

Commonly, aerial conidia are preferred as active ingredient for EPF-based pest control agents based on their high resistance against environmental factors and the comparatively high shelf life [34-37]. Very few products are based on blastospores (4.1%) or mycelium (2.3%) [37]. Usually, the fungal biomass is applied as a powder or oil dispersion with additional substances such as drying aids in order to improve the stability and shelf life of the product [38-41]. Consequently, large amounts of biomass are necessary. When aerial conidia are applied, a production is usually conducted in solid-state fermentation processes. However, solid-state fermentation is considered to have several disadvantages such as a slow growth rate, poor adjustability and to be labor-intensive [42; 43] compared to submerged fermentation strategies resulting in high cell densities of biomass in a large scale production [44]. It is known that *Metarhizium* spp. produce mycelium and propagules (blastospores) when cultivated in liquid media [45-47]. Even though blastospores are easier to produce and harvest on a technical scale than aerial conidia, they suffer from a higher sensitivity to environmental factors [36]. Furthermore, the majority of pests treated with fungal-based agents show a comparatively low mortality when conidia were directly applied in the field or on animals [26; 48]. A suitable formulation is not only able to stabilize living biomass for storage and application by creating a protective environment [49], encapsulation in biopolymer gels can additionally provide nutrients and other additives to improve growth, virulence and sporulation of the fungus in the field [50].

In order to further reduce the required dosage of tick control agents, the Attract-and-Kill approach is a promising control strategy, already successfully applied below ground [44; 51; 52]. Semiochemicals, such as CO_2, can lure ticks towards a control agent containing EPF as control agent [53]. Incorporation of aggregation pheromones can increase the efficacy by extending the contact time of the tick and the control agent [54]. Altogether, the development of a suitable formulation is inevitable to exploit the full potential of a biological control agent, tailored to the application against ticks.

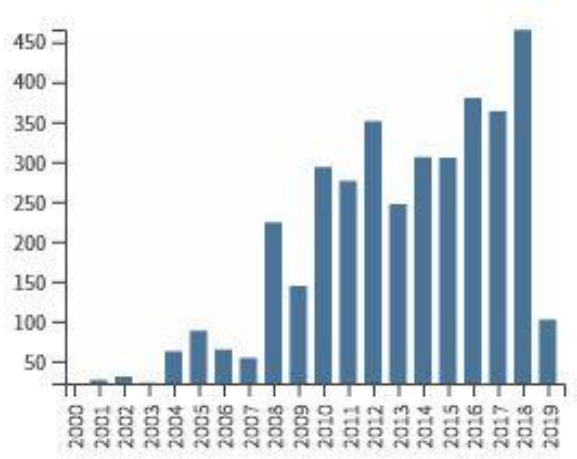


Figure 2.1 Sum of times cited by year over the past 20 years, using the queries '(tick OR ticks) AND ((entomopathogenic AND (fungus OR fungi OR fungal)) OR epf)' in the topic search criteria (Results found: 207, Sum of the Times Cited: 3,823, Average Citation per Item: 18.47, h-index: 30; source: Web of Knowledge: 28.05.2019).

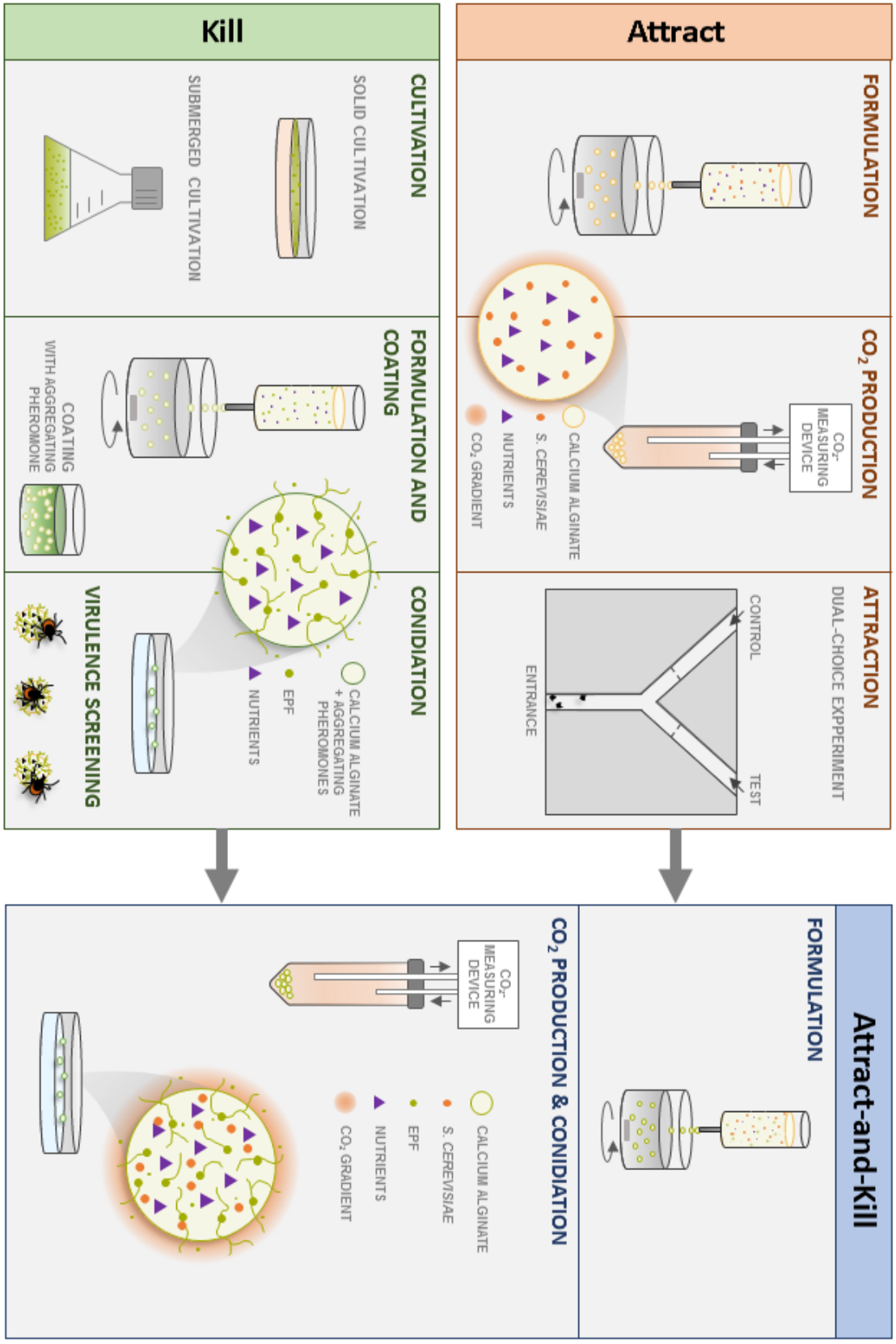


Figure 2.2 Overview on the conception of this dissertation dealing with the development of Attract-and-Kill formulations for biological control of ticks, especially *I. ricinus*. The work is divided into three central chapters. EPF = Entomopathogenic fungi.

3 Aims

The present work was part of the ZIM (Central innovation program of the middle class) project "BIOZEC" (No. 2426511CR4) which was funded by the BMWi (German Federal Ministry for Economic Affairs and Energy). An overview of the whole joint project is illustrated in figure A1 (supplementary material).

Different tick species, such as the common tick *Ixodes ricinus*, play an important role in the transmission of incurable diseases not only to domestic and farm animals but also to humans in Germany and worldwide. At present, ticks can only be controlled using unspecific acaricides, being usually harmful for human, animals and environment. Since no individual control measure is available on the market and due to the increasing number of risk areas for infection by tick bites every year, there is a significant demand for an effective biological control agent. Therefore, the overall aim of this work was to develop a biological tick control agent, attracting ticks by the release of carbon dioxide, to enable direct contact with a natural Kill component, as an entomopathogenic fungus, co-encapsulated in a biopolymer matrix.

The overall aim of this work was segmented into sub-aims and organized in three chapters:

Chapter 6.1: Attract

I. Development of an Attract formulation consisting of calcium alginate, supplemented with *S. cerevisiae*, granular corn starch and amyloglucosidase (AG), optionally supplemented with urea; for the application above ground. A release of CO_2 over two weeks was desired, which is why different AG concentrations and incubation temperatures were tested. The attractive effect of the formulation was tested for *I. ricinus* nymph with a dual-choice test in a Y-olfactometer setup.

Chapter 6.2: Kill

II. Examination of three highly virulent pre-selected *Metarhizium* spp. isolates on their conidiation at different temperatures on solid medium. Furthermore, the cultivation in submerged cultures to check for the production of high amounts of blastospores under different conditions was examined. For this purpose, the *Metarhizium* spp. isolates were cultivated in a nutrient rich medium at different temperatures and a range of initial

pH values to identify an isolate with high blastospore production and stability at a broad range of pH values.

III. Development of a Kill formulation, containing *Metarhizium* spp. blastospores, supplemented with granular corn starch, aiming at a high conidiation of the isolates on the bead surface.

IV. Drying of the Kill formulation with a high drying survival while maintaining the conidiation on the bead surface. Moreover, chitin powder from shrimp shells was evaluated as alternative nutrient source and its influence on conidiation as well as drying stability for the isolates.

V. Hematin was coated on the bead surface to examine its suitability as aggregation pheromone with maintaining conidiation on the bead surface and virulence to enhance the contact time between tick and bead.

VI. Examination of the virulence of newly formed aerial conidia on the bead surface against *I. ricinus* nymphs and adults under laboratory conditions in consideration of the influence of the supplemented nutrient.

Chapter 6.3: Attract-and-Kill

VII. Development of an Attract-and-Kill formulation, containing both *S. cerevisiae* cells and *M. pemphigi* X1c biomass, supplemented with starch and optional AG, aiming at a constant CO_2 release and a sufficient conidiation on the bead surface for tick infection.

4 State of the Art

4.1 *Ixodes ricinus*

Ticks are temporary and obligatory bloodsucking arthropods belonging to the class of arachnids. Along with mites, they constitute the subclass Acari. Generally, ticks are divided into two major families, the Ixodidae or hard ticks, and the Argasidae or soft ticks [6]. The entire dorsal surface of the Ixodidae, in contrast to the Argasidae, is covered with a rigid chitinous shield [55]. Within the hard ticks, *Ixodes* is the largest genus, including 217 species [3]. These ectoparasites play an important role in the transmission of over 100 diseases not only to domestic and farm animals but also to humans [1]. Regarding livestock, each time a tick bites it causes stress and weakens the immune response affecting the productivity of the animal. This results in losses in the production of meat and milk, increased morbidity and in many cases mortality, causing high annual economic losses [2-5]. Among the most important diseases transmitted by ticks are Lyme borreliosis (*Borrelia burgdorferi*), Tick-borne encephalitis (TBE, TBE virus) and Babesiosis (*Babesia* spp.) [6-9], whereas TBE is known to be the most important tick-transmitted human viral disease in Europe and Asia with up to 10,000 human cases annually [10] and with an increasing number of risk areas every year [56]. Vaccines, available on the market, protect only against infections by viruses vectored by ticks [57]. *Ixodes ricinus*, also known as castor bean tick, is vector of 95% of all tick-transmitted pathogens in Europe. Furthermore, it is known to be the most important vector of these diseases in Germany, causing 90% to 95% of all tick bite incidences in humans [11]. *I. ricinus* is a host-unspecified, generalist tick that feeds on more than 300 different vertebrate species [58]. In the USA, *Ixodes scapularis* is an important carrier of TBE and Lyme borreliosis to humans. *Dermacentor*, *Rhipicephalus* and *Amblyomma* [6] belong to other important disease vectors, especially to pets and farm animals.

The life cycle of *I. ricinus* contains three stages as illustrated in figure 4.1: larva, nymph and adult. For molting as well as oviposition by female adults, a preceding blood meal is necessary, whereas each stage feeds on different hosts. Larvae and nymphs preferably infest rodents, such as mice and squirrels [59-63], but also ground-foraging birds, lizards and cloven hoofed animals [64; 65]. Mature ticks usually feed on larger mammals, such as roe deer [66]. Although humans can be infested by all stages, nymphs are the most frequent parasite [8]. Especially the wide

host range leads to a high intra-species transmission of several infections, since hosts can be infested by a multitude of individuals [67].

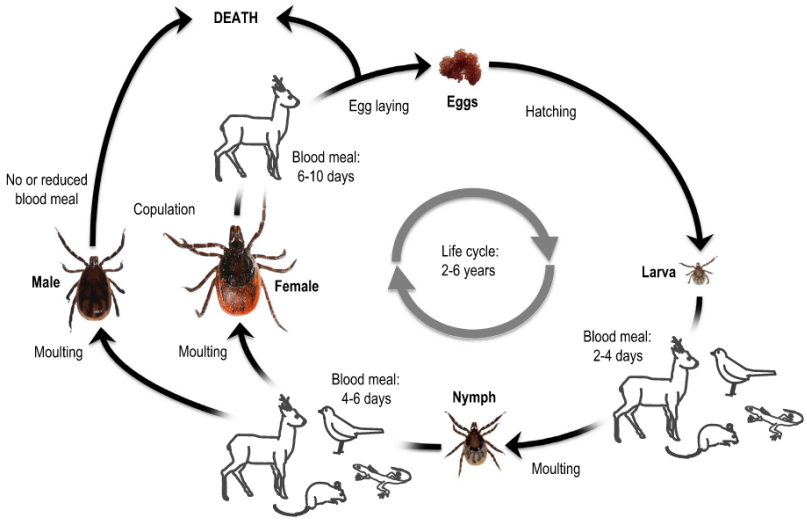


Figure 4.1 Life cycle of *I. ricinus.* [68]

The selection of the host depends on the habitat, the behavior of both host and tick as well as the prevailing climatic conditions [63]. To improve the chance of finding a host (questing), ticks climb in the vegetation to an elevated height, whereas the three tick stages show different questing heights: Larvae and nymphs can predominantly be found between 0 cm to 70 cm, whereas adults can be observed up to 150 cm, depending on the vegetation [69; 70]. The difference in the questing heights may be caused by the higher sensitivity of immature tick stages to ambient humidity [71].

I. ricinus occurs in forests, but also in parks and gardens and can be active also during winter, depending on temperatures and photoperiodicity [7; 72-74]. During this time, ticks usually do not infest hosts. The highest questing activity is connected to an ambient temperature above 7 °C, usually reached in the time from February to October [75]. High temperatures connected to a low relative humidity reduce off-host tick activities, due to the low tolerance towards desiccating conditions [76].

4.2 Encapsulation in calcium alginate

The encapsulation of microorganisms in calcium alginate is a well-established method suitable for several applications [30]. Alginate is a non-toxic and biodegradable, hydrophilic polysaccharide extracted from brown seaweed [77]. The two components β-D-mannuronic acid and α-L-guluronic acid are present in different arrangements and are 1,4-linked [78; 79]. Bivalent cations, such as calcium, bind to the carboxylate group of polyguluronic acid through ionic interactions and form a three-dimensional, semi-permeable hydrogel. The binding takes place according to the "egg box model" [80]. An overview of the chemical structure of sodium alginate and the molecular processes during formulation is shown in figure 4.2.

Calcium alginate beads can be formed by dripping the sodium alginate, supplemented with different components, into a cross-linking solution containing divalent cations. The gelation takes place from the outside to the inside due to ionotropic gelation [49]. To ensure complete crosslinking and stable structure of the bead, a sufficient incubation time is necessary.

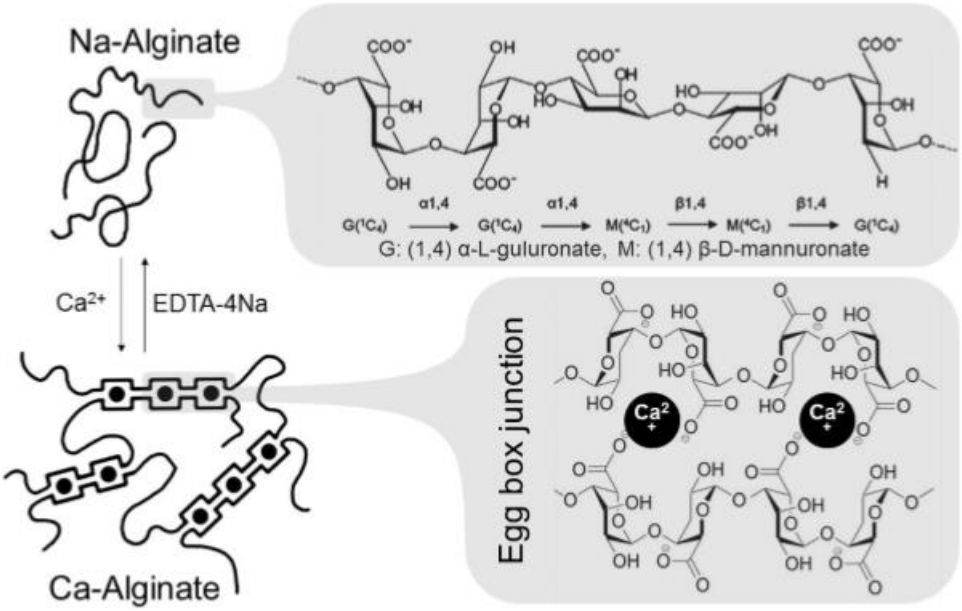


Figure 4.2 Molecular processes during ionotropic gelation of calcium alginate [49].

The encapsulation in the biopolymer matrix can reduce various environmental stress factors for the encapsulated microorganisms, such as UV light, dryness and temperature [37; 81; 82]. Co-encapsulation of further additives can have a positive effect on growth, drying survival and storage stability [50].

4.3 Attract

4.3.1 Scent perception of ticks

To find a potential host, *I. ricinus* possesses an organ located in the dorsal surface of the lowest segment of the front pair of legs. The so-called Haller's organ consists of an anterior pit and a posterior capsule, both with multipurpose and single-pore sensilla (figure 4.3).

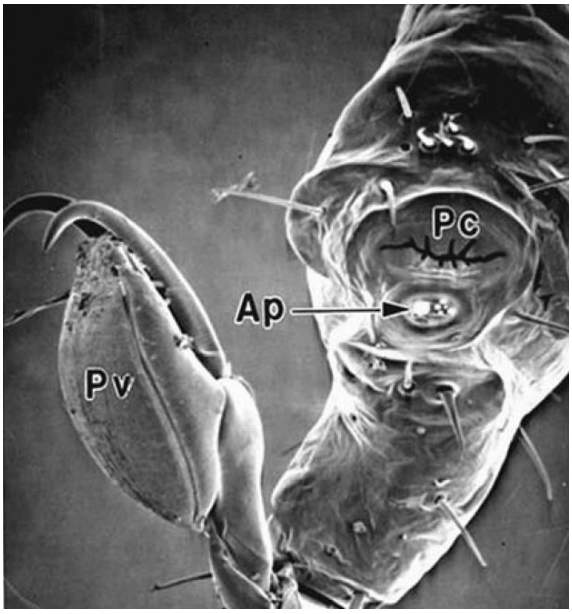


Figure 4.3 Scanning electron micrograph illustrating the dorsal surface and terminal organ of the front leg of *Dermacentor variabilis.* Ap, anterior pit of Haller's organ; Pc, posterior capsule of Haller's organ; Pv, pulvillus [55].

The tick uses this highly complex sensory apparatus as primary organ for determining host location, determining host odors [83], recognizing pheromones [84; 85], and other sensory functions, such as detection of temperature [55; 86]. The sensilla in this organ respond to thermal energy and to elevated levels of CO_2, NH_3, H_2S, as components of breath and body odor [53] and other odorants from warm-blooded animals that come within range, facilitating host location and contributing to successful attachment [8; 87].

Promising results on the attractive effect of CO_2 on ticks were already obtained in studies that applied CO_2 traps, containing dry ice or CO_2 gas bottles [88-92]. Schulze *et al.* [93] demonstrated an attracting effect of a CO_2 trap on *Ixodes scapularis* in the field. Also Gherman *et al.* [94] could improve the traditional method of flagging (collection of ticks from the environment with a cotton flag) by CO_2 dispersed into the white flannel. A study by van

Duijvendijk *et al.* [95] verified an activation of *I. ricinus* nymphs due to applied CO_2. Even other tick species, such as *Amblyomma* or *Dermacentor* spp., were mainly attracted when CO_2 baits were applied [93; 96-98].

Figure 4.4 Adult *Ixodes* spp. tick questing [99].

The questing *I. ricinus* locate hosts in open spaces of forests, brushland, or grassland habitats. To enable attachment, the tick climbs vegetation, spread their first pair of legs (figure 4.4) containing the Haller's organ, and wait for the host [55]. Ticks use their forelegs in a manner similar to how insects use antennae.

4.3.2 *Saccharomyces cerevisiae*

The microorganism *Saccharomyces cerevisiae*, known as baker's yeast, is exploited in a broad range of application fields, including chemistry and food industries, health care and research [100; 101]. *S. cerevisiae* is a unicellular eukaryote and a chemoorganotrophic facultative aerobic organism [102]. *S. cerevisiae* can generate energy from its principal energy source glucose via aerobic metabolism (cell respiration) or anaerobic metabolism (fermentation), both preceded by cytosolic glycolysis, the general pathway for conversion of glucose to pyruvate [103]. Under aerial conditions, pyruvate is oxidized to CO_2 via cell respiration and oxygen is reduced to water. Energy in the form of ATP is generated [104]. Under anaerobic conditions and due to high glucose concentrations pyruvate is converted to ethanol and CO_2 via acetaldehyde. The alcoholic fermentation is shown schematically in figure 4.5.

S. cerevisiae can metabolize glucose to ethanol and CO_2 under aerobic conditions [105]. This regulatory phenomenon is called 'Crabtree effect' and is mediated by a critical glucose concentration from 0.1 $mg \cdot mL^{-1}$ [106]. At high glucose concentrations a repression of mitochondrial function is induced, leading to a less efficient energy production, compared to

respiration [107], but at the same time to a faster liberation of energy and thus more rapid growth [108]. After consumption of glucose, ethanol is assimilated as an energy source. The development of this phenomenon is due to an adaptation to high glucose concentrations [103; 109].

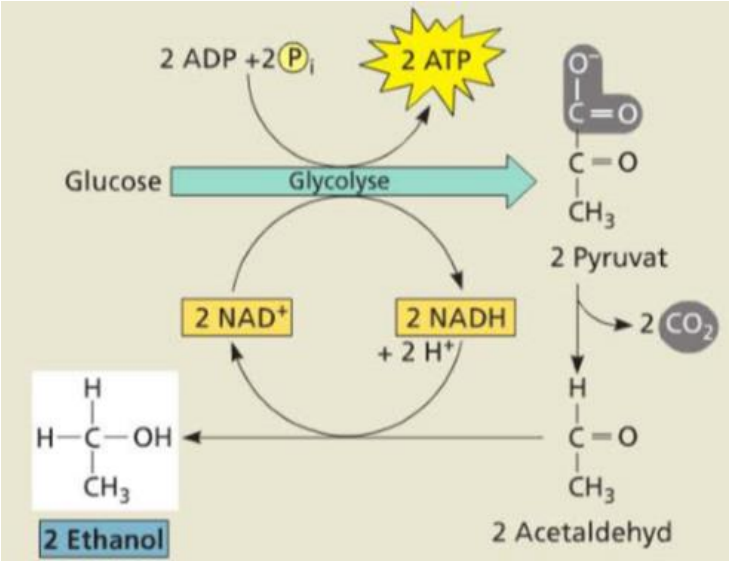


Figure 4.5 Fermentation in *S. cerevisiae* [104].

S. cerevisiae is known to have a relatively high resistance to high glucose concentration as well as ethanol toxicity [110; 111], even though ethanol can retard the growth rates of yeasts [112]. A study of Aguilera *et al.* [113] reported a tolerance of 4% (v/v) ethanol for *S. cerevisiae*. This was supported by Ansanay-Galeote *et al.* [114]. In this study, a final viability of the cells was at least 85%, also when *S. cerevisiae* cells were exposed to ethanol concentrations up to 6% (v/v). Balakumar *et al.* [115] even reported a survival of a concentration of 10% ethanol, added to the medium. The survival of higher concentrations due to slow adaption is conceivable. It can be further presumed that the production of ethanol is inducted by *S. cerevisiae* as a consequence to defend its niche from other microorganisms [109; 116].

4.3.3 Formulation of attractants

S. cerevisiae

The microorganism *S. cerevisiae* is able to produce CO_2 by utilizing glucose. For an application as attractant producing component in a biological control agent, a supply of nutrients within a bead formulation with a biopolymer, such as calcium alginate, is necessary. Due to their low molecular weight, monosaccharides cannot be encapsulated permanently in calcium alginate because it is not retained by the polymer matrix [49; 117]. Since an encapsulation of monosaccharides is not possible due to the low molecular weight [49], starch can be supplemented alternatively. Starch is the main storage material of plants and is composed of glucose units which are glucosidal α-1,4-linked (figure 4.6).

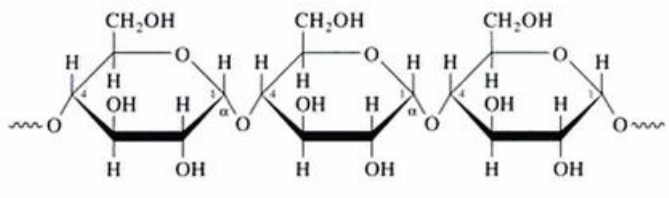


Figure 4.6 Chemical structure of starch [118].

As already demonstrated by Humbert *et al.* [119], supplemented starch, as a carbon source with high molecular weight, remains in the bead and furthermore enhances the structure of the hydrogel network [120]. Because *S. cerevisiae* lack the enzymatic equipment for the assimilation of starch, the supplementation of an amyloglucosidase (AG) is needed to make the nutrient source available (figure 4.7).

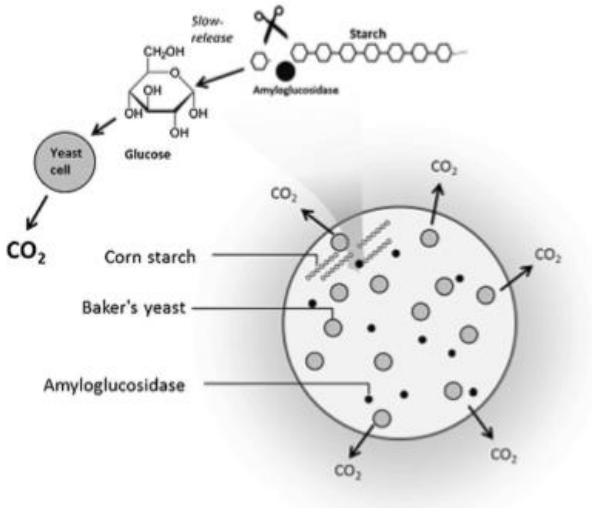


Figure 4.7 Schematic illustration of an Attract formulation with controlled CO_2 release [119].

The co-encapsulation with EPF biomass is another conceivable and already reported method to make starch available for the encapsulated *S. cerevisiae* cells [44; 51; 52].

Urea supplementation

Urea is a natural substance that is excreted as a metabolic product through urine and sweat of mammals. Pure urea is a white, crystalline, odorless, non-toxic and non-hazardous solid that dissolves well in water and ethanol and is one of the most widely produced chemicals [121]. It is being used in many fields such as medicine, fertilizers, food and environmental protection [122].

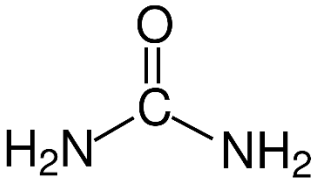


Figure 4.8 Chemical structure of urea [123].

S. cerevisiae is reported to utilize urea as nitrogen source [124]. Urea is degraded in two steps, with a urea carboxylase and an allophanate hydrolase (figure 4.9) [125]. Because the sensilla of the Haller's organ, ticks react to both CO_2 and NH_3 as components of body odor [53] the supplementation of urea may lead to an increase of the CO_2 production of the Attract beads. The resulting release of NH_3 may further enhance the attractive effect on ticks.

$$\text{Urea} + \text{ATP} + HCO_3^- \xrightleftharpoons{Mg^{2+}\,K^+} \text{Allophanate} + \text{ADP} + P_i$$

$$\text{Allophanate} \longrightarrow 2NH_3 + 2CO_2$$

Figure 4.9 Two step degradation of urea in *S. cerevisiae* cells [125].

Due to its low molecular weight, an effective encapsulation in pure calcium alginate is not possible [117]. Nonetheless, urea can be supplemented in both polymer and crosslinking solution to attain a defined concentration within the beads prepared by ionic gelation.

4.4 Kill

4.4.1 Control strategies for *I. ricinus*

Acaricides from various chemical groups, e.g. organophosphates, carbamates, pyrethroids or macrocyclic lactones (such as doramectin, ivermectin or moxidectin) are used worldwide for tick control [15] However, at present, there are no available control measures for ticks with chemical acaricides in the field in Germany, due to related disadvantages, such as negative impact on non-target animals, increasing development of resistances of the target organisms [8; 126-128]. In Germany, their application is predominantly restricted to veterinary medicine, as spot-on or collar, while the use of such biocides in larger areas is not carried out for ecological as well as economic reasons. Current control agents follow different approaches.

One product for the control of ticks within a restricted area is the "Ixogon tick roll" (Roekel & Dalsum B.V., Amsterdam, Netherlands). The product is based on mice equipping their nest with permethrin impregnated cotton (chemical acaricide), to kill attached ticks on their body. In a related concept, a host-targeted bait box was deployed, containing a bait attractant and fipronil-treated felt wick, passively treating small mammals with the acaricide when entering the box [129; 130]. However, both methods do not affect all mice species and other tick hosts usually do not get in contact with the product. Another product, working with chemical control is a clip treated with specific pheromones and acaricides that was developed against tropical tick species [131]. This bag works according to the Attract-and-Kill strategy and is attached to the ear or tail root of cattle. However, it is only effective against special tick species of the genus *Amblyomma*, which do not occur in Europe.

Other options to prevent a direct contact between host and tick is the application of specific repellents. Especially for domestic animals, several studies were conducted on the effectiveness of essential oils and other natural substances. Substances as turmeric, cinnamon or thymol showed repellent effects towards different tick species, including *I. ricinus* [16-18]. Nevertheless, a high dosage application on human or animal skin can lead to an allergic contact dermatitis [19].

The development of biological control strategies for ticks has been overlooked for a long time. Biological methods in tick control have become increasingly prominent only within the last decade [8; 22; 23]. Besides entomopathogenic nematodes and parasitoids, acaricidal fungi have proved to be suitable antagonistic organisms which possess the ability to infect ticks. Acaricidal

fungi often also belong to the group of entomopathogenic fungi (EPF), which is why this general term is used as follows. Due to the high genetic variability, the development of resistances cannot be expected [132]. The most promising fungi are *Beauveria* spp. and *Metarhizium* spp. whereas the fungal species *Metarhizium anisopliae* and *Beauveria bassiana* are already proved to be virulent against ticks under optimal laboratory conditions [22; 31; 32]. A comparatively low mortality was frequently observed when the fungi were applied directly in the field or on animals (e.g. cattle as host) [26; 48]. This suggests that although the conidia adhere to the surface of the tick, in many cases they do not germinate or penetrate the cuticle or only induce a sub-lethal infection [133]. However, due to the large application area and the high dosages of aerial conidia usually required for the control of pests in the field [134], the application of unformulated EPF is mainly uneconomic. First approaches towards an Attract-and-Kill strategy for ticks were published in previous studies [38; 135], but not for the control of *I. ricinus.*

Usually, EPF isolates show a relatively high specific virulence for a small group of hosts [133]. While the application of high concentrations of EPF can lead to an influence on non-target arthropods, EPF are already present in the soil. Consequently, the use of EPF will not introduce foreign pathogens into the environment [136]. The living biomass in a control agent is exposed to environmental factors, such as extreme temperatures, dryness or UV light, when applied in the field resulting in high losses in viability. Therefore, a suitable formulation, especially for the encapsulation of EPF biomass is inevitable for an effective, cost efficient control agent against *I. ricinus*.

4.4.2 Aggregation pheromones

Ticks use behavior modifying substances, known as semiochemicals, including pheromones (communication) and allelochemicals such as allomones (defense) and kairomones (attractants for host identification and location, such as CO_2 and NH_3). The best studied semiochemicals are those for aggregation, attraction and sexual behavior [55], whereby the substances can have a varying influence on the behavior, specific for each tick species and development stage of the tick.

A known aggregating substance for hard ticks is hematin. It consists of a central iron atom and a porphyrin ring (figure 4.10) and is soluble only at high pH values and tends to self-aggregate at low pH values [137]. Hematin is a degradation product of blood [138].

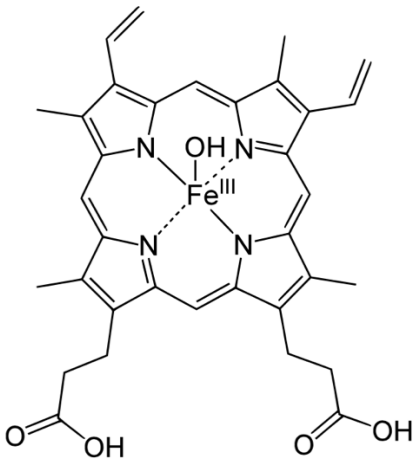


Figure 4.10 Chemical structure of hematin.

A significant effect of hematin as an aggregating substance on ticks of the species *I. scapularis* could be demonstrated [54; 139]. A quantitative effect on *I. ricinus* could not be shown so far.

The incorporation of aggregation pheromones like hematin on the bead surface was thought to increase the contact time of the tick with the formulation and thus the probability of infection with a co-encapsulated entomopathogenic fungus.

4.4.3 Formulation of entomopathogenic fungi

A recent survey confirmed that that aerial conidia are the most commonly used biomass for pest control [41]. Very few products are based on blastospores (4.1%) or mycelium (2.3%). Usually, biomass is applied as powder or oil dispersions with additional substances as drying aids in order to improve stability and shelf life of the product [37]. In comparison, encapsulation in calcium alginate beads is a well-established method for the protection of fungal biomass from environmental factors, such as temperature, extreme pH values or low water contents [140; 141] and was demonstrated not to interfere with vegetative growth and conidiation of EPF [142]. Nonetheless, drying of blastospores is, compared to aerial conidia, still a challenging task. Previous studies have shown that the addition of skimmed milk powder can increase the survival of *B. bassiana* blastospores [143]. This result was also corrobated for blastospores of *M. flavoviride* [144]. Studies by Jackson *et al.* [145] and Chong-Rodríguez *et al.* [146] showed

a increased survival during drying processes of *B. bassiana* and *Paecilomyces fumosoroseus* blastospores, respectively when diatomaceous earth was supplemented. An enhanced drying survival of *M. anisopliae var. acridum* blastospores was reported in a study by Leland *et al.* [36]. In that study the survival was significantly increased with a resulting germination of 20% of the blastospores, when the biomass was left in growth medium instead of washing with distilled water or PEG solution, prior to a fast-drying process on silica gel.

Not only the supplements but also the final a_w value of the formulation is reported to be an important factor in the drying process. Conidia and blastospores with a lower water activity exhibit lower conidiation rates after drying than those with higher a_w values [81]. The cultivation time and the sampling point of the culture can have further impact on the drying stability of biomass, due to the composition of the cells [147; 148]. Especially the polyol content within cells, that was reported to affect drying stability, can vary during cultivation [149; 150].

A dried bead formulation furthermore facilitates application and storage of the control agent. Even though the use of conidia clearly predominates over blastospores, suitable formulations have the potential to make blastospores applicable and exploit their advantages, above all faster germination, faster speed-to-kill and cheaper production [44].

4.4.4 *Metarhizium* spp.

Isolates of the genus *Metarhizium* belong to the family of Clavicipitaceae, which are classified as Ascomycota. A characteristic of these fungi is the asexual reproduction with spores. The classification is based on morphological characteristics of hyphae, aerial conidia and blastospores [8]. The genus is known to infect and kill a wide range of insects and arthropods. Different *Metarhizium* spp. isolates have been found worldwide in soil and could already be isolated from plant roots [151], insects [152] and ticks [23]. The commonly known species *Metarhizium anisopliae* was first described by Metschnikoff in the Ukraine in 1879. The EPF was isolated from infected beetles of the species *Anisoplia austriaca* (Southern Grain Leaf Beetle) and thus got its name and was then placed in the genus *Metarhizium* introduced by Sorokin in 1883. *M. anisopliae* was already used as a biological pesticide shortly after its discovery from 1880 to 1890 in the former Soviet Union against the beetle *Cleonus punctiventris* [153]. This makes *M. anisopliae* one of the first described EPFs to be used for pest control. There are over 40 different products based on *Metarhizium* spp. that are

applied against different types of pests. These include locusts, cockroaches, mosquitoes and also ticks [30; 41].

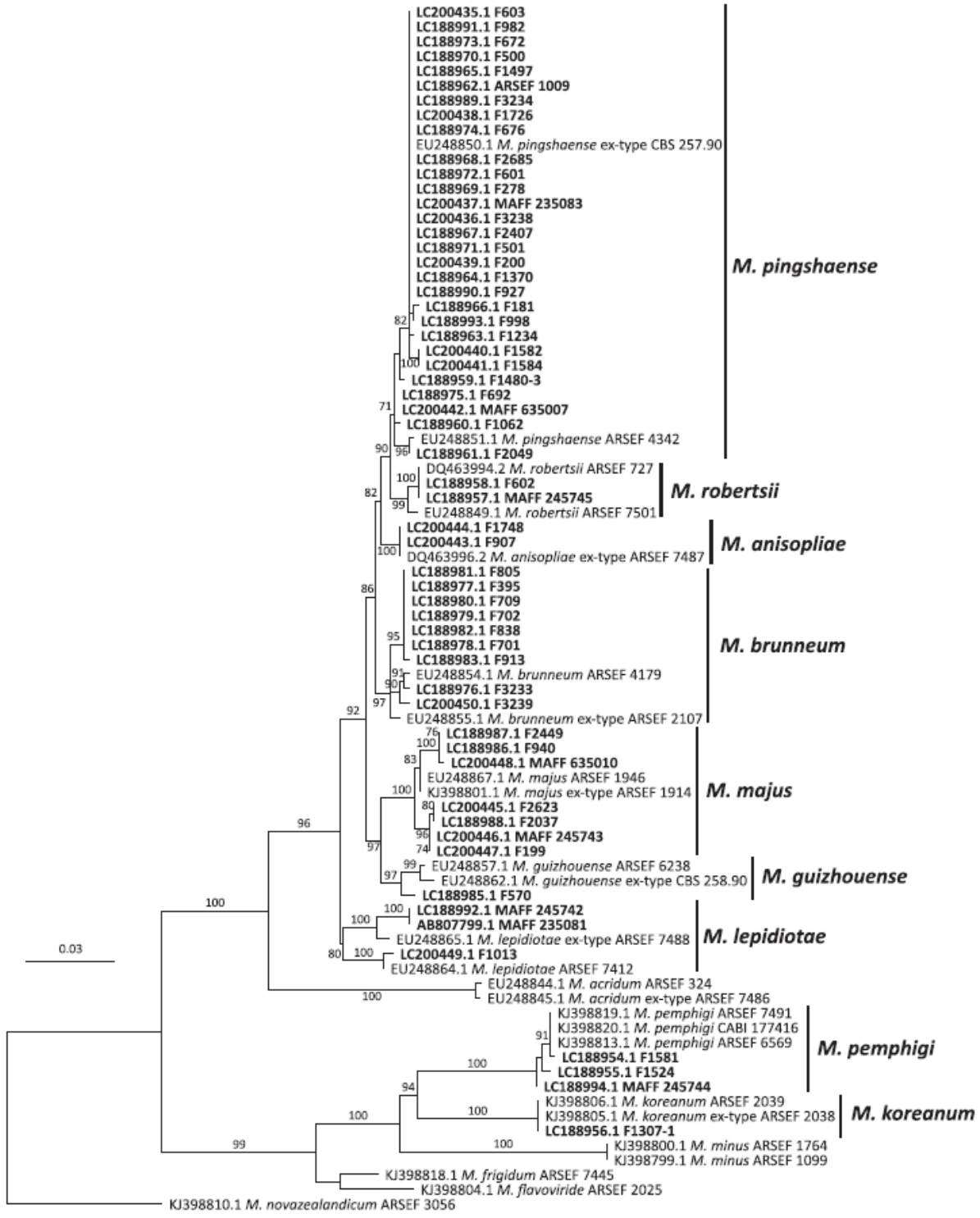


Figure 4.11 Phylogenetic tree of the genus *Metarhizium* [154].

Today the genus *Metarhizium* belongs to the order of hypocreales. The pedigree was revised by molecular methods by Bischoff *et al.* [155], resulting in a division of the genus into 12 different species. It has been found that former *M. anisopliae* isolates contain different species, including *M. brunneum, M. anisopliae, M. pingshaense* and *M. robertsii* (figure 4.11). Other known species are *M. pemphigi, M. flavoviride, M. album* and *M. frigidum*. Therefore, the assignment of isolates to the different species in the literature is often unprecise, not completely clarified and partly outdated [155]. Consequently, a screening for virulence is mandatory, to ensure the applicability of a *Metarhizium* spp. isolate as control agent for a certain pest.

Cultivation

For the cultivation of EPF, a distinction can be made between two general production processes, solid cultivation and submerged cultivation. It is commonly known from the literature, that *Metarhizium* spp. can form different morphologies, depending on the surrounding medium [156-159]. The application of EPF as control agent usually requires a large amount of biomass with a high vigor. Therefore, an optimized suitable cultivation method in order to produce biomass in a cost-efficient way is needed.

On solid medium, EPF produces small asexual spores (<10 µm) called aerial conidia. Characteristic is the thick hydrophobic cell wall, which offers protection against abiotic stress factors and provide drying resistance and storage stability [34; 35; 37; 158]. Aerial conidia, as the natural infection unit of the EPF, are most commonly used in commercial formulations. Their germination on solid medium can take up to 24 h [160].

Solid phase cultivation has historically been the most widely used cultivation method. On a smaller scale it is a simple method with little technical effort. Usually, substrates as rice and barley, but also by-products and waste products are used as nutrients. The biggest disadvantage of solid phase cultivation is its low suitability for upscaling for industrial applications. It is either a simple technique requiring a large number of personnel or a technique requiring expensive equipment. Large amounts of substrate are required which causes problems during sterilization. In addition, the bioreactors for solid cultivation require lots of space because EPF only grow on the surface of the medium [82].

In submerged culture, EPF form mainly mycelium [47; 157] and thin-walled blastospores by budding from hyphae of the mycelium (figure 4.12) [161; 162]. Blastospores are vegetative cells which, in contrast to conidia, do not have a formal cell wall and are consequently more sensitive in terms of storage stability and drying resistance [82; 158]. On the other hand, their

faster germination is advantageous. Blastospores do not represent the natural infection unit, but it has been shown that they can be equally or even more virulent than conidia [33; 37]. Fungal mycelium is the second vegetative growth form of EPF. It consists of branched hyphae and can grow filamentous or pellet-like, whereas filamentous growth is an optimal basis for blastospore production [163; 164]. A high blastospore concentration further can be obtained for instance by increasing the osmotic pressure of the medium [47; 157]. The optimal temperature for submerged cultivation of *Metarhizium* spp. was reported in a range between 25 and 28 °C [36; 45; 156; 161].

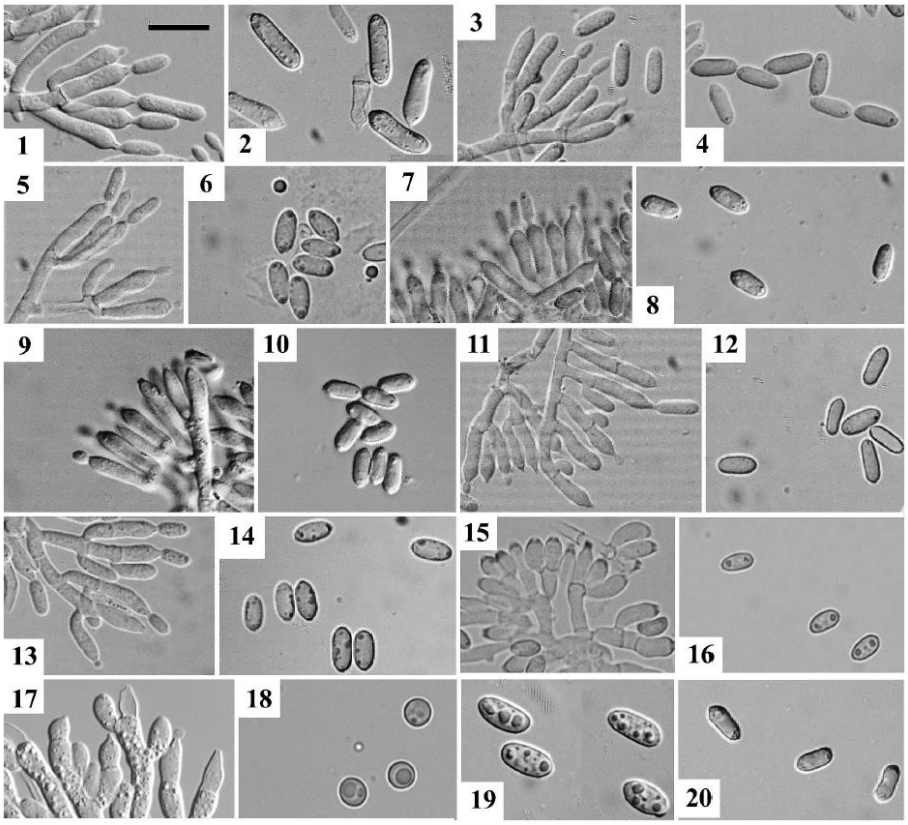


Figure 4.12 Microscopic images of different *Metarhizium* spp. isolates. [155].

Besides the amount of biomass, the vigor is an important parameter that can be influenced by culture conditions [36; 158]. The ecological fitness of the spores is closely related to their endogenous components, e.g. the content of polyhydroxy alcohols (polyols). An increased polyol content is associated with germination at low water activities (a_w), faster germination and improved drying tolerance [150]. The endogenous polyol content can be manipulated by medium parameters, as pH value or a_w value of the medium [165]. The culture age has further impact on endogenous components and the properties of the formed biomass [82]. A negative

effect of a cultivation under unfavorable conditions (medium, pH value, temperature) on the virulence of the EPF was reported by Fernandes *et al.* [81].

Mode of action

A unique feature of entomopathogenic fungi is their ability to infect insects and ticks via their cuticle. The natural infection unit of EPF are aerial conidia. The conidia adhere to the cuticle through hydrophobic interaction and also through the formation of mucus and adhesive proteins [166]. The cuticle of hard bodied ticks, such as *I. ricinus*, provides natural protection from adverse environmental factors [55]. It consists mainly of tanned proteins and chitin, as well as short-chain fatty acids. It has a low water activity, only hardly available nutrients for pathogens and a specific colonization by microbes serving as protection against pathogens. The composition of the epicuticle plays an important role in identifying the correct host. As a consequence, EPF are adapted to the composition of the cuticle and are therefore equipped with a particularly high number of proteases and chitinases [167].

The conidia adhering to the cuticle germinate and form penetration structures, such as an appressorium resulting in a penetration hypha (figure 4.13). Enzymatic and mechanical processes are involved in the penetration of the cuticle. Important enzymes are the mentioned proteases and chitinases, as well as esterases and lipases to soften the structure of the tick cuticle and facilitate an infection [168]. In comparison, EPF show significantly more proteases and chitinases than plant-pathogenic fungi. This difference is even bigger in fungi with a broad host spectrum than in specialists [166]. As soon as the penetration hyphae reaches the hemolymph of a host, blastospores are produced [167; 169].

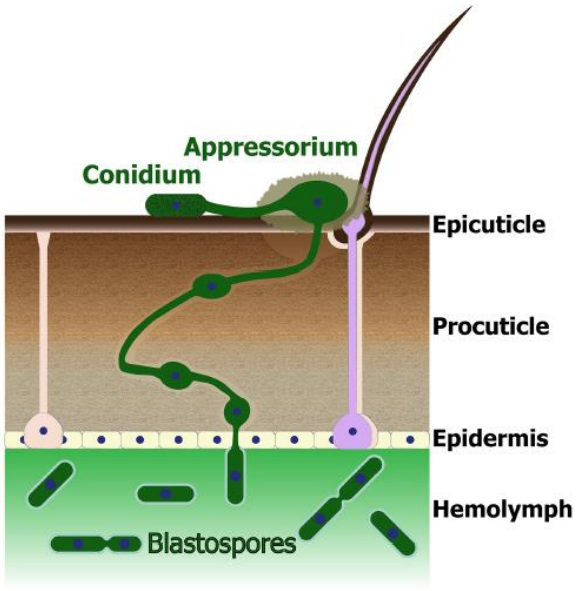


Figure 4.13 Diagrammatic representation of cuticle penetration by *Metarhizium* spp. using an appressorium along a seta [127], glandular duct (beige), and trichogen cell (purple) followed by budding off of blastospores in the hemolymph. [166]

The death of the host finally occurs through a combination of different factors. The consumption of available nutrients as well as invasion and physical destruction of the organs are main reasons [170]. Additionally, toxic substances are produced by *Metarhizium* spp., such as destruxins [160]. After death, the fungus continues saprotrophically growth resulting in the formation of new infectious aerial conidia on the host surface [169].

A screening for a suitable isolate is of major importance for the development of a biological control agent, due to the high host specificity of fungi. EPF show a very high genetic variability between isolates of the same species. Since different isolates furthermore show individual growth behavior, a selection process under biotechnological aspects, such as cultivability, is necessary for a large-scale production. Even though blastospores are easier to produce and harvest on a technical scale than aerial conidia, they suffer from a higher sensitivity to environmental factors. Therefore, the screening of isolates for beneficial properties as well as for a suitable formulation is obligatory at the beginning of the development process of an efficient tick control agent.

5 Materials and methods

5.1 Attract

5.1.1 *Saccharomyces cerevisiae*

Baker's yeast strain *S. cerevisiae* H205 (commercially available as compressed baker's yeast under the brand name "VITAL-AROM") was provided by Deutsche Hefewerke GmbH, Nürnberg, Germany.

5.1.2 Bead preparation

Amyloglucosidase

As amylolytic enzyme for starch degradation, the amyloglucosidase (AG) preparation Panzym® HT 300 (Novozymes A/S, Bagsværd, Denmark) containing 300 AG units·mL^{-1} was used. By definition, one AG unit·mL^{-1} is the amount of enzyme cleaving 1 µmol of maltose per minute (conditions: 10 mg mL^{-1} maltose, pH 5.0, 37 °C, 30 min) [171].

Encapsulation

Encapsulation was carried out based on a protocol of Humbert *et al.* [119]. A 4% (w/w) sodium alginate (Manugel GMB, Batch No. G7708901; FMC Biopolymer, Philadelphia, PA) solution was prepared, stirred for at least 1 h for homogenization and heat sterilized (6 min at 121 °C). The sodium alginate solution was thoroughly mixed with native granular corn starch (CIF GmbH, Siegburg, Germany). Then *S. cerevisiae* biomass was added as well as ultrapure water. The final concentrations were 2% (w/w) sodium alginate, 20% (w/w) granular corn starch and 15% (w/w) *S. cerevisiae* biomass. Optionally, AG (0.05 – 1 U·g^{-1}) was added to the sodium alginate matrix solution. The stirred matrix solution was transferred into a 20 mL syringe and dripped through a drain tube (Ø=0.90*40 mm Sterican; B. Braun Melsungen AG, Melsungen, Germany) into 180 mM calcium chloride. The beads were kept in the cross-linking solution for 20 min, then separated and washed with ultrapure water. For beads supplemented with urea, 12.5% (w/w) was added to both crosslinking and polymer solution. Beads containing urea were not washed to prevent loss of urea. All calcium alginate beads were prepared under sterile conditions with autoclaved materials. During preparation, all materials were kept on ice.

Optionally, beads were air-dried under the laminar air-flow of a clean bench for 24 h. Dry beads were slowly rehydrated in petri dishes containing 1.5% (w/w) water agar at 7 °C for 24 h.

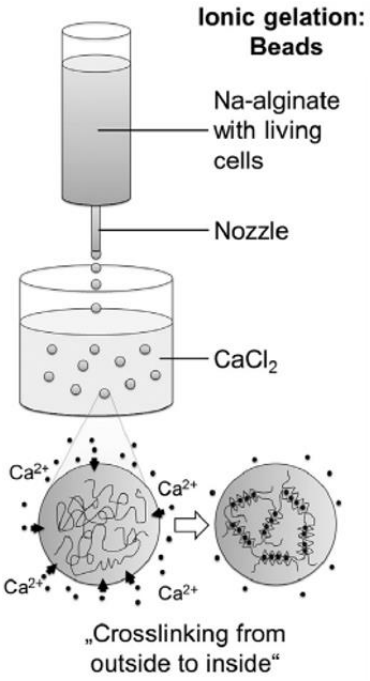


Figure 5.1 Encapsulation based on the dripping method: Production of beads by ionic gelation [49].

5.1.3 Determination of CO_2 release

For the measurement of the CO_2 production of the beads, the released amount of CO_2 was quantified using a carbon dioxide meter with pump-aspirated sampling (Vaisala CARBOCAP® GM70; Vaisala Oyj, Helsinki, Finland). The CO_2 release rates were determined like previously described by Vemmer *et al.* [52]. In this work, the amount of CO_2 produced by 1 g moist beads in 1 h was measured in a closed tube with a volume of 50 mL at 25 °C. The data are represented as difference between ambient air and the CO_2 release rate of the beads and illustrated as mL CO_2 produced per g of moist or rehydrated beads in 1 h.

5.1.4 Y-olfactometer

In order to determine the attractivity of beads, the behavior of *I. ricinus* nymphs was tested in a dual-choice experiment using a Y-olfactometer (teflon, self-constructed by IS Insect Services GmbH, Berlin, Germany). Between two and four nymphs were applied at the entrance of the Y-olfactometer and the movement was screened for 3 min. The decision for one side (‘control’

with fresh air or ‘test’ with attractant) was noted, when a mark at 3 cm was crossed by a tick (figure 5.2). The flow in the test side either contained CO_2 produced by Attract beads or a defined CO_2 concentration. The laminar flow was adjusted at 47.8 $mL \cdot min^{-1}$ with 77-80% relative humidity and a CO_2 concentration of the ambient air between 520-650 ppm at 23 °C.

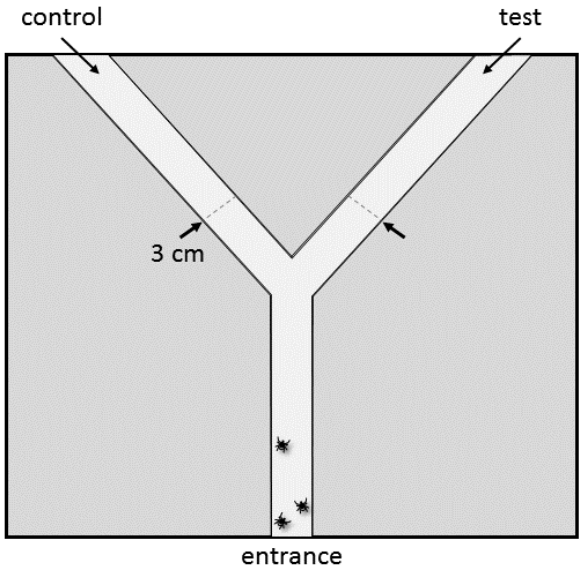


Figure 5.2 Schematic drawing of the experimental setup of the Y-olfactometer.

5.2 Kill

5.2.1 *Metarhizium* spp.

The *Metarhizium brunneum* isolates Ca8II and Cb16III were obtained from Goettingen University (Prof. Stefan Vidal, Agricultural Entomology). *Metarhizium pemphigi* X1c was received from the University of Hohenheim (Prof. Ute Mackenstedt, Parasitology). All fungal isolates were cultivated on malt-peptone agar (MPA; 3.0% (w/w) barley malt extract, 0.5% (w/w) peptone from casein and 1.5% (w/w) agar) in the dark at 25 °C. The produced aerial conidia were used for cultivation on solid media as well as for inoculation of submerged cultures and encapsulation in calcium alginate beads.

5.2.2 Solid state cultivation

Metarhizium spp. isolates were cultivated on solid medium adapted from Krell *et al.* [172], containing 1.5% (w/w) agar, 4.0% (w/w) ANiPept (protein hydrolysate from animal by-products, ANiMOX GmbH, Berlin), 5.0% (w/w) glucose and 7.0% (w/w) polyethylene glycol 200 [31]. The initial pH value was adjusted to 6.0 with 1 M HCl. All dishes were inoculated with 10^3 aerial conidia and incubated at 15 °C, 25 °C or 30 °C for three weeks.

5.2.3 Submerged cultivation

Metarhizium spp. isolates were cultivated in 75 mL medium in 250 mL shake flasks with baffles for 210 h at 25 °C and 175 rpm (IKA KS 4000 ic control, Staufen, Germany). The medium was adapted from Krell *et al.* [172] with 4.0% (w/w) ANiPept, 5.0% (w/w) glucose and 7.0% (w/w) PEG and an initial pH value for all cultures adjusted to pH 6.0 with 1 M HCl. Precultures were prepared with cryopreserved aerial conidia from the solid cultivation and cultivated for 48 h. Submerged cultures were inoculated with blastospores from the preculture to an initial concentration of 10^6 blastospores·mL^{-1}. The blastospore concentration of submerged cultures was counted with a Thoma cell counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda Koenigshofen, Germany) under a light microscope (Photomicroscope; Carl Zeiss AG, Oberkochen, Germany).

Analysis of biomass formation

To determine the aerial conidial concentration of the solid cultures, an area of 1 cm^2 was punched out of the medium with a scalpel and placed in 1 mL of 0.1% (w/w) Tween-80. Aerial conidia were counted, and the concentration calculated similar to blastospores in submerged cultures (see above). Dry weight of total biomass of submerged cultures was determined by drying in dried and pre-weighted 1.5 mL centrifuge tubes. Therefore, 1.5 mL culture broth was pipetted into the tube and centrifuged for 10 min at 27,000 g. The supernatant was removed, and the tubes dried at 60 °C for 72 h. After cooling the tubes down to room temperature, the dry weight was determined gravimetrically.

Glucose measurement with high performance liquid chromatography

Media samples from submerged cultures were centrifuged for 20 min at 27,000 g in a 1.5 mL centrifuge tube to remove biomass and particles. The supernatant was transferred in a new

1.5 mL centrifuge tube and centrifuged again. The remaining supernatant was transferred to HPLC vials. Glucose was quantified by high-performance liquid chromatography (HPLC) using the Primaide system (Hitachi, Chiyoda, Japan) equipped with a NUCLEODUR® 100-5 NH_2-RP (5 µm) column (Macherey-Nagel GmbH, Düren, Germany) and ultrapure water/acetonitrile as eluent at a ratio of 21:79 and a flow rate of 1 $mL \cdot min^{-1}$. The injection volume was 10 µL. Pure glucose was used as analytical standard for the calculation of the glucose concentration in the samples.

5.2.4 Bead preparation

Preparation of blastospores

Blastospores were separated from the culture broth after submerged cultivation by sterile filtration through autoclaved paper dishes (Qualitative filter paper, 401, 12 – 15 µm pore size, VWR, Germany) as described above. The collected blastospores were centrifuged for 10 min at 2,700 g to separate blastospores from the medium. The pellet was re-suspended in 1 mL 0.9% (w/w) NaCl and centrifuged again to remove residual medium. Finally, the pellet was dissolved in 0.5 mL 0.9% (w/w) NaCl and blastospore concentration was determined with a Thoma cell counting chamber as described above.

Encapsulation

The encapsulation was conducted analogous to chapter 5.1.1. Prior to encapsulation, a 4.0% (w/w) sodium alginate (Manugel GMB, Batch No. G7708901; FMC Biopolymer, Philadelphia, PA) solution was prepared with ultrapure water and heat sterilized for 6 min at 121 °C. For preparation of calcium alginate beads under sterile conditions, 4.0% (w/w) sodium alginate solution was thoroughly mixed with either 15% (w/w) sterilized native granular corn starch (CIF GmbH, Siegburg, Germany) or 15.0% (w/w) chitin powder from shrimp shells (Sigma-Aldrich Chemie GmbH, Munich, Germany) as well as 2.0% (w/w) inactivated and ground yeast (Deutsche Hefewerke GmbH, Nürnberg, Germany). Then, *Metarhizium* spp. blastospores were added to the suspension up to a final concentration of 10^6 $blastospores \cdot g^{-1}$. The solution was diluted with ultrapure water to a final sodium alginate concentration of 2.0% (w/w). After stirring, the matrix solution was dripped into a stirring sterile 2.0% (w/w) $CaCl_2$ solution through a 20 mL syringe equipped with a cannula (Ø=2.10·80 mm, Sterican; B. Braun

Melsungen AG, Melsungen, Germany). Prepared beads were separated and rinsed with ultrapure water.

Drying of beads and determination of CFU

If not stated otherwise, beads were separated and dried for 24 h. under the laminar air flow of a clean bench at ambient temperature and 35 – 50% relative humidity to a water activity (a_w value) of 0.3 – 0.4. The a_w of the beads was measured, using a water activity meter (LabMASTER-aw, Novasina AG, Switzerland).

To determine the survival of encapsulated blastospores from dried beads, ten beads were dissolved in 10 mL of a buffer containing 0.05 M Na_2CO_3 and 0.03 M citric acid solution adapted from Mater *et al.* [173] on a rotary shaker for 60 min at 50 rpm and 25 °C. The number of colony forming units (CFU) was evaluated by a plate counting method on MPA, incubated at 25 °C for 48 – 72 h. The survival of the encapsulated cells during drying of the beads was calculated as follows:

$$\% \text{ of survival} = (N/N_0) \cdot 100$$

With N: total CFU after drying and N_0: total CFU before drying of the beads.

Coating with hematin

A stock solution of 20% (w/w) hematin (Chemos GmbH & Co.KG, Altdorm, Germany) in 1 M NaOH was prepared. The stock solution was mixed in equal parts with Tween-80 and diluted with coating buffer (25% (w/w) 0.2 M tris(hydroxymethyl)aminomethane (Tris), 29.7% (w/w) 0.1 M HCl in ultra-pure water) to a final concentration of 0.5% (w/w) hematin. Dried Kill beads containing 15% (w/w) granular corn starch and *M. pemphigi* X1c blastospores were soaked in the solution for up to 60 s. After the soaking, beads were not washed. For the determination of hematin on the bead surface, ten beads were dissolved in dissolving buffer as described above, diluted in equal parts with 1 M NaOH and the concentration calculated based on the optical absorption at a wavelength of 387 nm (Photometer Genesys 10s UV-Vis Spectrophotometer, Fisher Scientific, Schwerte, Germany). The beads had a final surface concentration of 10–20 $\mu g \cdot cm^{-1}$ hematin.

5.2.5 Analysis of conidiation of encapsulated *Metarhizium* spp. on beads

Moist beads were placed on water agar (1.5% (w/w) agar) in petri dishes (Ø=94 mm) and incubated at 25 °C for four weeks. After every week, the surface concentration of aerial conidia was determined for ten beads. Therefore, each bead was placed in 1.0 mL of 0.1% (w/w) Tween-80 and mixed gently for 30 sec to detach aerial conidia from the surface. The concentration was calculated using a Thoma cell counting chamber as described above.

5.2.6 Screening for virulence

Ticks

Ixodes ricinus, *Dermacentor reticulatus* and *Rhipicephalus sanguineus* nymphs as well as adults of all three species were obtained from IS Insect services (Berlin, Germany). Unfed ticks were kept at ambient temperature and with saturated $MgSO_4$ solution to maintain a relative humidity > 90% in sealed plastic boxes (Clip & Close, Emsa GmbH, Emsdetten, Germany) in the dark until used in experimental designs.

Experimental setup

In order to screen for the virulence of the newly formed aerial conidia on the bead surface for tick nymphs, beads were prepared as described above and incubated on water agar (1.5% (w/w) agar) at 25 °C for two weeks and then placed in ELISA-stripes (F8 maxisorp loose NUNC-immuno module, Thermo Fisher Scientific, Roskilde, Denmark) with one bead in each well (figure 5.3). For every *Metarhizium* spp. isolate, 30 wells were used. In each well, one nymph was added and then sealed with Parafilm M. Air holes were pricked into the Parafilm M with a small cannula zo allow air exchange. The ELISA-stripes were incubated at 25 °C in a sealed box with saturated $MgSO_4$ solution (relative humidity >90%) in the dark and monitored twice a week with a digital microscope (Keyence VHX-1000; Keyence Corporation, Osaka, Japan) to identify infected nymphs. Two control groups (nymphs without treatment and beads without active ingredient) were used.

Figure 5.3 Experimental setup of the virulence screening.

5.3 Attract-and-Kill

5.3.1 Bead preparation

Attract-and-Kill beads were prepared as described above for the Kill formulation containing 15% granular corn starch. In addition, 15% (w/w) *S. cerevisiae* cells and optionally AG was added in different concentrations. Different forms of biomass were co-encapsulated. For encapsulation of mycelium, it was separated from culture medium and blastospores by sterile filtration through autoclaved paper dishes (Qualitative filter paper, 401, 12 – 15 µm pore size, VWR, Germany). The filter was rinsed with 0.9% (w/w) NaCl to remove remaining blastospores. A concentration of 2% (w/w) mycelium was applied to the bead matrix instead of blastospores. Aerial conidia for encapsulation were collected from MPA agar, cultivated at 25 °C for three weeks, using 3 mL 0.1% (w/w) Tween-80. The final concentration of aerial conidia inside the beads was analogous to blastospores 10^6 conidia·g^{-1}.

Analysis of beads

The quantification of CO_2 produced by the beads was conducted as described in chapter 5.1.2. Conidiation on the bead surface was determined as described in 5.2.4

Determination of glucose content inside beads

Glucose concentration was determined with HPLC as described above. Beads were dissolved as described in 5.2.4, centrifugated at 27,000 g for 20 min and the supernatant analyzed as described in 5.2.3.

5.3.2 Ethanol determination in beads

To determine the ethanol content inside the beads, an Ethanol Assay kit (Megazyme International, Wicklow, Ireland) was used. Attract-and-Kill beads were prepared without EPF biomass. 0.5 g beads were a dissolved t each timepoint in 10 mL dissolving solution as described in chapter 5.2.3. Solutions were centrifuged for 10 min at 2,700 g for 10 min. The supernatant was removed fort ethanol determination.

The determination of ethanol was conducted according to the manual, using a spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, Waltham, USA) and disposable micro cuvettes (Biosigma, Cona, Italy)

5.3.3 Ethanol stability

A defined amount of 10^4 blastospores was incubated in a final volume of 1 mL of different ethanol concentrations in ultrapure water (0 – 0.4 $g \cdot mL^{-1}$) for 10 min. A volume of 100 µL of each sample was plated on MPA and incubated at 25 °C for 72 – 96 h. CFU was calculated as described above.

5.4 Statistical analysis

Statistical analysis was carried out with the software SPSS Statistics V22.0 (SPSS, Chicago, IL). All data are given as mean values ± standard deviations (sd) or as mean values ± standard error (se) and were checked for normality and homogeneity of variance using Shapiro-Wilk and Levene test. The level of significance was set at $p<0.05$.

Means for CO_2 production, attractivity of the formulation, drying survival and ethanol survival were tested for significant differences by one-way analysis of variance (ANOVA) followed by a Games-Howell post-hoc test with Welch correction for non-homogeneity, if the requirements of homoscedasticity of variance were not met.

Repeated measures ANOVA was conducted for cultivation experiments (blastospore concentration and dry weight), conidiation on beads containing encapsulated EPF, virulence screening, glucose content in beads as well as ethanol content in beads to compare the effects of the treatment across time with time and treatment as independent variables. The sphericity of the matrix assumption was assessed with the Mauchley sphericity test; if the outcome of the test was significant, the F values were corrected using the Greenhouse-Geisser approach. When the requirements for homoscedasticity of variance were not met, a Games-Howell post hoc test was used to establish differences in means.

6 Results and discussion

6.1 Attract

Ticks, such as *I. ricinus* are known to be activated or attracted by CO_2 gradients [94; 95]. In view of an active movement of the ticks towards the CO_2 source, the required amount of a control agent in the field can be reduced when this agent is in close proximity with the attractant. An artificial CO_2 source can lure ticks into a defined area what facilitates the treatment with control agents.

6.1.1 CO_2 production of encapsulated *S. cerevisiae* in calcium alginate

Originally, beads containing *S. cerevisiae*, native granular corn starch and amyloglucosidase (AG) were developed by Humbert *et al.* [119] for the attraction and control of wireworms in soil. This formulation was based on pre-studies from Vemmer *et al.* [52] and used exclusively below ground, so far. In this work, CO_2 producing beads were adapted and for the first time applied for the attraction of arthropods living above ground.

The microorganism *S. cerevisiae* naturally assimilates monosaccharides, such as glucose, for growth and metabolism. CO_2 is a by-product of this process and was early reported to attract ticks as *I. ricinus* in the field [88]. Due to their low molecular weight, monosaccharides cannot be encapsulated permanently in calcium alginate because it is not retained by the polymer matrix [49]. As already demonstrated by Humbert *et al.* [119], supplemented starch, as a carbon source with high molecular weight, remains in the bead and furthermore enhances the structure of the hydrogel network [120]. Because *S. cerevisiae* does not bring the enzymatic equipment to assimilate starch, an AG was co-encapsulated to make the nutrient source available for the encapsulated cells.

Influence of the amyloglucosidase concentration on the CO_2 production

In a first step, the CO_2 production of moist beads containing different concentrations of AG was compared at a constant incubation temperature of 25 °C to identify the optimal concentration for an application of the beads above ground. To ensure tick attraction, a minimum concentration of 0.2 $mL \cdot (g \cdot h)^{-1}$ CO_2 for the Attract beads was intended for a period of two weeks. The CO_2 production of four formulations with different AG concentrations is illustrated in figure 6.1.

There was a significant influence of the AG concentration on the CO_2 production at every time point (0 d $F_{3,11}$=90.755; p<0.05, 7 d $F_{3,10.507}$=49.067; p<0.05, 14 d $F_{3,9.292}$=11.494; p<0.05, 21 d $F_{3,11,022}$=4.656; p<0.05). The CO_2 production decreased for all formulations significantly over the incubation time of three weeks (p<0.05). Freshly prepared beads containing AG produced CO_2 concentrations higher than 0.2 mL·(g·h)$^{-1}$, whereas the control showed a production of 0.14 mL·(g·h)$^{-1}$, probably as a result of remaining nutrients in the cells. After seven days, only beads containing 0.05 and 0.1 U·g^{-1} AG exceeded the minimum CO_2 concentration, whereas the CO_2 production of beads prepared with 1.0 U·g^{-1} AG already decreased to a concentration of 0.17 mL·(g·h)$^{-1}$. After two weeks, the nutrients inside all formulations were probably already depleted and as a consequence none of them reached the defined minimum of 0.2 mL·(g·h)$^{-1}$ CO_2. Beads containing 0.05 U·g^{-1} AG showed the significant highest production beyond 0.1 mL·(g·h)$^{-1}$ with 0.15 mL·(g·h)$^{-1}$ after 14 days (p<0.05).

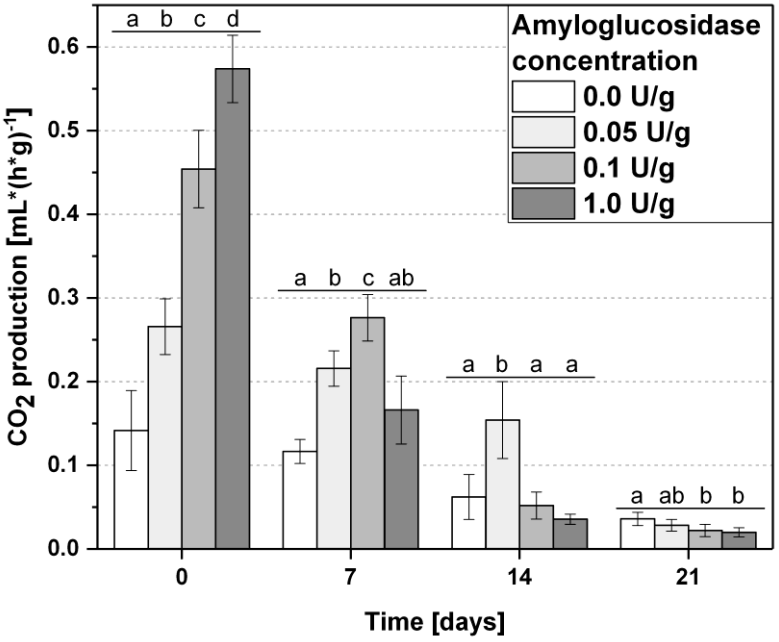


Figure 6.1 Influence of the amyloglucosidase concentration on the CO_2 production of *S. cerevisiae* encapsulated in calcium alginate supplemented with corn starch over three weeks at 25 °C. Different letters above bars indicate significant differences according to one-way ANOVA with Games-Howell post hoc test at p<0.05 (n=5; mean ± sd).

It is known from the literature that *S. cerevisiae* is not able to metabolize starch when the cells are not biotechnologically modified [52; 174; 175]. Therefore, a supplementation with AG is inevitable to attain adequate concentrations. The obtained CO_2 concentrations in the presented experiment are in good agreement with a study by Humbert *et al.* [119]. The faster decrease of

the CO_2 production of the formulation with 1.0 $U \cdot g^{-1}$ AG very likely resulted from the faster cleavage of starch by the relatively high concentration of encapsulated AG, leading to an earlier depletion of the nutrient source. For further experiments, a concentration of 0.1 $U \cdot g^{-1}$ AG was chosen, due to its relatively consistent, sustained CO_2 release, even though the desired CO_2 concentration was not attained for the entire two weeks.

Influence of temperature on the CO_2 production

Drying of microorganisms is a common method and usually required for a long-time storage to maintain a high viability of the cells [176]. Therefore, not only moist, but also dried and rehydrated formulations were examined for their CO_2 production at different temperatures to ensure practicability not only under ideal conditions but also in the field. Since the incubation temperature has a significant influence on the metabolism of encapsulated *S. cerevisiae* cells, the CO_2 production of moist as well as dried and slowly rehydrated beads was investigated at different temperatures in a range between 4 °C and 30 °C. The measured values and their statistical significance are summarized in figure 6.2.

There was a significant influence of the incubation temperature on the CO_2 production of moist as well as of dried and rehydrated beads ($F_{9,16.176}$=124.487; $p<0.05$). The CO_2 production increased nearly linearly from 0.15 $mL \cdot (g \cdot h)^{-1}$ to 0.47 $mL \cdot (g \cdot h)^{-1}$ (moist beads) or from 0.1 $mL \cdot (g \cdot h)^{-1}$ to 0.44 $mL \cdot (g \cdot h)^{-1}$, respectively (dried and rehydrated beads) with the temperature between 4 °C and 25 °C ($p<0.05$). However, there was no significant differences in the CO_2 production of beads between 10 °C and 18 °C (moist: p=0.059, rehydrated: p=0.413). Moreover, a further increase in incubation temperature up to 30 °C had no significant effect on the CO_2 production, equally whether moist (p=0.894) or dried and rehydrated beads (p=0.249) have been considered.

When comparing moist and dried and rehydrated beads, significant differences in the CO_2 production were solely noticeable for 4 °C and 25 °C ($p<0.05$). At 10, 18 and 30 °C, no significant differences were measured (p=0,72, p=0,201, p=0,978). However, there was a tendency for a slightly higher CO_2 production of moist beads than of dried and rehydrated beads at all incubation temperatures investigated.

The results in figure 6.2 illustrate that temperatures greater or equal 10 °C lead to an adequate minimal CO_2 production of 0.2 $mL \cdot h^{-1} \cdot g^{-1}$ to ensure applicability in the experimental setup of the Y-olfactometer for both, moist as well as dried and rehydrated beads. Consequently, it can be assumed, that the *S. cerevisiae* cells as well as the AG present a sufficient activity at lower

temperatures, even though the activity optimum for both is set at higher temperatures. *S. cerevisiae* is reported to grow between 3 °C and 45 °C with an optimum at 32 °C [177] whereas the encapsulated AG has a temperature optimum of 60 °C [178; 179]. As a result of a lower activity at moderate temperatures, an extended nutrient supply in the bead is supported and therefore a long-lasting CO_2 release is ensured [119]. The temperature dependent activity of co-encapsulated *S. cerevisiae* cells and AG correlating with the CO_2 release of beads was previously demonstrated by Humbert *et al.* [119], however for moist beads exclusively. The results are in good agreement with the release rates depicted in figure 6.1. Surprisingly for both, moist and dried and rehydrated beads, a significant difference was measured only at 4 and 25 °C. Due to the drying process and a related loss of viable cells [180], a reduction of the CO_2 production was expected. These similar release rates are most probably caused by the high quantity of encapsulated *S. cerevisiae* cells within the beads. When high amounts of living cells are encapsulated, a limitation in the supply of nutrients is supposed to occur. Through a lack of nutrient supply, not all living cells produce high amounts of CO_2. Therefore, a reduction of viable cells during drying is thought to solely lead to a minor reduction in CO_2 production. Even though drying of microorganisms is a common method, damaging effects of a drying process especially on encapsulated cells are known [120; 181-183], like compression by a shrinking polymer matrix. Further factors during drying that have a direct effect on the viability, like osmotic and oxidative stress, an increase of intracellular pH and salt concentrations, may lead to an additional lasting damage of the cells [180; 184]. When considering the determination of the colony forming units before and after the drying process (unpublished data), a significant reduction of viable *S. cerevisiae* cells was measured, whereas damaged cells which are not able to propagate are not monitored with this method even though their metabolism might still be working [185] what therefore may lead to a maintenance of the CO_2 production. This was also demonstrated by Humbert *et al.* [51], whereas the dried and rehydrated beads presented in at study only achieved a CO_2 release rate of 0.1 $mL \cdot h^{-1} \cdot g^{-1}$. A harsh rehydration procedure was conducted for the measurement of CO_2 release rates in that study. As already reported in the literature, a slow and therefore gentle rehydration leads to a much higher viability of living cells [186; 187]. Poirier *et al.* [187] pointed out a critical a_w range between 0.117 and 0.455 that a rehydration process needs to overcome slowly to induce a high cell survival. Due to the application of dry beads above ground without previous rehydration, a slow sorption of water is given and therefore high cell viability is supposed to be provided when beads are applied in

the field, whereas the supplemented corn starch maintain the moisture, as it is reported to possess a high water binding capacity [188; 189].

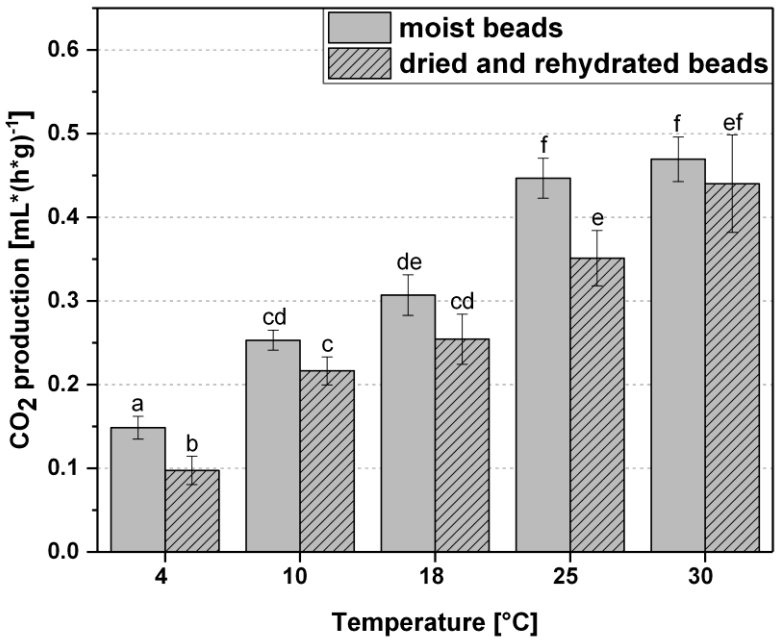


Figure 6.2 CO_2 production of moist and dried and rehydrated calcium alginate beads containing *S. cerevisiae*, corn starch and 0.1 U/g amyloglucosidase incubated at different temperatures. Different letters above bars indicate significant differences according to one-way ANOVA with Games-Howell post hoc test at $p<0.05$ (n=5; mean ± sd).

The temperature-driven activity of encapsulated *S. cerevisiae* cells and the related CO_2 production follows the natural behavior of *I. ricinus* nymphs. These ticks show an increasing active movement at temperatures above 7 °C to 10 °C, whereas temperatures over 24 °C combined with a relative humidity below 80% lead to a withdrawal of the ticks and less activity due to the danger of dehydration [190]. Consequently, at low temperatures with low tick activity, fewer nutrients are metabolized within the beads and therefore a longer CO_2 release is provided.

The beads in figure 6.3 are either moist or dried and rehydrated to a water activity of 0.99 under optimal conditions. In the field, drying and rehydration processes take place during the day, so that the examination of the bead behavior at different water activities is necessary. Figure 6.3 presents the CO_2 production as well as the moisture content of the beads as a function of the water activity.

The graphs for the CO_2 production of dehydration and rehydration were nearly congruent. It is noticeable that the CO_2 production dropped from 0.31 to 0.02 $mL \cdot (g \cdot h)^{-1}$ between a_w 0.962 and 0.871 for dehydration and raised from 0.01 to 0.36 $mL \cdot (g \cdot h)^{-1}$ between a_w 0.895 and 0.962 during rehydration. In this range, a moisture content between 10.8% and 26.9% was determined, implying a threshold for the CO_2 production and consequently a limitation for the application of the beads in the field. The water activity defines the amount of available water in the surrounding medium and has a large influence on the growth of living cells. Examinations on the growth of yeast, such as *S. cerevisiae* depending on moisture content or water activity was mainly conducted in context of food technology. Exemplary, a study of Termini *et al.* [191] support the findings presented in figure 6.3 as they could not report any growth for the tested osmotolerant yeast strains below a water activity of 0.760 at 30 °C, whereas the optimum a_w for growth was in range of 0.913 to 0.998.

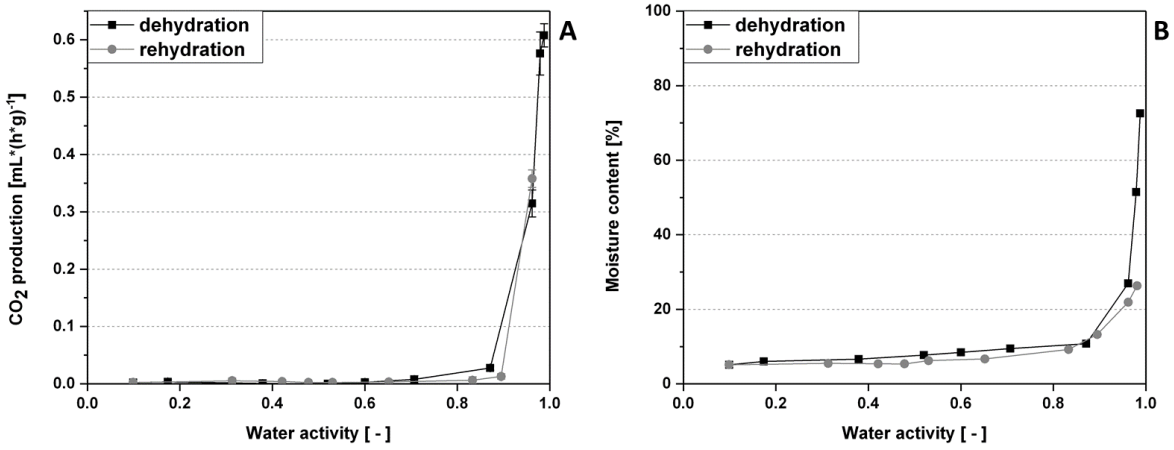


Figure 6.3 CO_2 production and moisture content during drying and rehydration of encapsulated *S. cerevisiae* in calcium alginate supplemented with supplemented corn starch. (CO_2 production: n=5, moisture content: n=10; mean ± sd).

Activity as well as shelf life highly depends on the water content of the cells [192; 193]. Therefore, the processes of drying and especially rehydration and a good moisture retention need further investigation to enable an application of the formulation in the field.

An Attract formulation, applicable above ground was developed. The formulation containing *S. cerevisiae* cells, granular corn starch and 0.1 $U \cdot g^{-1}$ AG releases a concentration of more than 0.2 $mL \cdot h^{-1} \cdot g^{-1}$ CO_2 at temperatures above 10 °C for a minimum of one week at 25 °C.

6.1.2 CO_2 production of Attract beads with supplemented urea

Urea is a natural substance that is excreted as a metabolic product through urine and sweat and can be decomposed by urease to CO_2 and ammonia (NH_3). The sensilla of the Haller's organ are reported to respond to elevated levels of these substances [53]. *S. cerevisiae* can utilize urea as sole nitrogen source by degrading it in NH_3 and CO_2 [124]. Therefore, urea was supplemented to the Attract beads containing 0.05 $U \cdot g^{-1}$ amyloglucosidase in order to increase the CO_2 production (figure 6.4). There was a significant influence of the composition of the formulation on the CO_2 production of moist beads (0 d: $F_{2,12}$=56.354; $p<0.05$, 1 d: $F_{2,12}$=62.514; $p<0.05$, 2 d: $F_{2,12}$=45.978; $p<0.05$, 3 d: $F_{2,12}$=25.097; $p<0.05$, 4 d: $F_{2,12}$=23.971; $p<0.05$). Beads containing *S. cerevisiae* supplemented with starch, amyloglucosidase and additional urea attained the highest CO_2 release at every timepoint when compared with the other two formulations. The production decreased from 0.56 $mL \cdot (g \cdot h)^{-1}$ CO_2 for fresh beads to 0.24 $mL \cdot (g \cdot h)^{-1}$ CO_2 after four days of incubation but still with the significant highest release rate ($p<0.05$). The other two formulations did not reach a CO_2 production above 0.22 $mL \cdot (g \cdot h)^{-1}$.

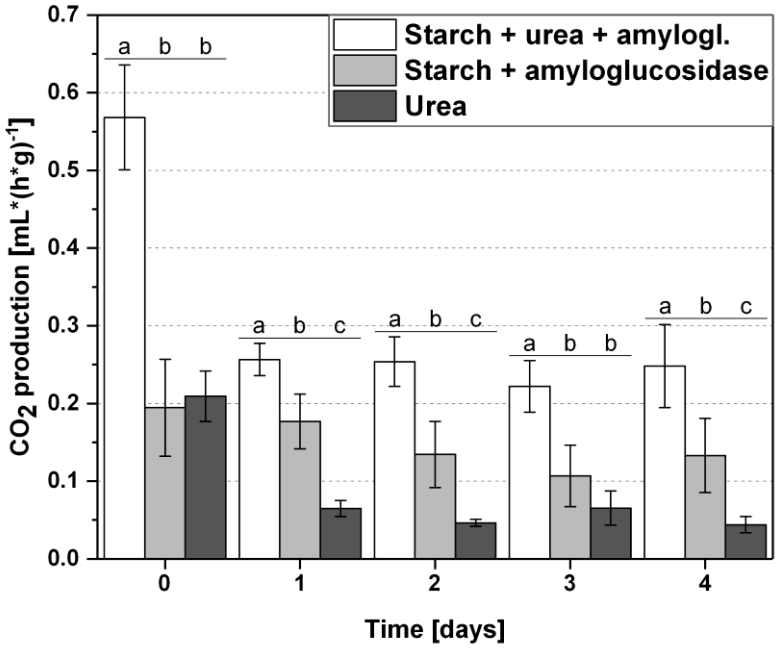


Figure 6.4 CO_2 production of moist calcium alginate beads containing *S. cerevisiae*, corn starch, urea and 0.05 $U \cdot g^{-1}$ amyloglucosidase incubated at 25 °C. Different letters above bars indicate significant differences according to one-way ANOVA with Games-Howell post hoc test at $p<0.05$ (n=5; mean ± sd).

It is noticeable that the beads achieve a significantly higher CO_2 production compared to the normal Attract beads only by adding urea. A significant increase in CO_2 production is also apparent in comparison to figure 6.1. The production decreases significantly within one day, either because most of the urea has already been degraded or because it diffuses out of the bead. Even if the capsules have not been stored in a liquid atmosphere, condensed water in the vessels allowed diffusion out of the bead. A strong decrease within 24 h from 0.21 to 0.06 $mL \cdot (g \cdot h)^{-1}$ CO_2 can also be observed for the beads, which exclusively contain S. *cerevisiae* and urea. In order to improve the retention of urea within the capsules, an alternative formulation material could be selected for encapsulation. Promising results from the fertilizer industry were achieved with starch-based formulations [194; 195]. A coating of the bead aiming at a reduction of pore size would additionally be conceivable.

However, the supplementation of urea as additional nutrient for the encapsulated *S. cerevisiae* attained promising first results in further increasing the CO_2 release. An improvement of the attraction of *I. ricinus* due to the released NH_3 still needs to be verified.

6.1.3 Activity of beads towards *I. ricinus* nymphs

To examine the attractive effect of CO_2 produced by encapsulated *S. cerevisiae* cells, dried beads were slowly rehydrated and placed in a Y-Olfactometer. As illustrated in figure 6.5, the application of beads as well as free CO_2 had a significant effect on the movement of *I. ricinus* nymphs compared to the control side with fresh air ($p<0.05$). 10 g dried and rehydrated Attract beads established an average CO_2 concentration of 2240±615 ppm in the experimental setup of the Y-olfactometer. 82.6% of the *I. ricinus* nymphs were attracted and migrated towards the test side. To verify the attraction, a positive control with free CO_2 was conducted and in this case 74% of the nymphs were attracted.

Due to evolutionary development, ticks are conditioned to react to CO_2, because mammals release large quantities of CO_2 via breath and skin [196; 197]. The tick *I. ricinus* uses the Haller's organ, located at their front pair of legs, to detect CO_2 in the surrounding air [198]. Literature on the attractive effect of CO_2 on ticks is usually based on the application of CO_2 traps, containing dry ice or CO_2 gas bottles, that only provide a short-term emission and high costs [88-92]. For example, Schulze *et al.* [93] demonstrated the attracting effect of a CO_2 trap on *Ixodes scapularis* in the field. The results presented in figure 6.5 are contrary to a study by

van Duijvendijk *et al.* [95]. In that study, CO_2 only evoked a response of the *I. ricinus* nymphs but no active movement, which only observed when combining CO_2 and host odor. Regarding the experimental setup, a two to 20-fold higher concentration of CO_2 was applied in the system, that may have had already a repelling effect on the nymphs. CO_2 concentrations higher than 3000 ppm in the experimental setup of this study triggered a significant repelling effect on the *I. ricinus* nymphs (data not shown), supporting this hypothesis.

Not only the CO_2 release but also the high moisture content of rehydrated beads may attract ticks. *I. ricinus*, like other terrestrial arthropods, needs to maintain its water balance in an environment with an average relative humidity often below a threshold concentration, situated between 86 to 96% RH [199]. Below this concentration, ticks need to maximize active uptake of water vapor from the surrounding air [200]. Crooks *et al.* [201] showed a significant attraction along a moisture gradient, when ticks were dehydrated. Since ticks absorb moisture from the surrounding air, a preference towards moist objects can be observed and was also monitored during experiments conducted with the moist Attract beads. A combination with a Kill-component therefore seems beneficial for a control agent.

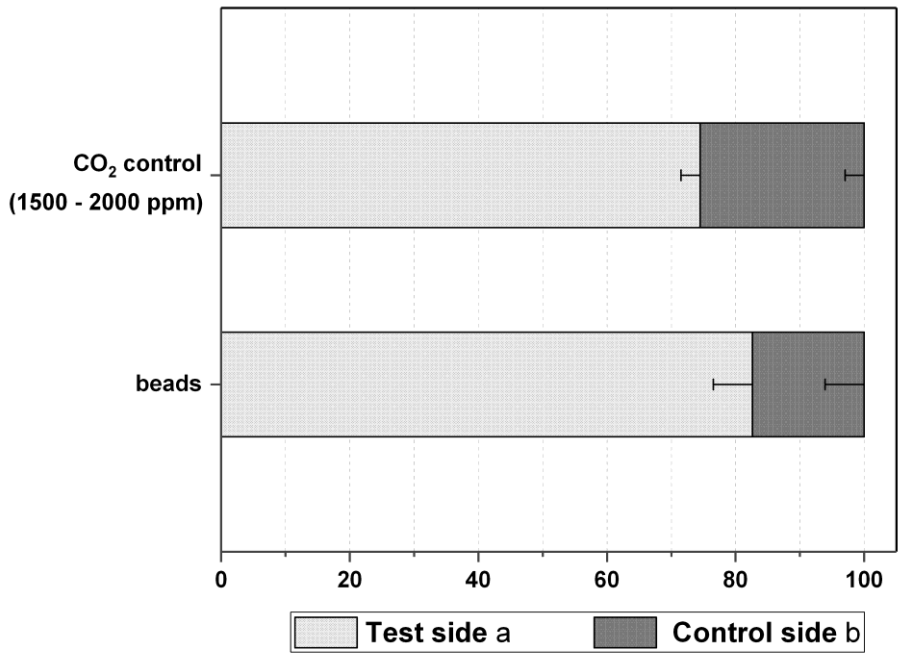


Figure 6.5 Attraction of *I. ricinus* nymphs by either 1500 – 2000 ppm CO_2 or 10 g dried and rehydrated calcium alginate beads containing *S. cerevisiae*, corn starch and 0.1 U/g amyloglucosidase at ambient temperature. Airflow of 47.8 mL·min-1 with 77-80% relative humidity. The control side contained fresh air. Different letters in legend indicate significant differences according to t-test at $p<0.05$ (n=30; mean ± sd).

Even though, the attraction of *I. ricinus* towards CO_2 is not completely clarified, the attraction of nymphs was shown in this experiment. Pursuing literature suggests promising results for the attractivity of the bead for luring several other tick species, such as *Amblyomma* spp. or *Dermacentor* spp., that are mainly attracted when CO_2 baits are applied [93; 96]. Attraction experiments with individuals of these species as well as *I. ricinus* adults need to be conducted in a following project. The supplementation with other odorous substances attractive for ticks, such as ammonium or acetate [196], can further enhance the attractive effect of the formulation. Nonetheless, the attraction of *I. ricinus* nymphs towards free CO_2 as well as towards the developed Attract formulation under laboratory conditions was successfully demonstrated in this work.

6.2 Kill

In a pre-screening, conducted by the University of Hohenheim, five *Metarhizium* spp. isolates were examined on their virulence against *I. ricinus* nymphs (supplementary material). The three most virulent isolates, *M. pemphigi* X1c, *M. brunneum* Ca8II and *M. brunneum* Cb16III were screened for their potential to serve as an active Kill-component in a tick control formulation.

6.2.1 Conidiation of *Metarhizium* spp. isolates on solid medium

Fermentation of entomopathogenic fungi on solid media is the conventional way to obtain aerial conidia for further application. It is known that most EPF can tolerate a wide range of temperatures, whereas optimal conditions for germination, growth and conidiation are specific for each isolate [202; 203]. The aim of this experiment was to get insights into the conidiation of the three pre-selected *Metarhizium* spp. isolates on solid medium at different temperatures (figure 6.6) and further on to find an isolate with a high conidiation under realistic conditions in the field. The isolates were cultivated on a medium adapted from Krell *et al.* [172] at 15 °C, 25 °C and 30 °C in the dark for 28 days.

For all three incubation temperatures, the *Metarhizium* spp. isolate had a significant effect on the aerial conidia production (15 °C: $F_{2,21}$=155.933; p<0.05, 25 °C: $F_{2,21}$=87.482; p<0.05, 30 °C: $F_{2,21}$=277.231; p<0.05) as well as on the interaction of treatment and time (15 °C: $F_{2.433,25.547}$=23.455; p<0.05, 25 °C: $F_{2.250,23.630}$=6.564; p<0.05, 30 °C: $F_{2.040,21.425}$=17.347; p<0.05) and the time solely (15 °C: $F_{1.217,25.547}$=133.757; p<0.05, 25 °C: $F_{1.125,23.630}$=79.210; p<0.05, 30 °C: $F_{1.020,21.425}$=72.192; p<0.05).

Regarding conidia production at 15 °C, *M. brunneum* Cb16III reached the highest aerial conidia yield with $3.5 \cdot 10^7$ conidia·cm^{-2} after 28 days which was significantly higher than for the other two *Metarhizium* spp. isolates (p<0.05). There was no significant difference between *M. bunneum* Ca8II with $0.9 \cdot 10^7$ conidia·cm^{-2} and *M. pemphigi* X1c with $0.9 \cdot 10^7$ conidia·cm^{-2} (p=0.903), respectively (figure 6.6 A). At 25 °C, conidiation was increased for all isolates when compared to 15 °C. In comparison, *M. pemphigi* X1c showed the significant lowest concentration ($2.2 \cdot 10^7$ conidia·cm^{-2}) at 25 °C after 28 days (p<0.05). Between the isolates *M. brunneum* Ca8II and Cb16III no significant difference was observed (p=0.802) (figure 6.6 B). Regarding incubation at 30 °C, *M. brunneum* Ca8II showed the significant highest conidiation with $4.7 \cdot 10^7$ conidia·cm^{-2} (p<0.05) after 28 days, while *M. pemphigi* X1c showed the significant lowest concentration with $0.2 \cdot 10^7$ conidia·cm^{-2} (p<0.05) (figure 6.6 C).

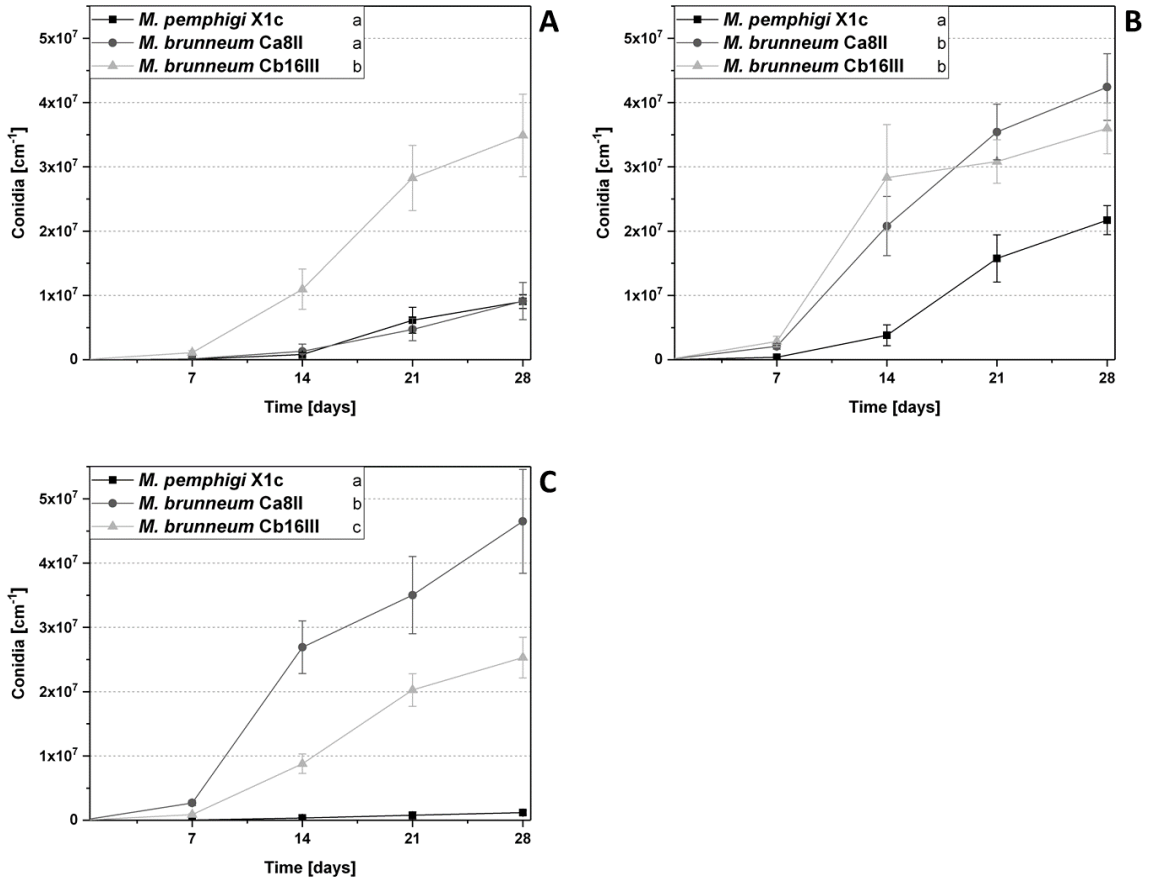


Figure 6.6 Conidia concentration during cultivation of three *Metarhizium* isolates at A: 15 °C, B: 25 °C and C: 30 °C on solid medium for four weeks. Different letters in the legend indicate significant differences according to RM-ANOVA with Games-Howell post hoc test at $p<0.05$ (n=8; mean ± sd).

The results clearly demonstrate a significant faster conidiation on the selected medium for the *M. brunneum* isolates compared to *M. pemphigi* X1c at 25 and 30 °C. The conidiation after 2 weeks of incubation at 25 °C ($2.8 \cdot 10^6$ - $2.8 \cdot 10^7$ conidia·cm^{-2}) are comparable to results obtained by Gao *et al.* [159] with a concentration between $1.4 \cdot 10^6$ to $2.4 \cdot 10^6$ conidia·cm^{-2} for *M. anisopliae* after two weeks. Since germination as well as conidiation is affected by the composition of the medium [159; 204], differences in germination and conidiation are not surprising. Regarding the different conidiation within the isolates, further studies of Ouedraogo *et al.* [203] and Nishi *et al.* [202] assumed a connection between the temperature optimum and the phylogenetic position and the regional origin of the tested *Metarhizium* isolates. This was also confirmed by Wang *et al.* [205]. Since the fungi tested in this screening were either isolated from soil (*M. brunneum*) or a dead tick (*M. pemphigi*) in Germany, an adaption to a higher temperature as e.g. 30 °C was not to be expected. This finding is in good agreement with a study of Davidson *et al.* [206] reporting only little or no growth at all for *Metarhizium* isolates

incubated at 30 °C and 35 °C. For *M. pemphigi*, growth was already decelerated at 30 °C in this study. Considering average meteorological data from Germany [207], conidiation of the fungi in a temperature range between 15 °C and 30 °C is required to be applicable in the field in Germany. This was proven for all three isolates, at least under laboratory conditions.

6.2.2 Liquid cultivation of three *Metarhizium* spp. isolates

Cultivation at different temperatures

Entomopathogenic fungi like *Metarhizium* spp. grow in two different morphologies, namely blastospores and mycelium, when cultivated in submerged cultures [156; 157], an established way to generate large quantities of fungal biomass. In the development of a pest control product, cost-effectiveness is a major factor. Submerged cultivation of EPF allows a simpler scale up and thus a reduction of costs, compared to a solid state cultivation process. In order to find a suitable candidate for blastospore production, the three isolates were incubated at three different temperatures. Figure 6.7 shows a significant influence of the *Metarhizium* spp. isolate on the blastospore production (15 °C $F_{2,18}$=122.437; p<0.05, 25 °C $F_{2,18}$=228.792; p<0.05, 30 °C $F_{2,18}$=8.991; p<0.05) as well as the total dry weight (15 °C $F_{2,12}$=177.262; p<0.05, 25 °C $F_{2,12}$=127.848; p<0.05, 30 °C $F_{2,12}$=416.054; p<0.05). Furthermore, the combination of isolate and time (blastospores: 15 °C $F_{5.032,45.285}$=19.612; p<0.05, 25 °C $F_{6.227,56.045}$=16.443; p<0.05, 30 °C $F_{7,62.997}$=3.676; p<0.05, dry weight: 15 °C $F_{6.082,36.494}$=26.717; p<0.05, 25 °C $F_{5.475,32.847}$=7.127; p<0.05, 30°C $F_{3.705,22.229}$=34.994; p<0.05) as well as the time by itself (blastospores: 15 °C $F_{2.516,45.285}$=132.180; p<0.05, 25 °C $F_{3.114,56.045}$=92.982; p<0.05, 30 °C $F_{3.5,62.997}$=4.498; p<0.05, dry weight:15 °C $F_{3.041,36.494}$=296.785; p<0.05, 25 °C $F_{2.737,32.847}$=101.934; p<0.05, 30 °C $F_{1.852,22.229}$=67.227; p<0.05) had a significant effect here.

Figure 6.7 illustrates the blastospore concentrations as well as the total dry weight for all three *Metarhizium* spp. isolates during cultivation at 15 °C, 25 °C and 30 °C. The three isolates showed significant differences in the blastospore concentration. At 15 °C and 25 °C, *M. brunneum* Cb16III showed the significant lowest blastospore production with. $4.77 \cdot 10^6$ blastospores·mL^{-1} compared to the other isolates. Even though *M. brunneum* Ca8II obtained the highest blastospore concentration at this temperature, *M. pemphigi* showed the significant highest total dry weight with 88.34 g·L^{-1} after 4 days (p<0.05). All isolates reached their individually highest blastospore concentration at 25 °C, but each at different time points.

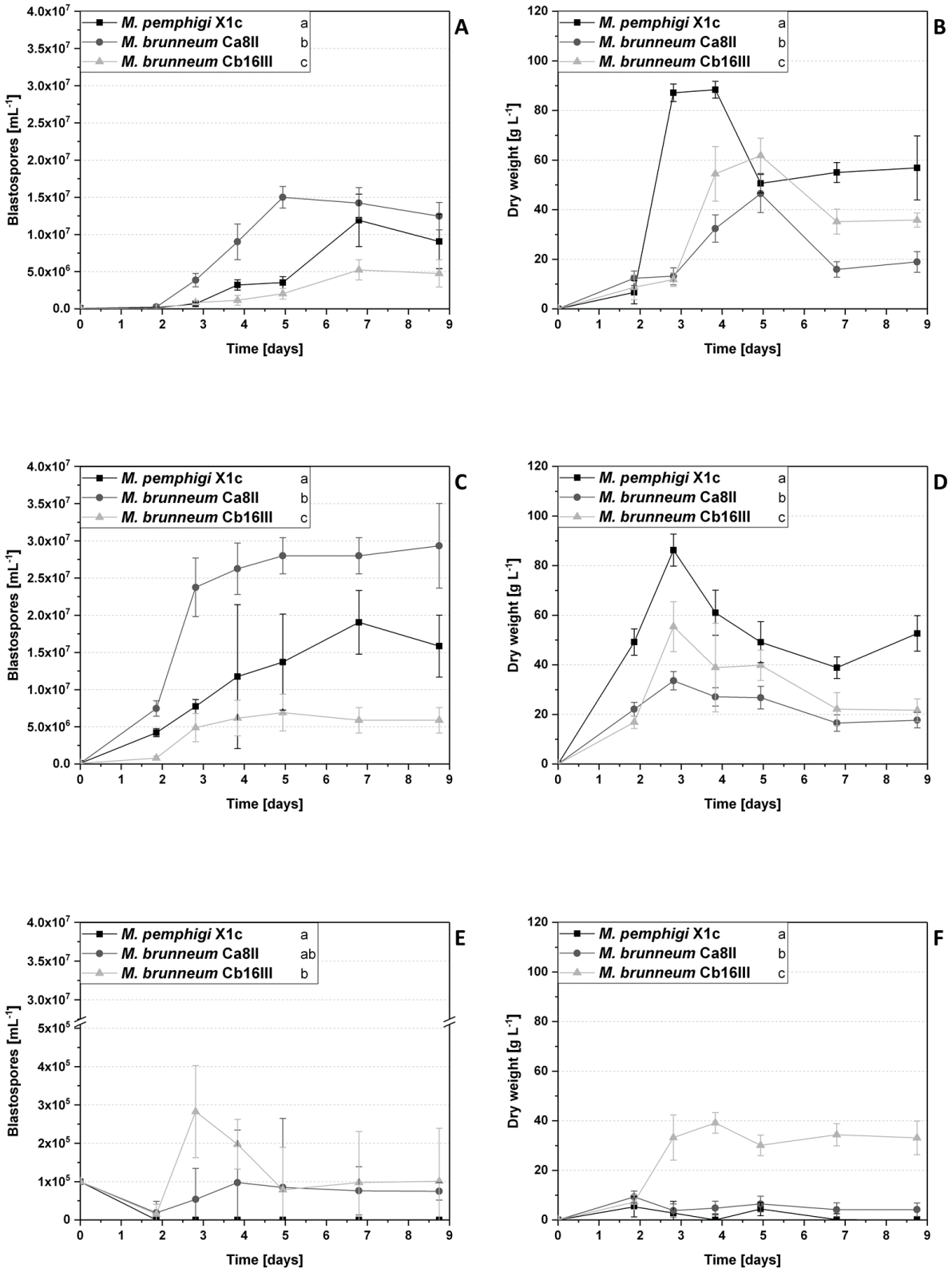


Figure 6.7 Liquid cultivation of three *Metarhizium* spp. isolates in shaking flasks at 15 °C (A, B), 25 °C (C, D) and 30 °C (E, F) for nine days. A, C, E: Blastospores, B, D, F: total dry weight. Different letters in the legend indicate significant differences according to RM-ANOVA with Games-Howell post hoc test at $p<0.05$ (blastospores: n=7, dry weight: n=5; mean ± sd).

M. brunneum Ca8II presented the significant highest overall concentration of $2.9 \cdot 10^7$ blastospores·mL^{-1} at 25 °C when the cultivation was stopped after nine days ($p<0.05$). In comparison, it is noticeable that *M. pemphigi* X1c had a lower increase in the blastospore concentration up to $1.9 \cdot 10^7$ blastospores·mL^{-1} but on the other hand attained the highest dry weight of 86 g·L^{-1} within the first three days. Surprisingly, *M. brunneum* Ca8II only reached less than half (34 g·L^{-1}) in the same time. Unlike on solid medium, *M. brunneum* Cb16III was found to be the least blastospore producing isolate at 25 °C with a maximum of $6.9 \cdot 10^6$ blastospores·mL^{-1} and a dry weight of 55 g·L^{-1} after three days. Regarding the total dry weight of all *Metarhizium* spp. isolates, it is notable that the dry weight increased within the first three days, followed by a measurable decrease to half of its weight. A correlation between the blastospore concentration and the total dry weight can be observed, especially when considering the curves for *M. pemphigi* X1c. A reduction of the blastospore concentration led simultaneously to a new increase in the dry weight.

Figure 6.7 E and F show that growth at 30 °C was only measurable for the isolate *M. brunneum* Cb16III. The isolate reached a blastospore concentration of $1.0 \cdot 10^5$ blastospores·mL^{-1} with a maximum total dry weight of 39.14 g·L^{-1} after four days, whereas the other two isolates generated a total dry weight up to a maximum of 9.3 g·L^{-1}, significantly lower compared to the results obtained at 15 °C and 25 °C.

In previous studies, the optimal temperature for submerged cultivation of *Metarhizium* spp. was reported in a range between 25 °C and 28 °C [36; 45; 156; 161]. This is consistent with the results obtained in this work. Regarding recent studies examining the growth of *M. brunneum*, the results obtained at 25 °C are in good agreement with Krell *et al.* [172]. In that study, a comparable medium was used and blastospore in concentrations up to $8.0 \cdot 10^6$ blastospores·ml^{-1} were produced with a *M. brunneum* isolate at 25 °C after 48 h which is in good agreement with $7.4 \cdot 10^6$ blastospores·ml^{-1} obtained in this study for *M. brunneum* Ca8II.

Comparing the results within the three isolates, *M. brunneum* Ca8II led to the highest overall blastospore concentrations, whereas *M. brunneum* Cb16III produced significantly less blastospores. It becomes clear that individual needs have to be considered, not only for different fungi, but also for different isolates within a certain species regarding culture conditions and medium composition.

M. pemphigi X1c presented a moderate blastospore concentration for 15 °C and 25 °C but the significant highest dry weight compared to the other isolates ($p<0.05$). The increase of the dry weight for all three isolates within the first days can be explained by the biology of the formation of blastospores by buddying from hyphae [161]. With an increasing blastospores production, mycelium growth seemed to decrease. Such phases of alternating production of mycelium and blastospores was already been observed before [162]. As shown by Krell *et al.* [172], the composition of liquid media for EPF cultivation can influence a shift from finely dispersed mycelium towards blastospores. Since the proportions of mycelium and blastospores are correlating to one another, a higher formation of blastospores and thus a significant increase in the blastospore concentration can be achieved by optimization of media composition [36; 157; 162] and process strategy [208]. Iwanicki *et al.* [209], for instance, obtained $4.0 \cdot 10^8$ and $5.9 \cdot 10^8$ blasospores·ml^{-1} within the same time for two isolates of *M. anisopliae* and *M. robertsii*, respectively with glucose concentrations up to 140 g·L^{-1}, nearly threefold higher than the glucose concentration in the medium used in this study. This indicates that a further raise of the glucose concentration may lead to a higher blastospore yield [157; 210]. On the other hand, Jackson *et al.* [211] used a glucose concentration of only 36 g·L^{-1} glucose in the medium and achieved concentrations up to $1.6 \cdot 10^8$ blasospores·ml^{-1} for *M. anisopliae* after four days of cultivation, whereby a reduction of the glucose concentration to 8.0 g·L^{-1}, only $6.5 \cdot 10^5$ blasospores·ml^{-1} were recorded. Figure 6.8 illustrates exemplary the remaining glucose concentration in the medium measured during the cultivation of *M. pemphigi* X1c at 25 °C. It can be noticed, that a concentration of 23.9 g·L^{-1} glucose remained in the medium after a cultivation period of nine days, compared to the initial concentration of 50 g·L^{-1} glucose in the medium. Since the fungi decelerated growth, a limitation in the glucose uptake is assumed, potentially caused by the lack of further nutrients or the accumulation of by-products [212]. This indicates that solely a further increase in glucose will probably not improve the blastospore concentration.

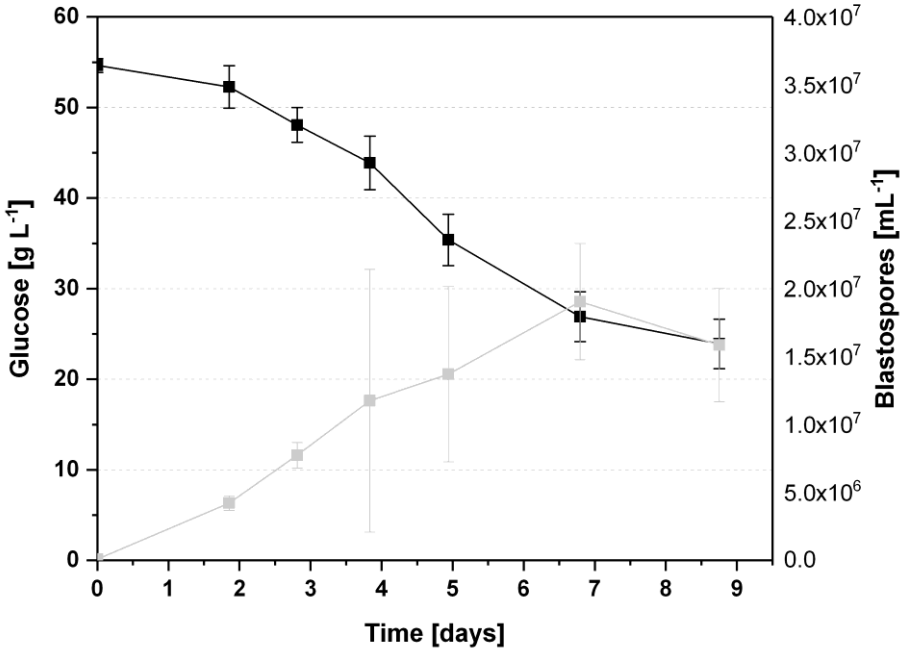


Figure 6.8 Comparison of glucose concentration of the medium as well as blastospore concentration during submerged cultivation of *M. pemphigi* X1c at 25 °C (n=5; mean ± sd).

Cultivation at different initial pH values

Regarding different growth conditions of EPF in submerged cultures, not only temperature but also the initial pH value can influence germination, total biomass and production of blastospores. As previously reported by Hallsworth *et al.* [149], EPF show growth at a larger pH range, probably due to a higher ability to regulate their cytosolic pH value. Therefore, growth at a broad range of pH values indicates a high pH tolerant isolate that can have advantages over other microorganisms when applied in the field. Hence, different liquid medium pH values were adjusted and their influence on the blastospore concentration as well as total dry weight was tested. Figure 6.9 reveals a significant influence of the *Metarhizium* spp. isolate on the blastospore production at different pH values (pH 3 $F_{2,18}$=77.542; p<0.05, pH 9 $F_{2,18}$=166.150; p<0.05) as well as the total dry weight (pH 3 $F_{2,12}$=53.282; p<0.05, pH 9 $F_{2,12}$=49.039; p<0.05). Furthermore, the combination of isolate and time (blastospores: pH 3 $F_{4.173,37.557}$=29.841; p<0.05, pH 9 $F_{5.077,45.694}$=47.562; p<0.05, dry weight: pH 3 $F_{4.922,29.531}$=27.726; p<0.05, pH 9 $F_{3.754,22.527}$=9.374; p<0.05) as well as the time by itself (blastospores: pH 3 $F_{2.087,37.557}$=73.463; p<0.05, pH 9 $F_{2.539,45.694}$=65.730; p<0.05, dry weight: pH 3 $F_{2.461,29.531}$=93.134; p<0.05, pH 9 $F_{1.877,22.527}$=60.764; p<0.05) had a significant effect here.

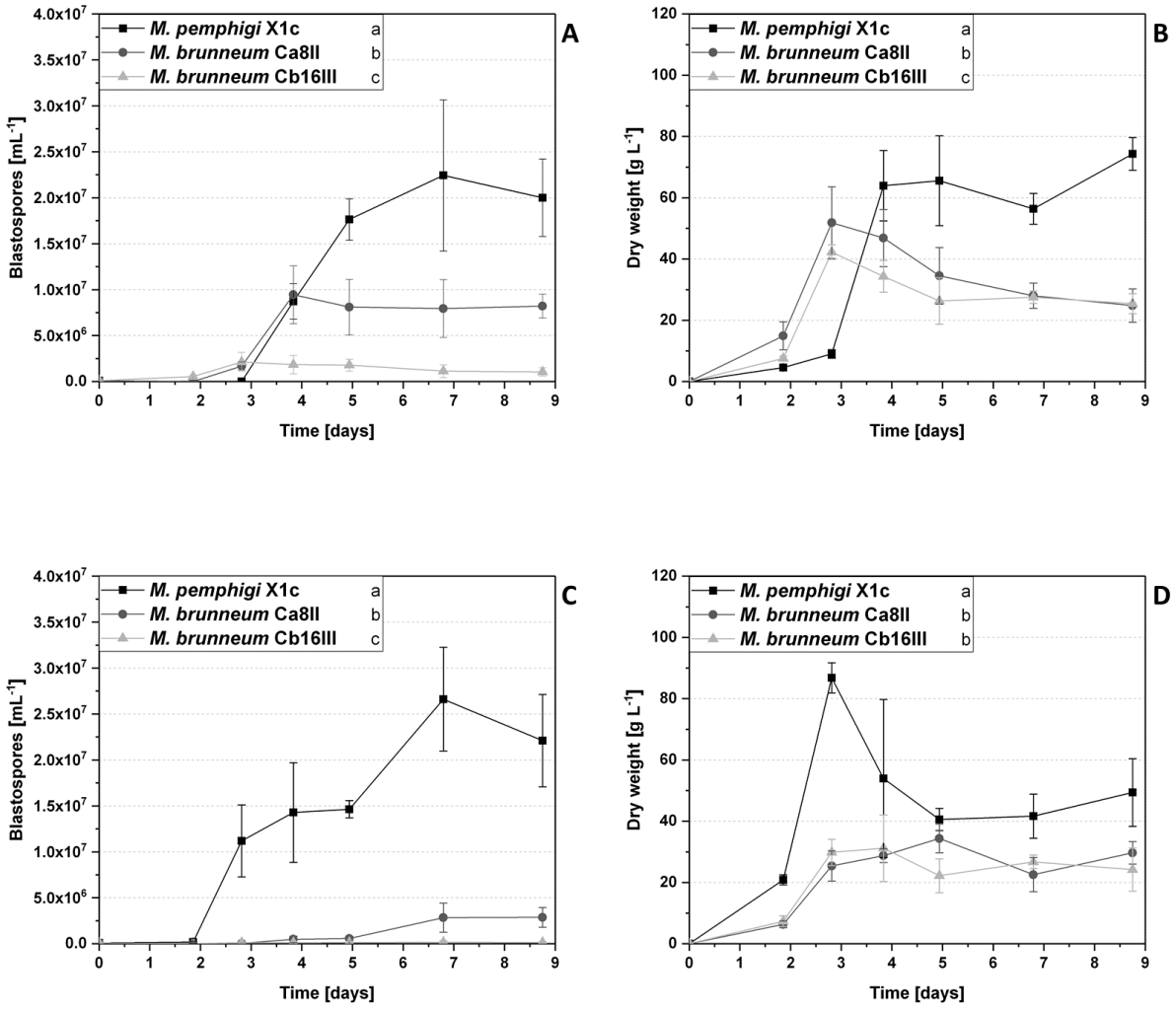


Figure 6.9 Blastospore concentration during liquid cultivation of three *Metarhizium* spp. isolates in shaking flasks at 25 °C and different initial media pH value 3 (A, B) and 9 (C, D) for nine days. A, C: Blastospores, B, D: total dry weight. Different letters in the legend indicate significant differences according to RM-ANOVA with Games-Howell post hoc test at $p<0.05$ (blastospores: n=7, dry weight: n=5; mean ± sd).

The three *Metarhizium* spp. isolates show a significant difference in their tolerance against different initial pH values, referring to the results illustrated in figure 6.9. Regarding both, total dry weight and blastospore concentration, *M. pemphigi* X1c showed the significant highest tolerance in a range of pH 3 to pH 9 ($p<0.05$). For all pH values (pH 4.5 and pH 7.5 in supplementary material) maximum blastospore concentrations between $1.89 \cdot 10^7$ to $2.66 \cdot 10^7$ blastospores·mL^{-1} and maximum total dry weights between 65.54 g·L^{-1} and 93.06 g·L^{-1} were obtained. For both *M. brunneum* isolates the blastospore production at pH 3 was higher than with initial pH 9. Since the EPF all tend to acidify the medium to create favorable

conditions for their growth (pH value during cultivation was measured), this result was as expected [213].

Generally, pH values in submerged culitvations of *Metarhizium* spp. are adjusted near neutral [45; 162] or not considered any further. Kleespies *et al.* [47] determined blastospore concentrations for three *M. anisopliae* isolates with an initial pH value between 4.5 and 8.0 in submerged culture for three days. The results of two isolates are in good agreement with the blastospore concentrations obtained in this study, even though those concentrations were reached in a shorter time. In contrast to this study, Kleespies *et al.* [47] inoculated with a ten-fold higher inoculum, leading to higher blastospore concentrations in a shorter time. On the other hand, the findings are contrary to those of Ypsilos *et al.* [161], who reported no growth at pH 3 for *M. anisopliae*. Other literature mainly focused on solid medium and reported growth over a wider range of pH (2.9 – 11.1) [149]. Issaly *et al.* [162] reported a positive effect of a pH regulation during cultivation of *M. flavoviride* due to pellet formation when the pH was not regulated. This phenomenon of pellet formation was not found for *M. pemphigi* X1c but for both *M. brunneum* isolates in this study, especially at pH 9, what may have led to the low blastospore concentrations. Further studies, such as Ypsilos *et al.* [161] found an increase in the polyol concentration for pH 5 – 10 within the blastospores. Even though this was not examined for the submerged cultures, polyols are described to be helpful for drying survival [150] and a high concentration within *M. pemphigi* X1c can be expected due to its stability at harsh culture conditions. Since blastospores are known to be very sensitive to drying processes, this should be taken into account for further experiments.

Even though, promising results for submerged cultivation of all three *Metarhizium* spp. isolates were obtained, *M. pemphigi* X1c presents the highest potential for further application as a biological control agent. The isolate offers not only the opportunity to achieve a shift towards a higher blastospore concentration (because of the highest dry weight) but also the chance for a higher activity when applied in the field due to its stability at a broad range of pH values. Nevertheless, this study exhibits some limitations. To enhance cultivation yields, factors, such as carbon/nitrogen ratio, oxygen levels or produced by-products should be considered.

A scale-up of the submerged cultivation of *M. pemphigi* X1c under optimal conditions up to a bioreactor volume five liters in a batch as well as fed-batch fermentation was checked by a project partner in context of the connected joint cooperation research project “BIOZEC”.

6.2.3 Conidiation of encapsulated *Metarhizium* spp. isolates on beads

Encapsulation in calcium alginate is a well-established method for the protection of fungal biomass from environmental factors, such as temperature, extreme pH values or low water contents [140; 141] and was demonstrated not to interfere with vegetative growth and conidiation of EPF [142]. The incorporation of different additives can result in further advantages for the EPF, such as drying stability or improved conidiation [50; 143; 214; 215].

Conidiation on moist beads

High dosages of aerial conidia (10^{13} – 10^{14} conidia/ha) are usually required for the control of pests in the field [134]. The direct application of non-formulated aerial conidia has several disadvantages, such as a lower shelf life, thus more frequent application and consequently higher costs. Following a different approach, a suitable formulation in the form of a bead that functions as a microfermenter can reduce the required initial biomass because encapsulated nutrients lead to a multiplication of a relatively small inoculum [50]. Therefore, blastospores of the selected EPF were encapsulated in calcium alginate beads together with different nutrients aiming at a multiplication of the biomass. The blastospores are supposed to germinate within the bead and in the next step grow out and produce virulent aerial conidia on the bead surface. Even though, blastospores are reported to be less resistant to environmental factors than aerial conidia [36; 158], they are easier to produce in high quantities and therefore more suitable for a large-scale production.

As illustrated by figure 6.10, there was a significant effect of the encapsulated *Metarhizium* spp. isolate (15 °C $F_{2,27}$=284.979; $p<0.05$, 25 °C $F_{2,27}$=111.423; $p<0.05$, 30 °C $F_{2,27}$=146.838; $p<0.05$) and the interaction of isolate and time (15 °C $F_{4.012,54.157}$=22.343; $p<0.05$, 25 °C $F_{3.744,50.549}$=10.878; $p<0.05$, 30 °C $F_{3.650,49.278}$=13.499; $p<0.05$) as well as time (15 °C $F_{2.006,54.157}$=84.148; $p<0.05$, 25 °C $F_{1.872,50.549}$=94.442; $p<0.05$, 30 °C $F_{1.825,49.278}$=29.369; $p<0.05$) on the conidiation on the bead surface.

In this experiment, native granular corn starch was added to the formulation. Starch is well known to maintain the structure of beads [120], to function as drying protectant [119], to bind water [189; 216], to serve as a long-lasting nutrient [50] and therefore may enhance aerial conidia production on the bead surface [215; 216]. In this study, a more than four hundred-fold multiplication of the initial biomass (10^6 blastospores ·bead^{-1}) was achieved for all three isolates within 28 days at 25 °C, referring to the produced aerial conidia on the bead surface.

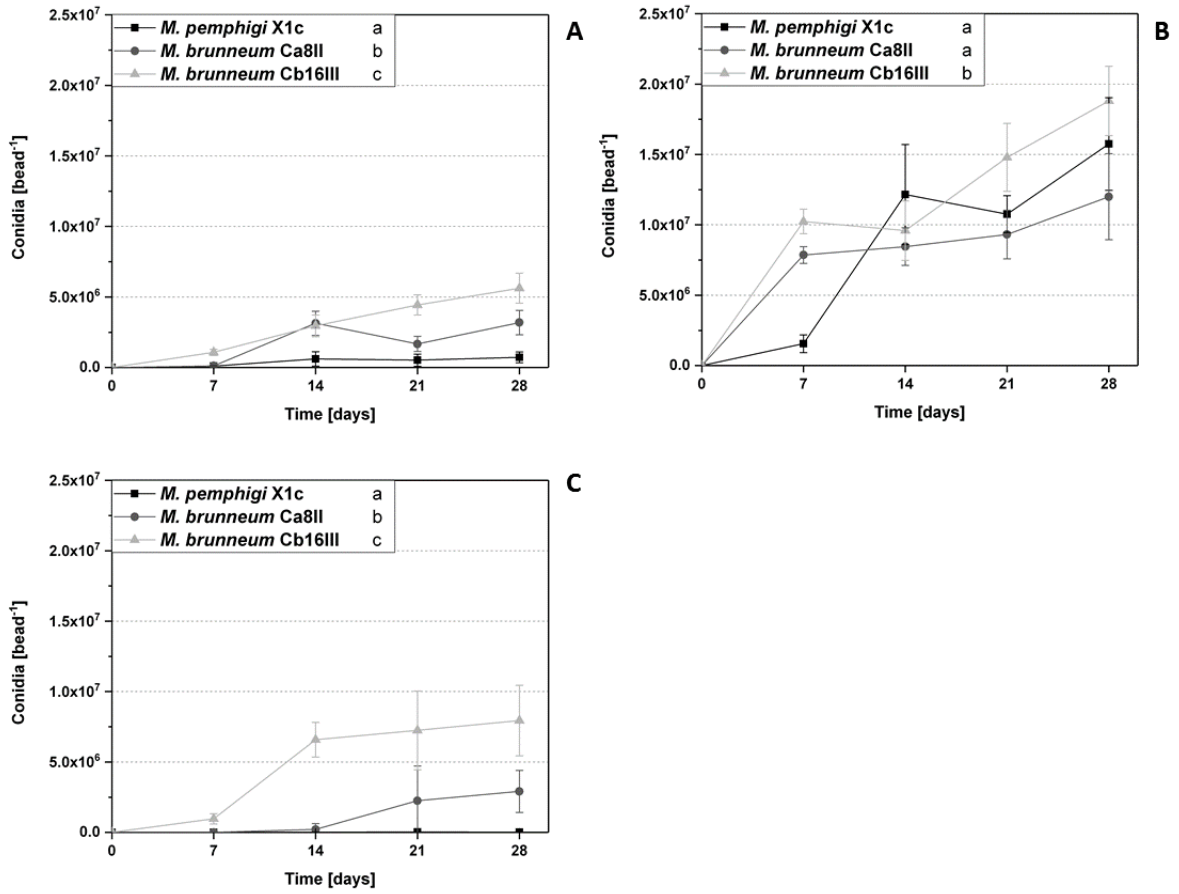


Figure 6.10 Conidiation of *M. pemphigi* X1c, *M. brunneum* Ca8II and Cb16III encapsulated in calcium alginate referring to the conidiation on the bead surface at 15 °C (A), 25 °C (B) and 30 °C (C) for four weeks. Different letters in the legend indicate significant differences according to RM-ANOVA with Games-Howell post hoc test at $p<0.05$ (n=10; mean ± sd).

All *Metarhizium* spp. isolates reached their highest conidiation when incubated at 25 °C (figure 6.10) with a concentration of $1.2 \cdot 10^7$ conidia·bead^{-1} (*M. brunneum* Ca8II) or even higher (*M. brunneum* Cb16III and *M. pemphigi* X1c). No significant difference was observed between the beads containing *M. pemphigi* X1c ($1.6 \cdot 10^7$ conidia·bead^{-1}) and *M. brunneum* Cb16III ($1.9 \cdot 10^7$ conidia·bead^{-1}) blastospores ($p=0.111$), when comparing the concentrations obtained after 28 days, although there was a significant difference between the isolates considering the interaction of isolate and time ($p<0.05$). Even though, *M. brunneum* Ca8II had a significant higher conidia concentration on the bead surface after seven days than *M. pemphigi* X1c, the conidiation decreased, and the isolate attained the significant lowest final conidiation of $1.2 \cdot 10^7$ conidia·bead^{-1}; $p<0.05$.

When the moist beads were incubated at 15 or 30 °C, the conidiation was decreased, compared to 25 °C. At 15 °C, beads containing *M. brunneum* Cb16II blastospores showed the highest conidiation with $5.61 \cdot 10^6$ conidia·bead^{-1} after four weeks. Similar to the conidiation on solid

medium (figure 6.1), both other isolates presented significant less conidiation ($p<0.05$) with $7.27 \cdot 10^5$ (*M. pemphigi* X1c) and $3.19 \cdot 10^6$ conidia·bead^{-1} (*M. brunneum* Cb16III).

The conidiation for *M. pemphigi* X1c reached an expectedly low concentration of $1.72 \cdot 10^4$ conidia·bead^{-1} when incubated at 30 °C, compared to the results obtained with solid cultivation. Also *M. brunneum* Ca8II only reached a concentration of $2.91 \cdot 10^6$ conidia·bead^{-1}. Regarding the decreased growth in submerged cultures at 30 °C, it can be assumed that *M. brunneum* Ca8II aerial conidia are more tolerant to higher temperatures than blastospores, leading to a faster germination [217; 218].

Figure 6.11 shows all three encapsulated isolates after four weeks of incubation at 25 °C and 30 °C.

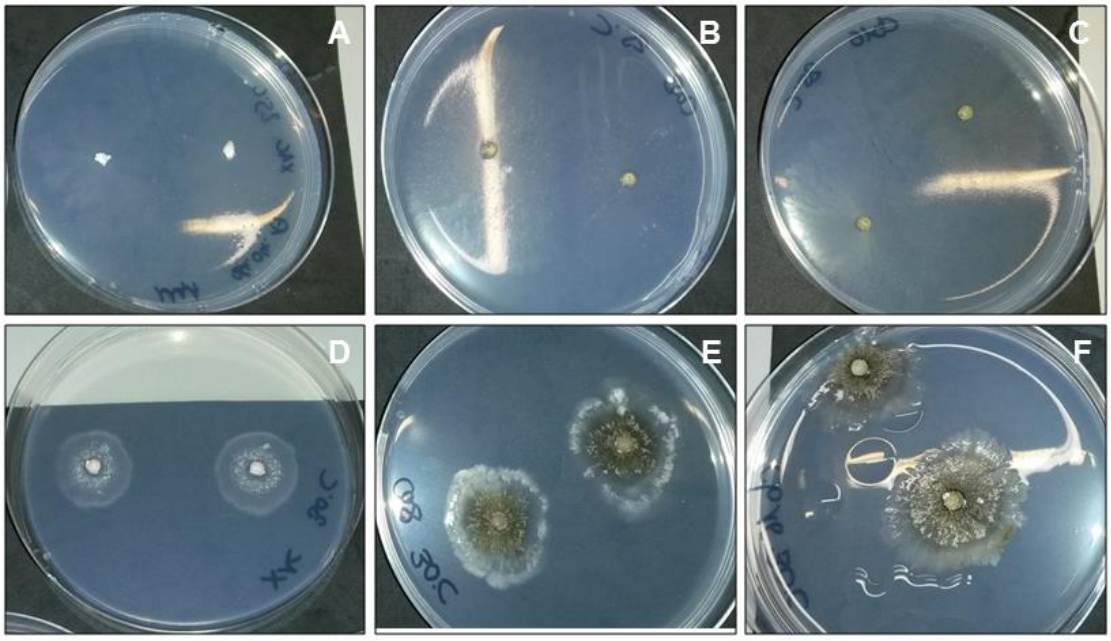


Figure 6.11 Moist beads containing *Metarhizium* spp. blastospores incubated for four weeks at25 °C (A, B, C) and 30 °C (D, E, F). A, D: *M. pemphigi* X1c, B, E: *M. brunneum* Ca8II, C, F: *M. bruuneum* Cb16III.

While beads incubated at 25 °C predominantly showed conidiation on the bead surface, beads incubated at 30 °C resulted in a significant higher radial mycelium growth for all three *Metarhizium* spp. isolates and thus an increased conidiation on the surrounding area around the bead. Since only the conidia concentrations on the bead surface were determined, the actual concentration of conidia formed at 30 °C was higher, considering the conidia on the surrounding area.

However, the presented results show a significant difference in the production of aerial conidia on the bead surface within the three *Metarhizium* spp. isolates for all temperatures tested. Since

M. brunneum isolates are reported to be able to assimilate corn starch [219], it is not surprising that *M. brunneum* Ca8II and *M. brunneum* Cb16III showed a significant higher conidiation within the first seven days of incubation, whereas *M. pemphigi* X1c needed further 14 days to attain equivalent conidiation at 25 °C. *M. pemphigi* X1c was isolated from a dead ticks' body, hence a fewer adaption to the utilization of corn starch as nutrient is conceivable. Qualitative enzyme activity assays support this presumption of less metabolization by *M. pemphigi* (figure 6.11). Notwithstanding, the presented data on conidiation at 25 °C are in good agreement with the concentrations of newly formed *M. brunneum* conidia as reported in a previous study by Przyklenk *et al.* [50]. In contrast to this screening, Przyklenk *et al.* [50] applied dried beads containing aerial conidia instead of blastospores. Concerning germination within the bead and the conidiation on the bead surface, no significant difference between encapsulated aerial conidia and blastospores was observed (see also chapter 6.3.2). A further increase of the inoculum did not result in a higher growth rates or a higher conidiation (data not shown) as already shown for *M. brunneum* by Przyklenk *et al.* [50].

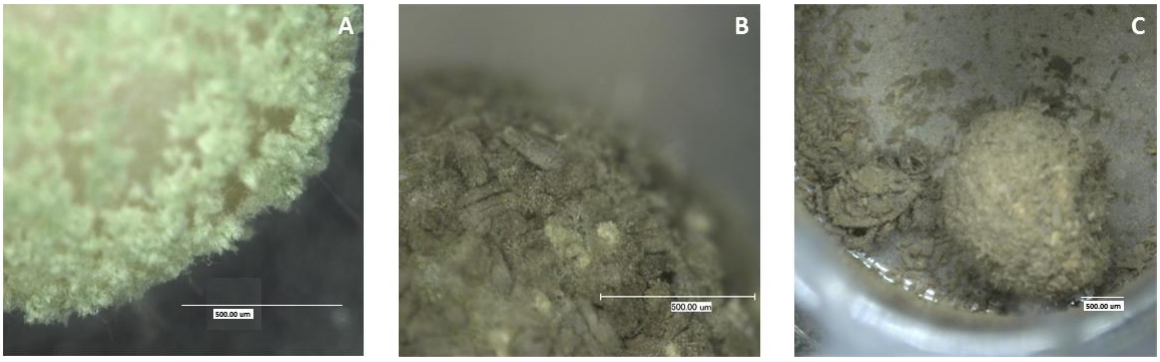


Figure 6.12 Microscopic image of newly formed *Metarhizium* spp. conidia on moist calcium alginate beads, incubated for three weeks at 25 °C. A: *M. pemphigi* X1c, B: *M. brunneum* Ca8II, C: *M. brunneum* Cb16III.

The findings illustrate an appropriate conidiation of more than $1.25 \cdot 10^4$ conidia·bead^{-1} (figure 6.12; equivalent to 10^5 conidia·cm^{-2} in the virulence pre-screening (supplementary material)) on the bead surface for all three isolates at 25 °C when granular corn starch was supplemented as nutrient. Conidiation at lower temperatures need to be improved to be applicable in the field, since the temperature in Germany in spring is to be expected between 10 °C and 28 °C [207].

Aiming at a further increase in conidiation, a quantitative test on petri dishes with agar containing different nutrients was conducted for the three *Metarhizium* spp. isolates (figure 6.13).

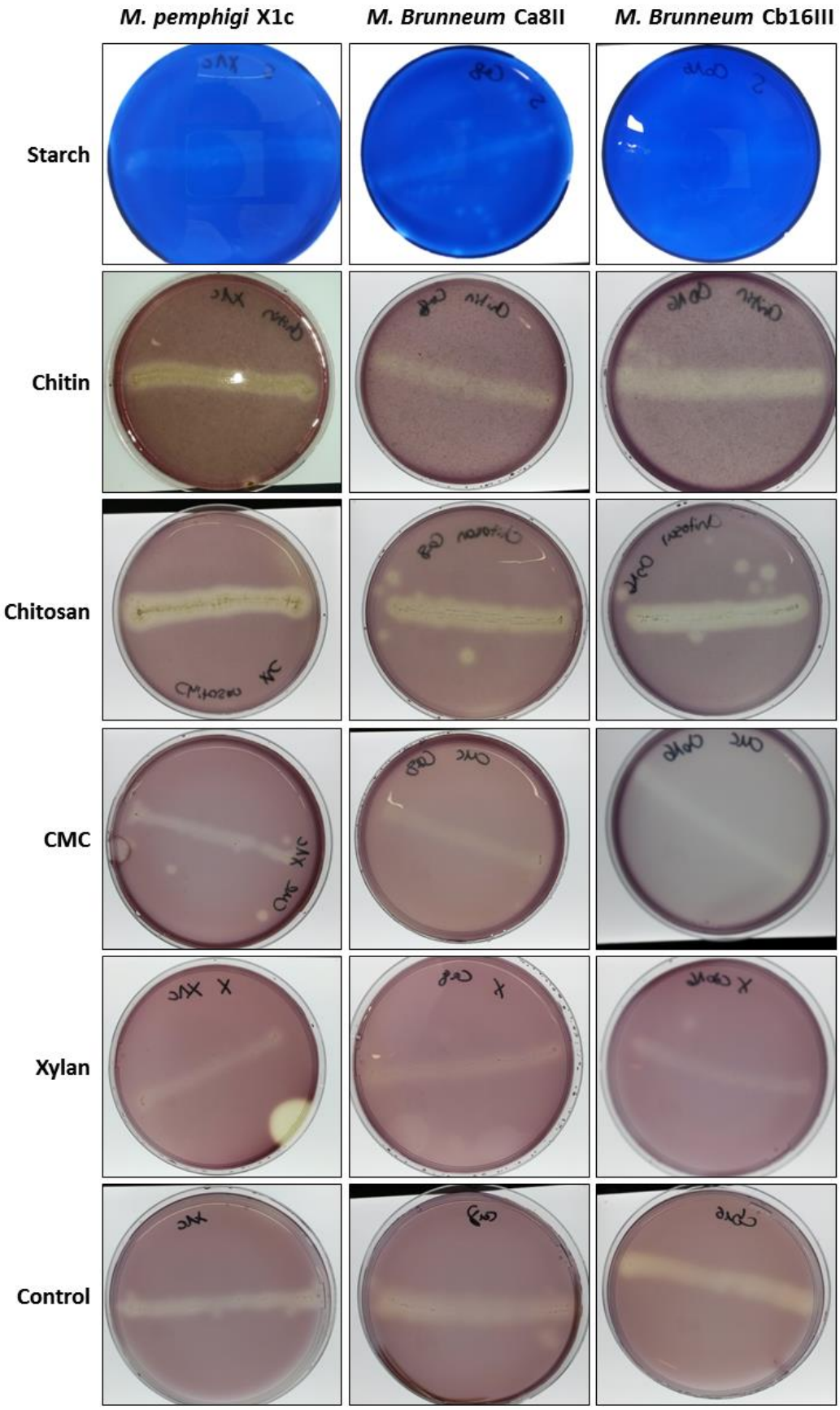


Figure 6.13 *Metarhizium* spp. isolates incubated on different media at 25 °C for 2 weeks, dyed with Lugol's iodine.

All petri dishes were incubated under the same conditions. As expected, the qualitative test showed only a low consumption of corn starch, whereas the three *Metarhizium* spp. isolates showed a utilization of supplemented chitin or chitosan. Microcrystalline cellulose (CMC) as well as Xylan showed no obvious difference to the control without any supplements.

To enhance conidiation on the bead surface, chitin as an industrial by-product was chosen as an alternative nutrient for encapsulation.

Conidiation on dried and rehydrated beads

Blastospores were encapsulated in calcium alginate beads together with either starch or chitin and dried gently in a laminar air flow. The conidiation on both, moist as well as dried and rehydrated beads was determined every week for 28 days. As illustrated by figure 6.14, there was a significant effect of the formulation (*M. pemphigi* X1c $F_{3,36}$=479.676; p<0.05, *M. brunneum* Ca8II $F_{3,36}$=140.596; p<0.05, *M. brunneum* Cb16III $F_{3,36}$=241.260; p<0.05) and the interaction of formulation and time (*M. pemphigi* X1c $F_{8.616,103.392}$=17.456; p<0.05, *M. brunneum* Ca8II $F_{5.251,63.008}$=11.035; p<0.05, *M. brunneum* Cb16III $F_{7.323,87.874}$=46.409; p<0.05) as well as time (*M. pemphigi* X1c $F_{2.872,103.392}$=240.695; p<0.05, *M. brunneum* Ca8II $F_{1.750,63.008}$=73.675; p<0.05, *M. brunneum* Cb16III $F_{2.441,87.874}$=169.583; p<0.05) on the conidiation on the bead surface.

Both, moist and dried and rehydrated beads containing chitin showed a significant higher conidiation compared to beads containing starch for all three isolates (p<0.05). Moist beads containing chitin and *M. pemphigi* X1c blastospores reached the highest overall conidiation with $4.68 \cdot 10^7$ conidia·bead^{-1} after 28 days of incubation, followed by beads with chitin and *M. brunneum* Cb16III blastospores ($3.93 \cdot 10^7$ conidia·bead^{-1}) For *M. brunneum* Ca8II containing beads, $2.60 \cdot 10^7$ conidia·bead^{-1} were determined. In comparison to moist beads containing starch, the conidiation could be increased at least by more than two-fold for all isolates, when chitin was supplemented instead.

Moreover, also dried and rehydrated beads containing chitin and *M. pemphigi* X1c revealed a higher conidiation of $3.03 \cdot 10^7$ conidia·bead^{-1} compared to both, moist and dried and rehydrated formulations containing starch (p<0.05). For beads containing the isolates *M. pemphigi* X1c and *M. brunneum* Ca8II, the process of drying and rehydration led to a significant reduction of the conidiation for both formulations (p<0.05), whereas it had no significant effect on the conidiation for beads containing *M. brunneum* Ca8II blastospores (starch p=0.20, chitin p=0.10).

A control bead consisting of alginate and blastospores without any further additive showed no conidiation for the dried and rehydrated beads and only $2.30 \cdot 10^5$ conidia·bead^{-1} for the moist formulation (data not shown).

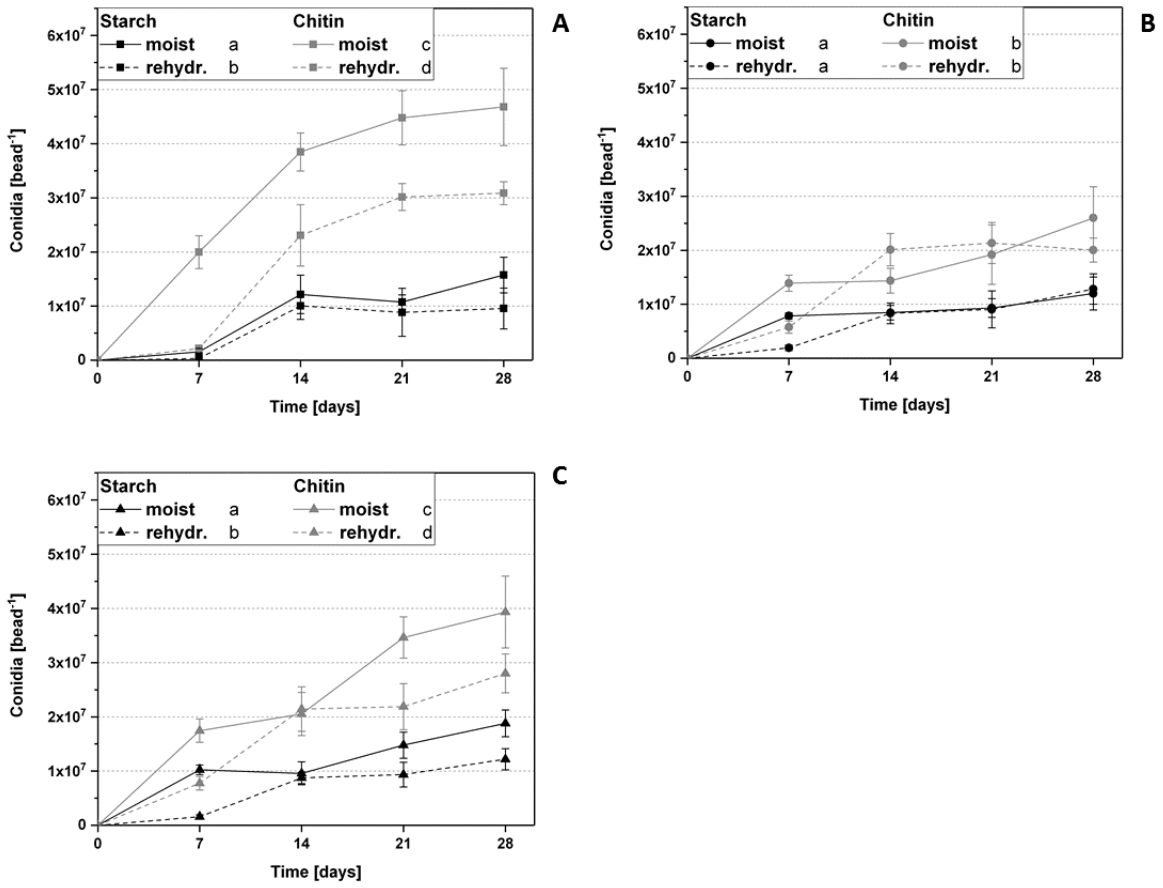


Figure 6.14 Conidiation of *M. pemphigi* X1c (A). *M. brunneum* Ca8II (B) and *M. brunneum* Cb16III (C) encapsulated in calcium alginate supplemented with corn starch or chitin on the bead surface at 25°C for four weeks. Different letters in the legend indicate significant differences according to RM-ANOVA with Games-Howell post hoc test at $p<0.05$ (n=10; mean ± sd).

For the determination of drying survival, moist as well as dried and rehydrated beads were dissolved, CFU determined and the drying survival calculated for all formulations. Considering the survival of *Metarhizium* spp. blastospores in all formulations containing nutrients, a significant higher drying survival was obtained compared to a control bead without additives ($p<0.05$). For the control containing *M. pemphigi* X1c blastospores, only 0.14% of the encapsulated biomass survived the process of drying and rehydration. The addition of starch to the bead led to a survival of 2.67% for *M. pemphigi* X1c, with no significant difference to *M. brunneum* Ca8II (5.18%, p=0.057) and Cb16III (5.02%, p=0.086). Beads containing chitin and *M. pemphigi* X1c reached the highest overall survival with 14.7% which was more than

ten-fold higher when compared to the control. Formulations with chitin and *M. brunneum* Ca8II attained 10.4% and *M. brunneum* Cb16III 12.3%, respectively. For all isolates, the supplementation of chitin led to a significant higher drying survival, compared to beads with granular corn starch ($p<0.05$).

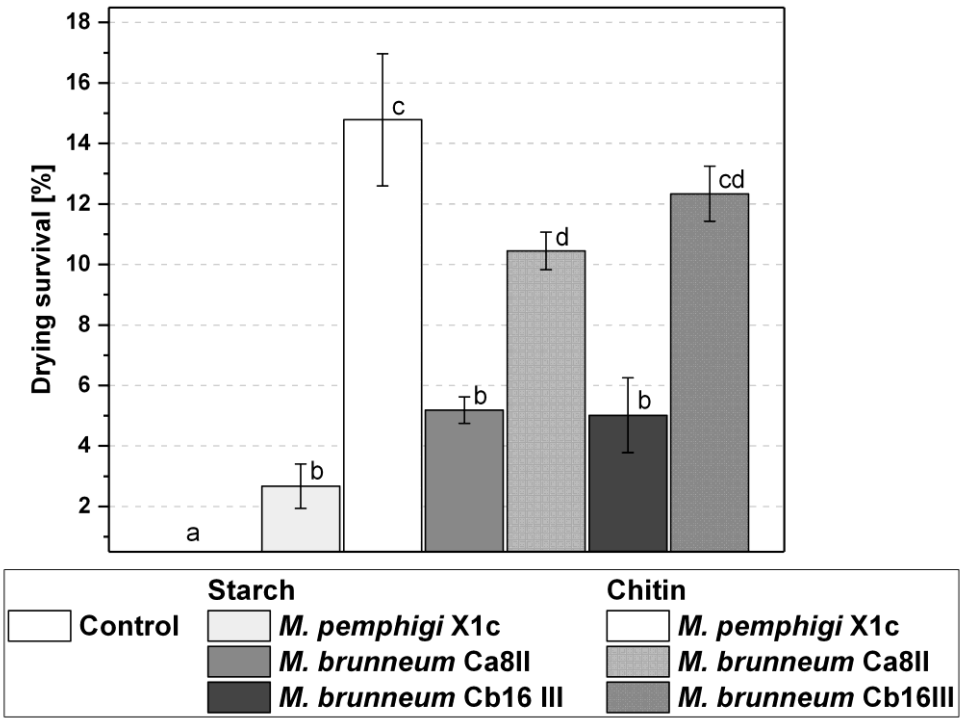


Figure 6.15 Drying survival of *M. pemphigi* X1c, *M. brunneum* Ca8II and *M. brunneum* Cb16III encapsulated in calcium alginate together with starch or chitin. Different letters above bars indicate significant differences according to RM-ANOVA with Games-Howell post hoc test at $p<0.05$ (n=10; mean ± sd).

The advantages of granular corn starch were already mentioned above. Chitin in comparison is known to be one of the most widely occurring polysaccharides, as it serves for structure formation of the cuticle not only in the majority of insects [220] but also in hard bodied ticks [55]. Most EPF are known to provide chitinase activity [221-223]. Therefore, chitin can likely serve as nutrient [224; 225] and enhance conidiation on beads [226]. Only marginal sorption properties for chitin are reported, compared to starch under optimal conditions [227; 228].

The presented results show a significant difference in the production of aerial conidia on the bead surface when comparing starch and chitin as nutrient. For both additives, assimilation by *Metarhizium* spp. was already reported [219; 222; 229]: Therefore, a high conidiation is not surprising. Nevertheless, a significant difference was revealed. Other than the *M. brunneum* isolates, *M. pemphigi* X1c was originally isolated from a dead tick body, so that a fewer adaption to the utilization of corn starch as nutrient compared to chitin as part of the tick cuticle

is conceivable. Fernandes *et al.* [81] showed relatively low amylase secretion for *M. anisopliae* compared to other EPF such as *Beauveria bassiana,* also supported by the qualitative enzyme assay in figure 6.13. Chitinase however, is part of the enzymatic equipment of EPF since it is needed for growth regulation [230], as their cell wall consists partly of chitin. A further benefit of a high chitinase secretion by EPF is to facilitate the infection of a potential host. The cuticle, consisting of chitin and other structure-giving substances [55] forms not only the primary surface for contact with conidia but also the main barrier for infection [222]. Therefore, extracellular chitinases with high activity are necessary for an efficient infection of the host by softening its cuticle due to assimilation of the incorporated chitin [231]. Conidiation on calcium alginate beads containing chitin was already shown for EPF such as *B. bassiana* [226] and *Erynia neoaphidis* [216]. For *Metarhizium* spp., only growth on solid medium containing chitin was demonstrated so far with the highest colony growth compared to other nutrients [224]. To the authors' best knowledge, this is the first report on conidiation of *Metarhizium* spp. blastospores encapsulated in calcium alginate beads supplemented with chitin as main nutrient.

Comparing the results, formulations containing chitin present a significant higher conidiation on the surface compared to beads supplemented with starch. Moreover. this formulation demonstrated a higher drying survival compared to the beads containing granular corn starch, probably due to size and stability of the encapsulated chitin flakes (<300 µm, sieved) that are known to be bigger than the starch granules (10-20 µm) [214]. This might prevent a compression during drying and therefore lead to reduced physical stress on the blastospores [50; 184]. Furthermore, a slightly slower drying based on the measured water activity was observed for beads containing chitin (data not shown). A slow drying reduces the drying stress as presented by [232; 233]. Dried beads containing chitin showed significant higher conidiation compared to moist beads containing starch. Even though, the initial biomass within the bead was significantly reduced during drying, supplemented nutrients can lead to higher conidiation due to the "microfermenter effect" of the beads: A fast consumption of nutrients results in a faster germination of the blastospores. It cannot be entirely excluded, that the EPF blastospores started germination directly after encapsulation what may lead to a higher vigor of the biomass prior to the drying process. Also, the harvesting time-point of the blastospores may have played a role. [148] showed a decrease in germination of aerial conidia with an increase of the incubation time. Even though, submerged cultures for encapsulation were only cultivated for five days, an effect of the cultivation time on the vigor of the biomass cannot be excluded. Not only leads a higher biomass concentration within the shake flasks to a reduced oxygen level in

the medium, but also a connection between the polyol content inside EPF and the cultivation time was demonstrated [149; 150]. A significant reduction of polyols within the blastospores after a cultivation time of 96 h can be hypothesized, based on results by [165]. Because a connection between the polyol-content within EPF biomass and the drying survival is reported [234; 235], this might also influence the results on drying survival of encapsulated *Metarhizium* spp. blastospores illustrated in figure 6.15.

The higher aerial conidia concentration after two weeks of incubation, however, suggests a faster assimilation of the chitin, compared to starch, because a fast secretion of extracellular chitinase by the EPF and consequently a fast germination leads to higher conidiation on the bead surface in a shorter time. It should also be noted that the real survival rates are probably higher since the conditions of capsule dissolution in this study are very harsh. A study of Faria *et al.* [233] demonstrated that a slow rehydration of dried EPF biomass, such as on water agar or in humid atmosphere, can prevent the imbibitional damage of water inflow during fast rehydration. This is the case when dried beads are directly dissolved without prior slow rehydration. An application on moist soil lead to a slow sorption of the beads and thus to a gentle rehydration.

Another known advantage of chitin compared to starch is the probability of fewer contaminations, as it cannot be used as carbon source by saprophytic organisms like *Penicillium* spp. [236]

The Kill formulation was successfully dried with a drying survival over 10% and not only maintaining the conidiation but also increasing it for all three *Metarhizium* spp. isolates, when chitin was supplemented as sole nutrient source.

Further screening experiments for the encapsulation of complex nutrients, like industrial waste-products such as grounded *Sitotroga* carcasses, resulted in promising conidiation on the bead surface. Another experiment was conducted with calcium gluconate instead of calcium chloride as crosslinker and led to a marginal increase of the conidiation, what is in line with results of Humbert *et al.* [214].

Moreover, the scale-up of the encapsulation process was already tested. A suitable method is jet-cutting which enables a large-scale production. Storage stability for beads containing *M. pemphigi* X1c blastospores was qualitatively examined for a period of six months. All beads showed conidiation on the surface after rehydration and incubation on water agar at 25 °C.

Influence of a hematin coating on Kill beads

Aggregating pheromones, such as hematin, are well known semiochemicals [55]. The coating of beads with these tick aggregating substances may lead to an enhanced infection of the ticks due to an increased contact time with the control agent. Previous studies related to this work showed a significant aggregating effect of 1 – 100 µg·cm-^{2} hematin for *I. ricinus* nymphs (personal communication, Dr. Hans Dautel, IS Insect Services GmbH, Berlin, Germany). To ensure a consistent germination, dried Kill beads exemplary containing *M. pemphigi* X1c blastospores, supplemented with starch as nutrient were soaked and coated with hematin for different periods (figure 6.16). There was no significant effect of the soaking ($F_{3,36}$=02.302; p=0.094) and interaction of soaking and time ($F_{3.477,41.724}$=1.606; p=0.197) but of the time solely ($F_{1.159,41.724}$=367.250; p<0.05) on the conidiation on the bead surface.

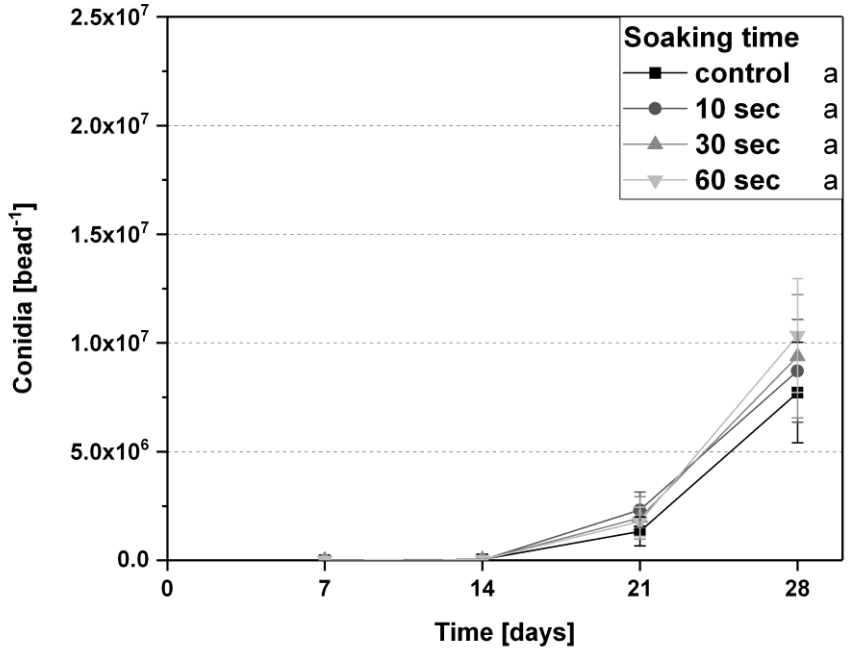


Figure 6.16 Conidiation of *M. pemphigi* X1c encapsulated in calcium alginate with starch at 25 °C for four weeks, coated with hematin for different soaking times. Different letters in the legend indicate significant differences according to RM-ANOVA with Games-Howell post hoc test at p<0.05 (n=10; mean ± sd).

All formulations revealed a conidiation between 7.72·10^{6} and 1.03·10^{7} conidia·bead^{-1} after incubation for four weeks at 25 °C. Since no significant differences between the conidiation on the control and the hematin-coated beads were determined, a coating with hematin does not seem to affect the encapsulated blastospores regarding the final conidia concentration on the bead surface.

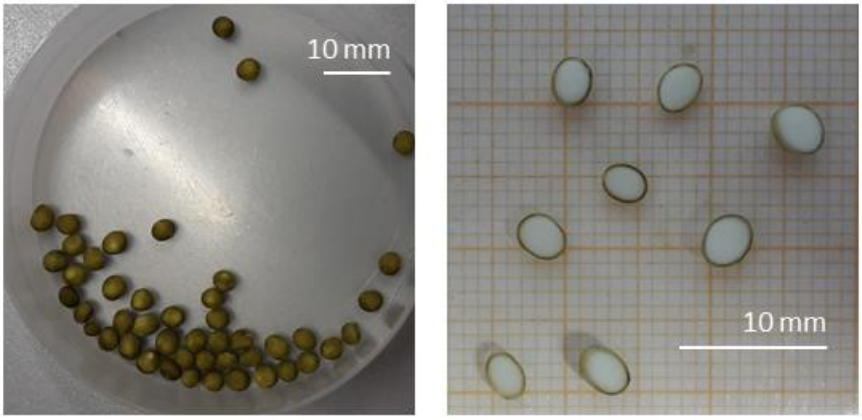


Figure 6.17 Calcium alginate beads containing starch, coated with hematin.

In a subsequent virulence test a prolonged contact time of *I. ricinu*s nymphs with the coated beads compared to the uncoated control beads could be observed. This aggregating effect of hematin is consistent with results of two studies that reported an aggregation of *I. scapularis* nymphs and adults [54; 139]. An influence of the green color on the bead surface due to the hematin on the behavior of *I. ricinus* nymphs was excluded. A higher average of dead *I. ricinus* nymphs due to a prolonged contact time with the beads was not determined.

6.2.4 Virulence of aerial conidia formed by encapsulated *Metarhizium* spp.

Virulence against nymphs

In order to examine the virulence of the encapsulated *Metarhizium* spp. isolates against *I. ricinus* nymphs, beads supplemented with granular corn starch were pre-incubated for 28 days and monitored for the effect of the newly formed aerial conidia against *I. ricinus* nymphs. Figure 6.18 illustrates that the treatment (=fungal isolate) ($F_{4,20}$=100.006; $p<0.05$) and the interaction of treatment and time ($F_{8.264,41.319}$=1937.750; $p<0.05$) as well as the time ($F_{2.066,41.319}$=7942.463; $p<0.05$) had a significant impact on the virulence of the beads.

For the experiment, two controls were used: a negative control without beads (control) and a second control, containing beads without EPF biomass (control bead). No significant difference was determined between the two control groups ($p=1.000$). Consequently, there was no effect of the formulation material on the average number of dead nymphs. Both groups showed an average number of 3.3% dead nymphs at the end of the experiment. All beads with EPF as active ingredient had a significant higher virulence compared to both control groups ($p<0.05$)

as demonstrated in figure 6.18. Beads containing *M. brunneum* Ca8II led to the lowest average number of 80% dead nymphs within the isolates. There was no significant difference between the beads containing *M. brunneum* Ca8II and *M. brunneum* Cb16III with 83% after 97 days of incubation (p=0.609). By contrast, the beads containing *M. pemphigi* X1c blastospores reached a significantly higher average number of 100% dead nymphs compared to both *M. brunneum* isolates (p<0.05). The virulent effect was already evident after ten days of incubation.

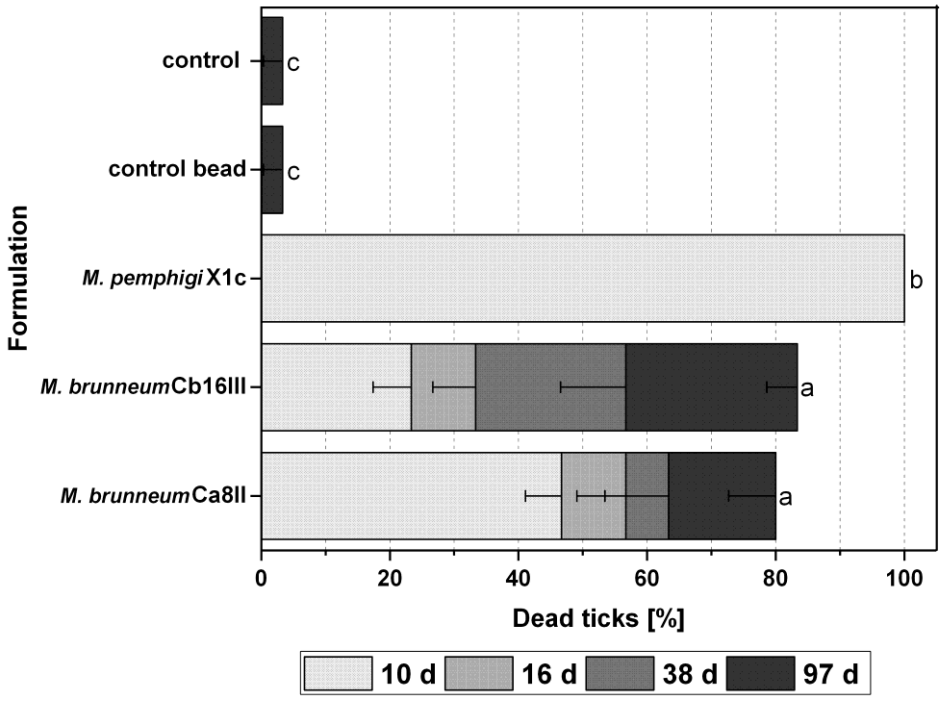


Figure 6.18 Examination of three *Metarhizium* spp. isolates encapsulated in calcium alginate with supplemented corn starch for virulence of their newly formed aerial conidia on the bead surface against *I. ricinus* nymphs for 97 days. Different letters behind bars indicate significant differences according to RM-ANOVA with Games-Howell post hoc test at p<0.05 (n=5; mean ± se).

The encapsulation of EPF in calcium alginate beads with integrated nutrients can have several advantages for a biological control agent, as mentioned above. Since the formulation material is in direct contact with the biomass, it has an immediate influence on the germination and growth and may influence the virulence of newly formed aerial conidia on the bead surface [133].

When comparing the virulence of aerial conidia from the different *Metarhizium* spp. isolates produced on the bead surface, it became apparent that *M. pemphigi* X1c showed a faster mode of action than the *M. brunneum* isolates (figure 6.18). Especially compared to the pre-screening (supplementary material), a significant increase of the virulent effect of *M. pemphigi* X1c was monitored. Since *M. pemphigi* X1c did not present a higher conidiation on the bead surface

compared to the other isolates (figure 6.10), the vigor of the encapsulated blastospores or the influence of the surrounding nutrients and biopolymer matrix had an influence on the different average numbers of dead nymphs. For both *M. brunneum* isolates, a lower virulence was determined for the formulation than in the pre-screening with non-formulated conidia. Even though blastospores for all three isolates were produced under the same conditions, the vigor of newly formed aerial conidia can vary due to the ability of the isolates to decompose encapsulated nutrients. As already reported by Faria *et al.* [237], aerial conidia in good condition with a fast germination are more virulent than debilitated aerial conidia. The germination of the encapsulated blastospores has a direct impact on the conidiation on the bead surface. Debilitated blastospores may have led to newly formed aerial conidia with a low vigor, resulting in a lower virulence of *M. brunneum*. In addition, it should be noted that the nymphs were able to avoid direct contact with the bead in this experiment as opposed to the virulence pre-screening trial. A physical contact with a sufficient number of conidia is mandatory to get infected, as reported by Jaronski [238]. The setup of the pre-screening provided a consistent dispersion of aerial conidia on the bottom plate and therefore a higher probability of permanent contact to the nymphs, whereas the beads provide high conidiation on a limited area (figure 6.19).

Figure 6.19 Microscopic image of infected *I. ricinus* nymphs with *M. pemphigi* X1c (A), *M. brunneum* Ca8II (B) or *M. brunneum* Cb16III (C).

A virulent effect of the newly formed conidia on the bead surface of all three encapsulated *Metarhizium* spp. isolates against *I. ricinus* nymphs could be demonstrated. Since *M. pemphigi* X1c turned out to be the most virulent isolate when formulated in calcium alginate, it was chosen for further experiments.

The virulence of the newly formed conidia on the moist beads containing *M. pemphigi* X1c blastospores supplemented with granular corn starch was also qualitatively proven for nymphs

of the species *Dermacentor reticulatus* and *Rhipicephalus sanguineus* (figure 6.20). To the author's best knowledge, there is no virulent effect of *Metarhizium* spp. on *D. reticulatus* published so far. A mortality of the related species *D. variabilis* of less than 20% was attained with *M. anisopliae* conidia in a study of Kirkland *et al.* [239]. The virulent effect of *Metarhizium* spp. isolates on *R. sanguineus* supports the results published in previous studies [32; 240-243]. A virulent effect of *Metarhizium* spp. conidia against *R. sanguineus* larvae was already demonstrated by Bernardo *et al.* [35]. The experiment showed 100% dead ticks after an incubation time of 14 days (n=50).

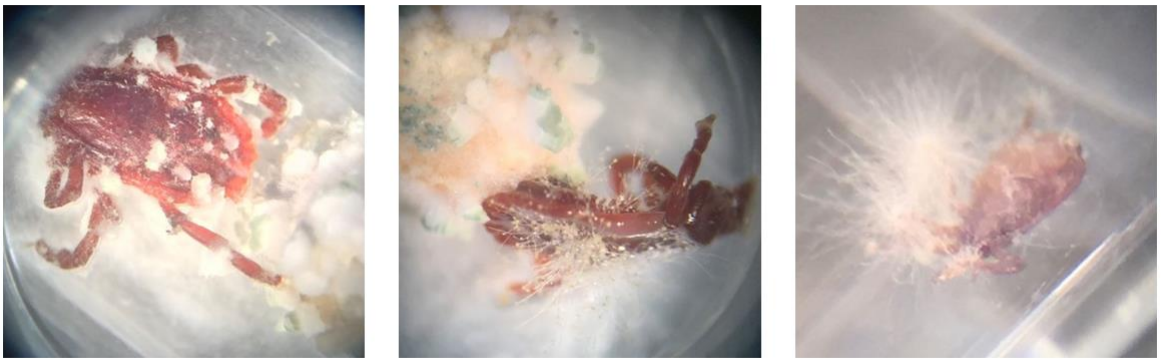


Figure 6.20 Microscopic image of infected *R. sanguineus* nymphs with *M. pemphigi* X1c from moist beads after incubation at 23 °C for 14 days.

Because blastospores are sensitive for drying, a consistent virulence of newly formed conidia even on dried and rehydrated beads needed to be ensured. Furthermore, the tick cuticle is mainly composed of proteins, chitin and several lipids [244]. A supplementation of these components in a bead formulation can result in a higher virulence of newly formed conidia due to a pre-conditioning of the fungus. A higher drying survival for blastospores encapsulated with chitin was already demonstrated in chapter 6.2.3. As demonstrated in figure 6.21, there was a significant effect of the treatment (=formulation) ($F_{5,24}$=672.800; $p<0.05$) and the interaction of treatment and time ($F_{6.535,\ 31.368}$=10.207; $p<0.05$) as well as time ($F_{1.307,31.368}$=45.407; $p<0.05$) on the average number of dead nymphs.

Figure 6.21 illustrates a significant lower average number of dead nymphs for the control without beads, compared to the groups treated with incubated beads containing EPF blastospores ($p<0.05$). As already shown in figure 6.18, the formulation without active ingredient had no effect on the nymphs compared to nymphs without any contact to beads. All treatments attained an average number of 100% dead *I. ricinus* nymphs within 97 days, whereas an average number of 90% was already reached after 10 days. Within the formulations, a

difference between moist and dried and rehydrated beads appeared but with no significant difference (starch: p=0.897, chitin: p=1.000). Furthermore, beads containing chitin as nutrient source did not show a higher average number of dead nymphs compared to the beads containing starch (moist: p=0.897, dried and rehydrated: p=1.000).

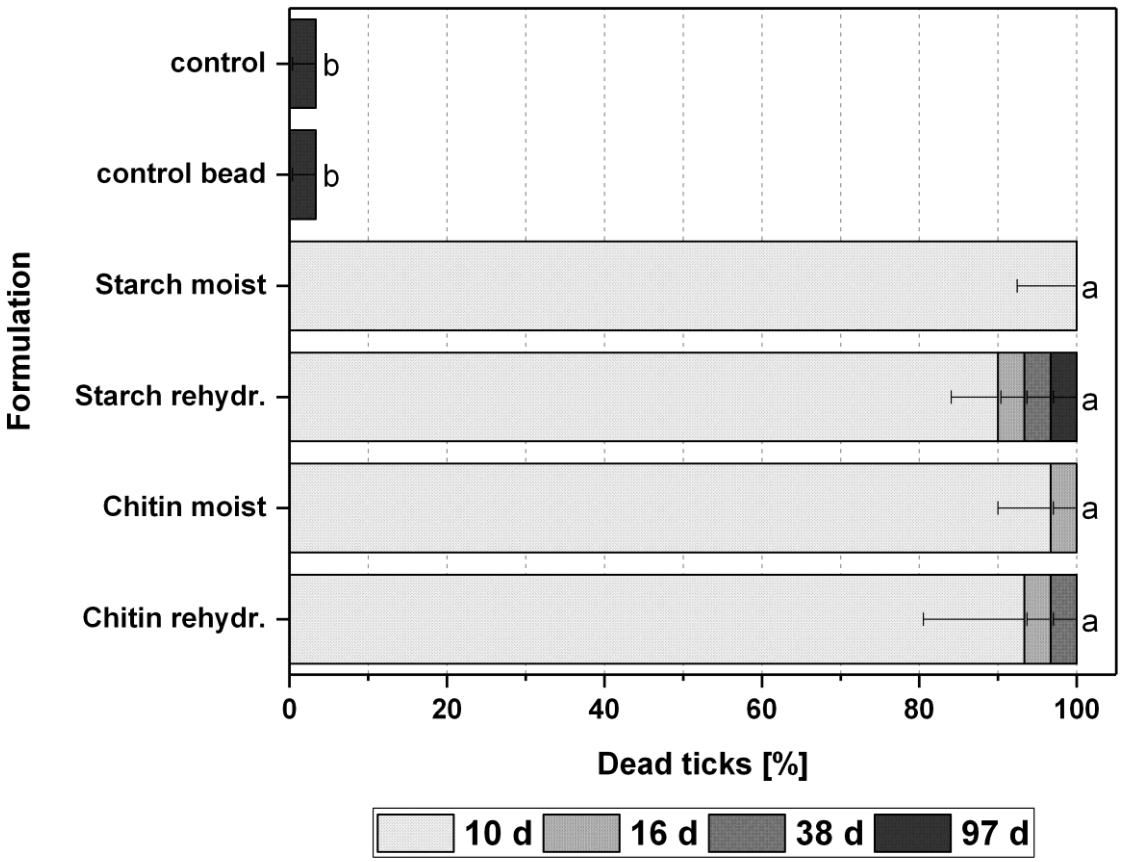


Figure 6.21 Virulence of newly formed aerial conidia on the bead surface of *M. pemphigi* X1c encapsulated in calcium alginate supplemented with corn starch or chitin against *I. ricinus* nymphs during incubation for 97 days at 23 °C. Different letters behind bars indicate significant differences according to RM-ANOVA with Games-Howell post hoc test at $p<0.05$ (n=5; mean ± se).

For infection, physical contact of the tick with a sufficient number of conidia is needed [238]. A concentration of aerial conidia on the bead surface sufficient for infection was given for all formulations, compared to the concentration of non-formulated conidia used in the pre-screening (supplementary material). A study of De Moraes *et al.* [245] demonstrated a higher proteolytic but not chitinolytic activity for *M. anisopliae*, when the EPF was cultivated in liquid cultures supplemented with 0.8% chitin. This is in line with results obtained by Beys da Silva *et al.* [244], who also reported a rise in lipase and protease concentrations for *M. anisopliae* in cultures containing chitin. Barreto *et al.* [246] showed an increase of intracellular chitinases

when chitin was added, whereas additional glucose or *N*-acetylglucosamine induced the secretion of extracellular chitinases.

A higher virulence for formulations containing chitin was expected but could not be shown in this experiment. Concerning former studies, a combination of chitin with other nutrients, such as *N*-acetylglucosamine or starch (as described in chapter 6.1) may enhance the virulent effect of newly formed conidia. In addition, the conidial concentrations were already high enough for an infection of *I. ricinus* nymphs.

Virulence against adults

Even though, nymphs and larvae are more common in the field than adults, a high effectiveness of the Kill formulation against all stages is desired. Figure 6.22 illustrates the average percentages of dead adults of the species *I. ricinus, D. reticulatus* and *R. sanguineus*. The beads containing *M. pemphigi* X1c blastospores, supplemented with granular corn starch, were pre-incubated for two weeks. The newly formed conidia on the bead surface resulted in 100% dead nymphs for both, *I. ricinus* after 38 days and *R. sanguineus* already after ten days, respectively. In comparison, 88% of the *D. reticulatus* adults were infected and killed.

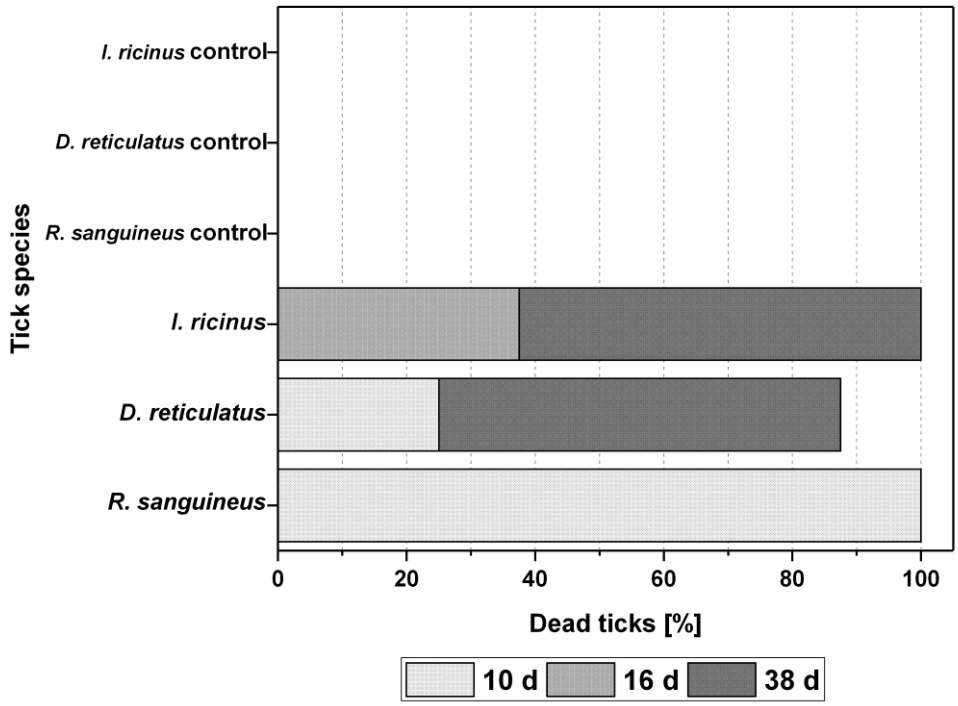


Figure 6.22 Virulence of newly formed aerial conidia on the bead surface of encapsulated *M. pemphigi* X1c against *I. ricinus, D. reticulatus and R. sanguineus* adults for 38 days (n=1).

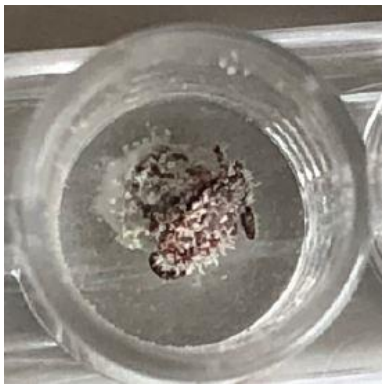


Figure 6.23 Microscopic image of infected *R. sanguineus* adult with *M. pemphigi* X1c.

R. sanguineus and *D. reticulatus* are not as common as *I. ricinus* in Germany, but have a worldwide distribution [247]. The two species are known to carry *Erhlichia, Francisella* and *Rickettsia,* as well as other bacterial parasites [248; 249], causing several diseases also in humans. Regarding the process of infection, tick larvae have fewer natural openings than adults [250], so that an infection could be complicated. Nevertheless, larva and nymphs appear to be more susceptible to conidia of entomopathogenic fungi than adults [28; 251]. Even though, the infection of adults with EPF is generally less efficient than for nymphs, it significantly reduces the fitness of all stages as well as egg oviposition [24; 26; 32; 252].

The Kill formulation enables the application of a relatively low amount of biomass that reproduces itself by means of a suitable formulation. It was demonstrated to have a virulent effect on both *I. ricinus* nymphs and adults under laboratory conditions and a further promising virulent effect against the ticks *D. reticulatus* and *R. sanguineus.* This formulation still shows some limitations, such as the missing quantitative examination for storability and field trials, but still provides a high potential for application in the field after some further development.

6.3 Attract-and-Kill

The most desirable formulation is a combination of both Attract- and Kill formulation to combine all advantages of the separate beads (chapter 6.1 and 6.2) to reduce efforts associated with a co-application. Therefore, different experiments were conducted to analyze the suitability of a co-encapsulation of *M. pemphigi* X1c and *S. cerevisiae* in calcium alginate.

6.3.1 CO_2 Production of *S. cerevisiae* co-encapsulated with *M. pemphigi* X1c

It is important to ensure a consistent attractive effect of the Attract-and-Kill formulation for ticks above ground. Since *S. cerevisiae* does not bring the enzymatic equipment to use starch as nutrient, the co-encapsulation EPF may solve this problem as previously reported for wireworms below ground [50-52]. Following the idea of a CO_2 production, depending on the EPF growth, ticks only get attracted, when the EPF starts growing, increasing the chance of a conidiation on the bead surface and thus infection. Because of the low AG secretion by *M. pemphigi* X1c as illustrated in figure 6.13, a co-encapsulation with AG was tested as well.

As illustrated in figure 6.24, there was a significant influence of the formulation on the CO_2 production at every time point during incubation of beads for 14 days. The highest CO_2 production was attained by the formulation containing 0.1 $U \cdot g^{-1}$ AG with 0.4 $mL \cdot (g \cdot h)^{-1}$ for freshly prepared beads, 0.32 $mL \cdot (g \cdot h)^{-1}$ after seven days incubation at 25 °C and 0.09 $mL \cdot (g \cdot h)^{-1}$ after 14 days. Except for the last measuring point, the CO_2 production was significantly higher compared to the other two formulations (0 and 7 days $p<0.05$, 14 days $p=0.051$). The beads containing *S. cerevisiae* cells and *M. pemphigi* X1c blastospores reached a nearly consistent CO_2 production between 0.06 and 0.12 $mL \cdot (g \cdot h)^{-1}$. The control bead, containing *S. cerevisiae* cells only, produced significant less CO_2 compared to the formulation with EPF at every timepoint ($p<0.05$)

The results support the findings of chapter 6.2, indicating a very low extracellular AG activity of *M. pemphigi* X1c. To exclude an influence of different biomasses in combination with AG, the CO_2 production for both, blastospores or aerial conidia as well as mycelium was measured. Figure 6.25 demonstrates, that beads with blastospores or aerial conidia led to a CO_2 production without significant difference) at all time points (0 days $p=0.059$, 7 days $p=0.960$, 14 days $p=0.129$, 21 days $p=0.314$. Beads with mycelium produced significant less CO_2 when freshly prepared but aligned the other treatments then within the first seven days ($p=0.05$).

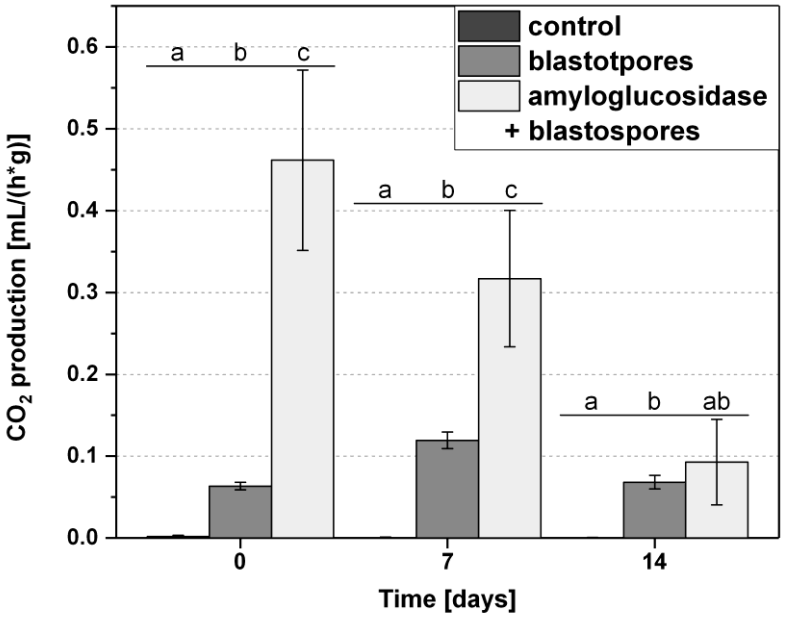


Figure 6.24 CO_2 production of *S. cerevisiae* encapsulated in calcium alginate supplemented with corn starch and co-encapsulated with either *M. pemphigi* X1c blastospores or blastospores and 0.1 $U \cdot g^{-1}$ amyloglucosidase for 14 days. Different letters in legend indicate significant differences according to one-way ANOVA with Games-Howell post hoc test at $p<0.05$ (n=5; mean ± sd).

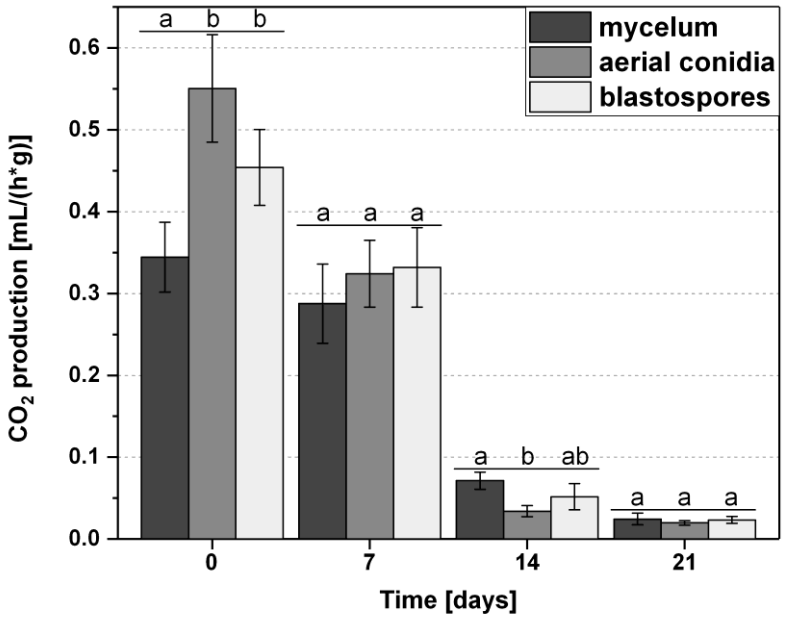


Figure 6.25 CO_2 production of *S. cerevisiae* encapsulated in calcium alginate supplemented with corn starch and co-encapsulated with *M. pemphigi* X1c and 0.1 $U \cdot g^{-1}$ amyloglucosidase for 21 days. Different letters in legend indicate significant differences according to one-way ANOVA with Games-Howell post hoc test at $p<0.05$ (n=6; mean ± sd).

The biomass form did not seem to have an additional positive effect on the CO_2 production of the formulation compared to formulations containing only AG illustrated in chapter 6.1. To attract ticks above ground under influence of environmental factors, a sufficient CO_2 production is inevitable. The results in chapter 6.1 as well as in figure 6.24 demonstrate that an Attract formulation without supplemented AG may attract ticks under defined laboratory conditions but will probably not produce enough CO_2 to attract nymphs in the field. Environmental factors like wind, as well as the natural diffusion of CO_2 will result in a dilution of the released CO_2 and thus in a reduction of attractivity. Therefore, only a formulation containing AG seems to be suitable for an above ground application regarding the CO_2 production, regardless of the supplemented *Metarhizium* pemphigi X1c biomass.

6.3.2 Conidiation of co-encapsulated *M. pemphigi* X1c

Not only the CO_2 production, but also the conidiation of *M. pemphigi* X1c on the bead surface is of great importance for the effectiveness of the formulation, as stated in chapter 6.2. Therefore, the different *M. pemphigi* X1c biomasses were examined for their conidiation on moist beads, when encapsulated with *S. cerevisiae* and 0.1 $U \cdot g^{-1}$ AG. The treatment (= encapsulated biomass) ($F_{2,27}$=9.278; $p<0.05$) and the interaction of treatment and time ($F_{3.776,50.838}$=5.351; $p<0.05$) as well as the time ($F_{1.883,50.838}$=137.673; $p<0.05$) had a significant impact on the conidiation on the beads.

Comparing the conidiation of beads containing different biomasses, it is conspicuous that blastospores led to the significant lowest amount of aerial conidia on the bead surface with an average number of $1.16 \cdot 10^6$ conidia·bead^{-1} after 28 days of incubation at 25 °C ($p<0.05$), as illustrated in figure 6.26. Between beads containing mycelium ($1.91 \cdot 10^6$ conidia·bead^{-1}) and aerial conidia ($2.39 \cdot 10^6$ conidia·bead^{-1}), no significant difference in conidiation was measured ($p=0.526$).

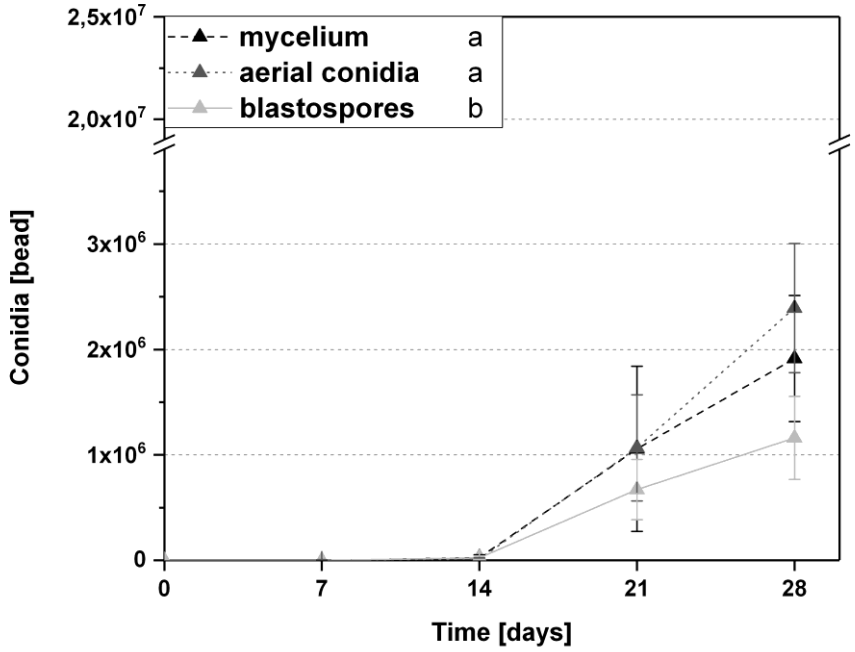


Figure 6.26 Conidiation of *M. pemphigi* X1c encapsulated in calcium alginate together with *S. cerevisiae*, supplemented with corn starch and 0.1 U g^{-1} amyloglucosidase referring to the conidiation on the bead surface at 25 °C for four weeks. Different letters in legend indicate significant differences according to one-way ANOVA with Games-Howell post hoc test at $p<0.05$ (n=10; mean ± sd).

Results presented in figure 6.10 demonstrate a more than ten-fold higher conidiation for a similar formulation containing blastospores but no AG. Despite the total amount of aerial conidia on the bead surface, the conidiation itself was delayed for several days, compared to the results presented in figure 6.10. Since the supplemented AG is the only difference within the formulation, different concentrations were examined on their influence on the conidiation on the bead surface. Figure 6.27 illustrates that the AG concentration ($F_{3,36}$=1004.076; $p<0.05$) and the interaction of concentration and time ($F_{6.257,75.080}$=33.689; $p<0.05$) as well as the time ($F_{2.086,75.080}$=55.140; $p<0.05$) had a significant impact on the conidiation on the beads. A ten-fold increase of the AG concentration up to 1 U·g^{-1} led to a significant reduction of the conidiation after 28 days of incubation ($0.69 \cdot 10^6$ conidia·$bead^{-1}$) compared to beads supplemented with 0.1 U·g^{-1} ($1.16 \cdot 10^6$ conidia·$bead^{-1}$) and 0.05 U·g^{-1} ($1.38 \cdot 10^6$ conidia·$bead^{-1}$) AG ($p<0.05$). Between 0.05 and 0.1 U·g^{-1} AG, no significant difference was measured ($p=0.095$) even though the average conidiation was marginally higher for the lower concentration of AG. In comparison, the control without supplemented AG reached a concentration of $1.71 \cdot 10^7$ conidia·$bead^{-1}$, analogous to the results attained in figure 6.10.

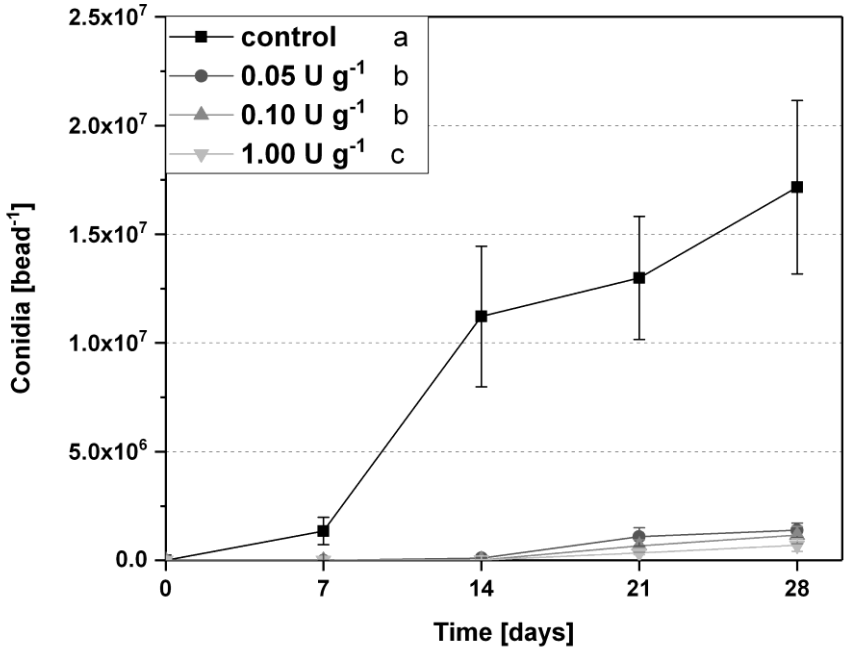


Figure 6.27 Conidiation of *M. pemphigi* X1c encapsulated in calcium alginate together with *S. cerevisiae*, supplemented with corn starch and different concentrations of amyloglucosidase referring to the conidiation on the bead surface at 25 °C for four weeks. Different letters in legend indicate significant differences according to one-way ANOVA with Games-Howell post hoc test at $p<0.05$ (n=10; mean ± sd).

The supplementation of AG at any concentration tested had a negative effect on the conidiation of the encapsulated fungus, regardless of the biomass form encapsulated. Even a relatively small concentration of AG led to a significant reduction in conidiation. Since, *S. cerevisiae* is well known and used to produce ethanol under different conditions [253; 254], an influence of this by-product was evident. For a better understanding of the procedures occurring inside the beads, a closer look at the ethanol production seemed to be a promising approach.

6.3.3 Ethanol production of *S. cerevisiae* in calcium alginate beads

S. cerevisiae is an ethanol producing microorganism. The intensity of the ethanol production depends mainly on the glucose concentration in the surrounding medium and the oxygen availability [102-104]. The AG concentration ($F_{2,12}=604.018$; $p<0.05$) and the interaction of treatment and time ($F_{5.167,31.001}=24.948$; $p<0.05$) as well as the time solely ($F_{2.583,31.001}=247.978$; $p<0.05$) had a significant impact on the ethanol concentration within the beads.

From figure 6.28 it can be seen that the control bead without supplemented AG showed no measurable ethanol at any time point whereas the ethanol concentration raised significantly with the increase of the AG concentration ($p<0.05$). Beads containing 0.05 $U \cdot g^{-1}$ reached an ethanol concentration of 0.15 $g \cdot bead^{-1}$, beads with 1 $U \cdot g^{-1}$ AG attained 0.40 $g \cdot bead^{-1}$ ethanol already after 14 days of incubation at 25 °C.

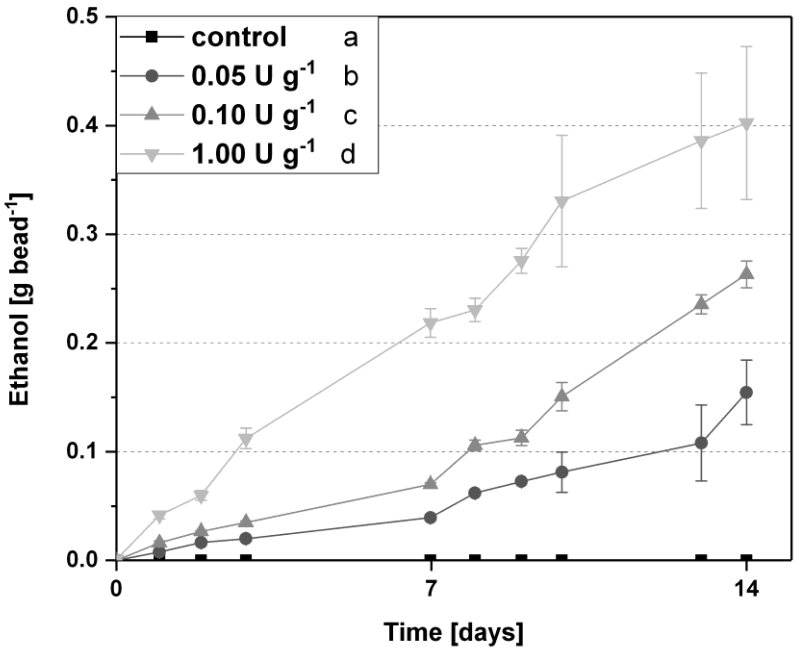


Figure 6.28 Ethanol concentration of encapsulated *S. cerevisiae* in calcium alginate supplemented with starch and different concentrations of amyloglucosidase for two weeks. Different letters in legend indicate significant differences according to one-way ANOVA with Games-Howell post hoc test at $p<0.05$ (n=5; mean ± sd).

High glucose concentrations lead not only to a higher CO_2 production but also to an additional production of ethanol, regardless of aerobic or anaerobic conditions, when a specific glucose concentration in the surrounding area is exceeded [105]. De Deken [106] demonstrated that already a concentration higher than 0.1 $mg \cdot mL^{-1}$ induces the so-called 'Crabtree effect'. In this experiment, one bead was dissolved in 1 mL buffer to determine the ethanol concentration inside the bead. A critical concentration was already exceeded a few minutes after preparation of the beads with 0.09 $mg \cdot bead^{-1}$ glucose as demonstrated in figure 6.29. During incubation at 25 °C, the concentration raises up to 0.87 $mg \cdot bead^{-1}$ glucose within 17 h. Regarding the encapsulated amount of initially 15% (w/w) starch, a maximum of around 4 $mg \cdot bead^{-1}$ glucose can be generated by cleavage with the supplemented AG. This concentration was reached inside

the control bead, without encapsulated *S. cerevisiae* cells, after eight days of incubation (figure 6.29).

The second graph shows the concentration of available glucose in the bead, when *S. cerevisiae* cells were co-encapsulated. The glucose concentration remains below a measurable concentration. *S. cerevisiae* is able to adapt to grow on high concentrations of glucose very fast [255; 256]. Glucose induces a repression of mitochondrial function and leads to a less efficient energy production, compared to respiration [107] but at the same time leads to a faster liberation of energy and thus enables more rapid growth [108]. Apart from that, *S. cerevisiae* is known to have a relatively high resistance to ethanol toxicity. The production of ethanol to defend its niche from other microorganisms is conceivable [109; 116].

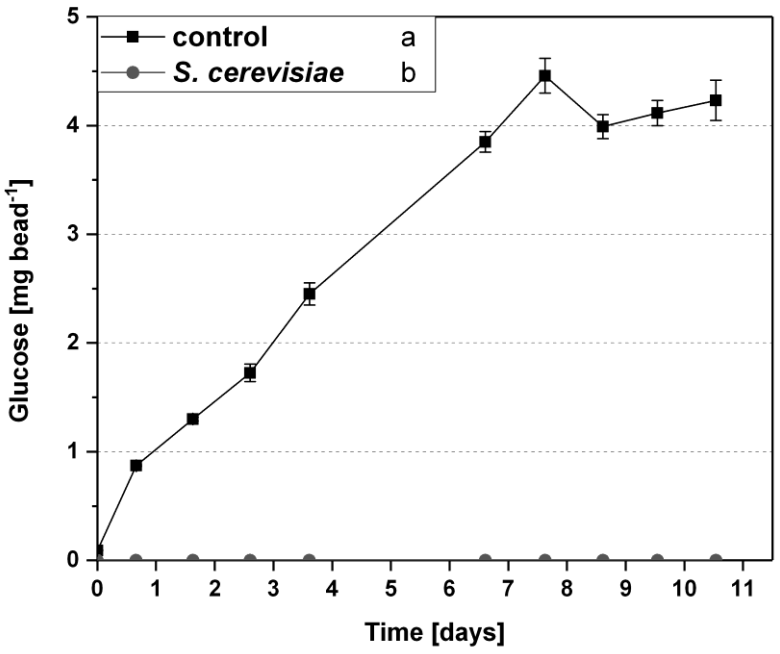


Figure 6.29 Glucose concentration in moist calcium alginate beads containing starch and 0.1 U·g^{-1} amyloglucosidase with and without *S. cerevisiae* as determined by HPLC measurement for eleven days (n=3; mean ± sd).

In view of the radius of the beads (around 1 mm), the diffusion of oxygen into the bead is limited [257]. It can be assumed, that the cells immobilized in the inner matrix of the bead grow under anaerobic conditions, regardless of the glucose concentration. This circumstance further enhances the production of ethanol and through the process of alcoholic fermentation, the CO_2 production is accelerated, but the total volume of released CO_2 is reduced [258]. A reduction of the diameter of the bead may reduce the process of fermentation in the yeast but also impairs

water retention within the bead, another important factor for the CO_2 production as pointed out in chapter 6.1.1.

Blastospores show a high sensitivity to stress factors compared to other biomass forms. The resistance against ethanol is very low, as demonstrated in figure 6.30. Already an incubation in an aqueous solution of 0.1 $g \cdot mL^{-1}$ ethanol led to a reduction of germinating blastospores of more than 50%. As illustrated in figure 6.28, beads containing 0.1 $U \cdot g^{-1}$ reached this concentration after seven days, beads supplemented with 1.0 $U \cdot g^{-1}$ AG already after three days. Even if the encapsulated blastospores were able to germinate within this time, a constant increase of the ethanol concentration as demonstrated in figure 6.28 will inhibit further growth. The water contained in the beads allows the ethanol to pass through the cell wall. Inside the blastospores the alcohol causes an unspecific precipitation of the proteins. Vital functions within the cell can no longer take place in this way [259].

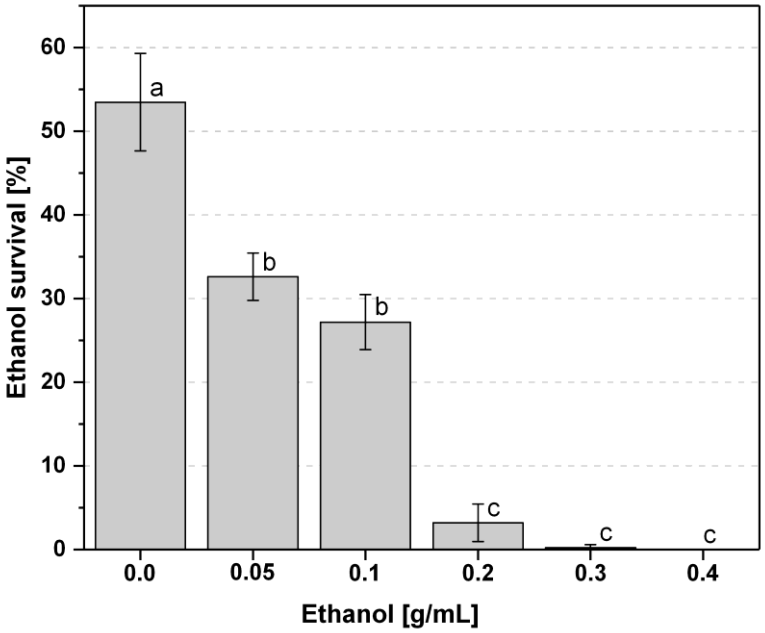


Figure 6.30 Survival of *M. pemphigi* X1c blastospores incubated with different concentrations of ethanol for 10 min at ambient temperature (n=5; mean ± sd).

Even though the produced ethanol impedes the germination and growth of co-encapsulated blastospores, it inhibits contamination by other microorganisms being present in the field, such as other fungi. The *S. cerevisiae* cells, as already stated, affect their surrounding environment to improve their ecological fitness.

The realization of a formulation, containing both, *S. cerevisiae* and *M. pemphigi* X1c biomass, would reduce costs by saving production steps and bead material. In addition, the application of a single formulation facilitates its use in the field and the microorganisms can take advantage of each other (like cleavage of starch through extracellular enzymes of a fungus). By contrast, the interaction of two co-encapsulated microorganisms can lead to a reduced efficiency, more precisely of CO_2 release and conidiation on the surface, due to occurring by-products, such as ethanol.

A co-encapsulation of both microorganisms was demonstrated but with resulting decreases in either CO_2 release or in conidiation on the bead surface due to the high ethanol concentration inside the beads. Even though the Attract-and-Kill approach is still generally imaginable, the formulation needs further investigation. Another possibility will be a co-application of both formulations, since ticks generally tend to move over the beads when actively searching for a blood meal. Therefore, an infection is presumable when both formulations are applied in a close area. An independent application of both formulations for tick control (e.g. the combination of the Kill formulation with other attractants or the combination of the Attract formulation with an independent Kill-component) is conceivable.

7 General discussion and conclusions

Different tick species, such as the castor bean tick *Ixodes ricinus*, play an important role in the transmission of serious diseases in Germany and worldwide. Besides the infestation of domestic animals, tick bites cause massive economic losses in the agricultural sector by the infection of livestock with several pathogens. Resulting infections in the host animal can cause decreases in productivity, an increasing morbidity and a higher mortality [2-5]. Several transmitted pathogens also pose a great danger to humans, inducing diseases such as Lyme borreliosis, Tick-borne encephalitis or Babesiosis [6-9]. In Germany, the risk of human infections is the most serious concern with the *I. ricinus* constituting the most important vector pathogens [11]. Other species as *Rhipicephalus* spp. or *Dermacentor* spp. are more rarely infesting humans but with an increasing percentage. At present, no individual control measure for ticks is available on the marked. Ticks can only be controlled using unspecific chemical acaricides [15], coming along with several disadvantages, such as negative impact on non-target animals, increasing development of resistances of the target organisms and risks for human, animals and environment [8; 126-128; 260]. Because of the constantly increasing number of risk areas for tick infection and a consumer-driven shift towards a more environment-friendly pest control, there is a significant demand for an effective biological control agent for ticks. To overcome several drawbacks of biological control agents, such as low persistence and activity in the field, detailed research into a suitable formulation containing active biological components is of utmost importance. This is why this work has focused on the development of a novel biologically based formulation to be used in an Attract-and-Kill approach to control ticks in the field.

The overall aim of this work was to develop a formulation which attracts ticks, especially of the species *I. ricinus*, by the release of carbon dioxide, to enable direct contact with a bead, coated with an aggregating substance to increase the exposure to a natural Kill component, preferably an entomopathogenic fungus. The following questions were addressed: Can encapsulated *S. cerevisiae* cells produce sufficient CO_2 over a defined period of time for an application above ground? What influence do supplemented amyloglucosidase, drying and rehydration processes as well as temperature have on the CO_2 release? Is it possible to further increase the CO_2 release by the supplementation of urea as nitrogen source? Is the released CO_2

from dried and rehydrated Attract beads attractive to *I. ricinus* nymphs? How do three highly virulent pre-selected *Metarhizium* spp. isolates conidiate on solid medium depending on the temperature? Which isolate produces the highest blastospore concentration in submerged cultivation regarding temperature and initial pH value? Which isolate shows the highest conidiation on beads, when blastospores were encapsulated with different nutrients? Can encapsulated blastospores be dried? Does a coating with aggregation pheromones affect the conidiation on the bead surface and enhance contact time between ticks and beads? Are the newly formed conidia on the bead surface still virulent against *I. ricinus* nymphs and adults? Are *S. cerevisiae* and EPF blastospores suitable for co-encapsulation? How do the co-encapsulated biological components inside the bead interact with each other, regarding CO_2 release and conidiation on the bead surface? The research questions are discussed below in a general context and objectives for future research are proposed. Several topics have already been discussed in detail in previous chapters, so wherever possible, reference is made to the relevant chapters to avoid redundancies.

CO_2 gradients play a decisive role in the host localization by ticks such as *I. ricinus* [88; 94; 95]. The use of artificial CO_2 sources has already been reported for monitoring of ticks in the field, but not on the basis of encapsulated *S. cerevisiae* cells. The idea of encapsulating *S. cerevisiae* together with nutrients in order to provide a long-term attracting effect on insect pests has been successfully established for wireworms below ground [52; 119; 219]. The organism *S. cerevisiae* naturally assimilates monosaccharides, such as glucose, for growth and metabolism, with CO_2 as a by-product. However, due to their low molecular weight, monosaccharides cannot be encapsulated permanently in calcium alginate because it is not retained by the polymer matrix [49; 117]. Against this, starch, as a carbon source with high molecular weight, remains in the bead and enhances the structure of the hydrogel network [120]. Because *S. cerevisiae* does not bring the enzymatic equipment to metabolize starch [54; 174; 175], a supplementation of amyloglucosidase was necessary to make the nutrient source available for encapsulated cells.

The Attract formulation, developed for the application above ground, was presented and discussed in **chapter 6.1**. The encapsulation of *S. cerevisiae* cells with granular corn starch and supplemented amyloglucosidase led to a release of adequate CO_2 concentrations over 0.2 $mL \cdot (g \cdot h)^{-1}$ over a minimum period of one week. The starch is decomposed into glucose molecules by the amyloglucosidase and can thus be assimilated by the *S. cerevisiae* cells. Depending on the amyloglucosidase concentration, the amount and duration of the CO_2 release

varies. Furthermore, it could be shown that the temperature has a significant influence on the CO_2 release. Lower temperatures reduce the activity of the cell metabolism as well as the enzyme activity and therefore lead to a reduced but also a prolonged release of CO_2 [119; 177-179]. The temperature-driven activity of encapsulated *S. cerevisiae* cells and the related CO_2 production follows the natural behavior of *I. ricinus* nymphs, showing an increasing active movement at temperatures above 7 to 10 °C [190]. Consequently, at low temperatures with low tick activity, fewer nutrients are metabolized within the beads and therefore a sustained CO_2 release is provided.

Drying of microorganisms is a common method for maintaining a high viability of the cells during long-time storage [176]. Even though, the drying process results in a loss of viable cells [120; 180-183], the CO_2 release of slowly rehydrated beads was only marginally affected compared to moist beads. When high amounts of living cells are encapsulated, a limitation in the supply of nutrients is supposed to occur, resulting in a lower CO_2 release compared to the same concentration of free cells, dissolved in liquid medium. A reduction of the cell concentration during drying consequently solely lead to a minor reduction in CO_2 production because the total number of well supplied cells remains more or less constant [51]. Nevertheless, a rehydration of the beads to a high water activity of more than a_w 0.95 was demonstrated to be inevitable for reactivation of the *S.* cerevisiae metabolism and thus a high CO_2 release [191]. As already reported in the literature, a slow and therefore gentle rehydration leads to a much higher viability of living cells [186; 187]. The supplemented corn starch may maintain the moisture, as it is reported to possess a high water binding capacity [188; 189].

Due to environmental factors there is a stronger diffusion of CO_2 above ground when compared with below ground applications, which is why a high release is necessary to attract ticks. It has been shown that the addition of urea can significantly increase a CO_2 release for a short period of time. *S. cerevisiae* cells can use urea as nitrogen source converting it to CO_2 and NH_3 [125]. The additional release of NH_3 could increase the attractiveness for ticks [55; 261; 262]. However, due to its low molecular weight, urea is still difficult to retain in the capsule and could easily be rinsed out in the field during rain, which requires more detailed research into a suitable formulation.

The attraction of *I. ricinus* nymphs by the developed CO_2 releasing beads was shown in chapter 6.1. Dried and rehydrated beads released a sufficient CO_2 concentration to attract ticks in a dual-choice experiment. Even if a luring effect by pure CO_2 is confirmed for some tick genera [88-90; 92], an attractive effect of CO_2 produced by *S. cerevisiae* is not guaranteed. The

volatiles, produced by yeast, such as, such as ethanol or acetaldehyde [263], are reported as attractant for baiting traps for different pests [264-266] but, however, can affect ticks in a both attractive or repellent way what needed to be investigated. The alternative of attracting ticks by traps containing dry ice was already reported, but with the limitation that these traps only provide a short-term emission combined with high costs [88-92]. A long-term solution to attract *I. ricinus* in the field has not been published so far. Other literature further suggests promising results for the attractivity of the CO_2 producing beads for several other tick species, such as *Amblyomma* or *Dermacentor* spp., that are mainly attracted when CO_2 baits are applied [93; 96].

The results show first promising steps on the way to a bead formulation, which releases CO_2 over a defined period of time in the field and could be used to attract ticks such as *I. ricinus*. The application to other pests moving above ground is also conceivable. The attractive effect allows to reduce the required dosage of kill components for pest control when this agent is in close proximity with the attractant. This not only protects the environment but also drastically reduces costs. Up to an applicable form, however, various limitations of this bead formulation have yet to be overcome in order to ensure their effectiveness in the field.

Therefore, future research needs to focus on the extension of CO_2 emissions in order to guarantee a long-term attractive effect. In addition, more attention should be paid on the influence of by-products formed by the *S. cerevisiae* cells on the formulation as well as the pH value inside the beads. The utilization of urea should be investigated more closely and its influence on the attractive effect for ticks should be examined. Other starches and amyloglucosidases should also be considered in future work in order to further increase the CO_2 emission. It has already been shown that a high moisture content is indispensable for a sufficient CO_2 emission. The desiccation of the beads above ground poses a bigger problem than during below ground application due to higher diffusion processes. By incorporating swelling and moisture absorbing agents, moisture retention within the beads can be improved and thus the CO_2 release ensured over a longer period of time.

The Kill formulation, developed for the application above ground, was presented and discussed in **chapter 6.2**. The choice of biomass form is a crucial factor in the development of a Kill formulation based on entomopathogenic fungi. Entomopathogenic fungi like *Metarhizium* spp. grow in two different morphologies, namely blastospores and mycelium, when cultivated in

submerged cultures [156; 157], as well as mycelium and aerial conidia, when cultivated on solid medium [202; 203], whereas optimal conditions for germination, growth and conidiation are specific for each isolate. Fermentation on solid medium is the conventional way to obtain aerial conidia for further application, whereas submerged cultivation not only allows a simpler scale up and thus a reduction of costs [44]. For all three selected *Metarhizium* spp isolates, *M. brunneum* Ca8II and Cb16II and *M. pemphigi* X1c, was shown that they form aerial conidia on solid medium within a wide temperature range, which is an important prerequisite for the application in the field. Also, all isolates showed a blastospore production depending on the set conditions. Since the proportions of mycelium and blastospores are correlating to one another, a shift from mycelium towards blastospore formation can be achieved by optimization of media composition [36; 157; 162] and process strategy [208]. Furthermore. *M. pemphigi* X1c was found to be the most tolerant isolate, when cultivated at different initial pH values. Differences in cell composition, depending on the pH value, can have a significant effect on their drying stability due to the changing polyol content inside the cells [150; 161]. In addition, a varying pH value within the cells becomes apparent during dehydration. A pH tolerant isolate can therefore be advantageous and achieve a higher drying survival rate. The stability during drying processes is an important factor since blastospores are known to be very sensitive to, compared to aerial conidia [158].

The transfer of an active ingredient in an applicable bead form can not only facilitates an application in the field but also improves its characteristics. By encapsulating EPF in calcium alginate, the biomass can be effectively protected against detrimental environmental factors [140; 141]. Furthermore, the addition of various additives, such as drying aids or nutrients, not only increases drying stability but also improves conidiation on the bead surface [50; 143; 214; 215]. Due to the supplemented nutrients, encapsulated biomass is supposed to germinate within the bead and in the next step grow out and produce virulent aerial conidia on the bead surface. Starch was demonstrated to provide stabilization of the bead structure in previous studies [120], function as drying protectant [119] and serve as nutrient for the EPF, resulting in a higher conidiation [215; 216]. The beads can be seen as microfermenters that provide everything to multiply a relatively small inoculum. Thus the usually required dosages for pest control in the field can be reduced [50]. Moist beads containing *Metarhizium* spp. blastospores and starch reached their highest conidiation on the bead surface at 25 °C. However, the presented results show a significant difference in the production of aerial conidia on the bead surface within the three *Metarhizium* spp. isolates. Even though *Metarhizium* spp. isolates are reported to be able

to assimilate starch [219], a qualitative test demonstrated a relatively low utilization as nutrient, especially for *M. pemphigi* X1c. It was isolated from a dead ticks' body, whereas the other two isolates were isolated from soil, hence a fewer adaption to the utilization of corn starch as nutrient is conceivable. To enhance the conidiation, chitin was chosen as alternative for starch. It serves for structure formation of the cuticle not only in the majority of insects [220], but also in hard bodied ticks [55]. Since EPF are reported to secrete chitinases, it can probably serve as nutrient [221-225]. Regarding the results, formulations containing chitin present a significant higher conidiation on the surface compared to beads supplemented with starch, whereas beads containing *M. pemphigi* X1c showed the highest conidiation on both moist as well as dried and rehydrated beads. The results support the assumption, that especially this species is more adapted to utilize components of the tick cuticle. Moreover, the formulation containing chitin demonstrated a higher drying survival for encapsulated blastospores compared to the beads containing granular corn starch, probably due to size and stability of the encapsulated chitin flakes that are known to be bigger than the starch granules [214], preventing compression during drying and therefore lead to reduced physical stress on the cells [50; 184].

In order to increase the probability of infection, the coating of an aggregation pheromone can extend the contact time between tick and bead [54; 139]. Hematin was attached to the bead surface by soaking. The coating had no negative effect on the conidiation of the EPF on the bead surface. A subsequent virulence test showed an extended contact time of *I. ricinus* nymphs with the coated beads, but had no effect on the virulence, compared to a control, probably due to the high conidia concentration on the surface.

A virulence test with moist beads was performed to check the efficacy of the beads against *I. ricinus* nymphs. A virulent effect of the newly formed conidia on the bead surface of all three encapsulated *Metarhizium* spp. isolates could be demonstrated. The virulence of the newly formed conidia on the moist beads containing *M. pemphigi* X1c blastospores supplemented with granular corn starch was also qualitatively proven for nymphs of the species *D. reticulatus* and *R. sanguineus.* Compared to moist beads, no significant difference in virulence was observed, when dried and rehydrated beads were applied. Also, the supplementation of chitin as nutrient had no effect, even though a higher virulence was expected due to a pre-conditioning of the EPF with the encapsulated chitin [244; 245]. A virulent effect of *M. pemphigi* X1c was further demonstrated on adults of the species *I. ricinus, D. reticulatus* and *R. sanguineus.* Even though, the infection of adults with EPF is generally less efficient than for nymphs, it significantly reduces the fitness of all stages as well as egg oviposition [24; 32; 252].

In order to increase the biomass yield, in this case for blastospores, further research should focus not only on the optimal media composition to prevent limitations but also on possible by-products that might be formed by EPF during cultivation and can inhibit growth [212]. The regulation of the pH value could also lead to an increase in the yield [162]. Furthermore, a quantitative examination for storability is necessary. For further improvement of the speed to kill of newly formed conidia, experiments with fresh beads without pre-incubation, need to be carried out in order to gain a more precise insight into the threshold conidia concentration on the surface needed for a sufficient infection of ticks and whether various additives influence this process. Finally, the execution of field trials is inevitable, in order to examine the effectiveness of the beads also in the field. First experiments were carried out in a semi-free area but did not provide any significant results. The evaluation was carried out by recovering the ticks from a leaf litter, which posed major problems. Also, the conidiation on freshly prepared beads containing blastospores was reduced in the field, compared to laboratory conditions, because the beads dehydrate very quickly and consequently the EPF lacks the required moisture for growth. Again, the supplementation of swelling agents can improve the performance of the developed bead in the field, here regarding the growth of EPF.

A suitable Kill formulation not only reduces costs, due to a relatively low amount of required biomass, but also provides further advantages for encapsulated EPF biomass, such as protection against environmental factors and thus a higher persistence in the field. The encapsulation of the total biomass instead of pure blastospores is likewise conceivable in order to further reduce production costs. The developed formulation can also be adapted to other EPF, such as *Beauveria* spp. and thus provides a high potential in application. A combination with an Attract formulation could furthermore reduce the required amount and make the application even more efficient. Regardless of the limitations, this work paves the way for the encapsulation of EPF blastospores as control agent above ground, not only for Ixodidae but also for other pests, naturally getting infected by EPF.

To bring together the benefits of the bead formulations described in **chapters 6.1** and **6.2**, a combination of both, the Attract and the Kill formulation is the most desirable solution, as described in **chapter 6.3**. A co-formulation of both microorganisms, *S. cerevisiae* and *M. pemphigi* X1c, supplemented with nutrients can reduce production and material costs

associated with a co-application. Since *S. cerevisiae* does not bring the enzymatic equipment to use starch as nutrient, the co-encapsulated EPF may solve this problem by secretion of amyloglucosidase to assimilate the supplemented starch, as previously reported for wireworms below ground [50-52]. It is essential for the functionality of the Attract–and–Kill formulation that the CO_2 production is maintained at a constant level, compared to the Attract bead. A CO_2 production depending on the EPF growth was aspiring as it directly connects its conidiation with the release of enzyme and thus glucose, resulting in a release of attractant by the encapsulated *S. cerevisiae*. Below ground, diffusion processes are slower compared to processes above ground, therefore a pest attracting CO_2 gradient can be generated and perpetuated with lower CO_2 concentrations. However, it was shown that the CO_2 release is too low for the application above ground, when no additional amyloglucosidase is supplemented. A formulation without supplemented amyloglucosidase may attract ticks under defined laboratory conditions but will probably not produce enough CO_2 to attract nymphs in the field, due to the influence of environmental factors, such as wind, and diffusion processes. When beads containing *M. pemphigi* X1c were supplemented with additional amyloglucosidase, however, a significantly reduced conidiation on the bead surface was observed, regardless of the type of biomass used. Beads following the Attract-and-Kill approach containing *S. cerevisiae* and EPF have already been successfully used below ground with constant conidiation [219], but with the difference that no additional amyloglucosidase was supplemented. The amyloglucosidase inside the bead releases large amounts of glucose, which can lead to the induction of a Crabtree effect in the encapsulated *S. cerevisiae* cells. Since, *S. cerevisiae* is well known and used to produce ethanol under different conditions, such as anaerobicity or high glucose concentrations [102-104; 253; 254], an influence of this by-product was evident. Already a glucose concentration higher than 0.1 $mg \cdot mL^{-1}$ induces ethanol production in *S. cerevisiae* [105; 106], whereas a critical concentration inside the beads was already exceeded a few minutes after preparation of the beads, even though the preparation was conducted on ice. The consumption of the complete glucose released within the beads by the encapsulated *S. cerevisiae* cells was demonstrated. This is possible due to a fast adaption of this microorganism to high glucose concentrations [255; 256]. The Crabtree effect leads to a faster liberation of energy and thus enables more rapid growth [108], then again is *S. cerevisiae* known to have a relatively high resistance to ethanol toxicity, what further offers evolutionary advantages over other microorganisms [109; 116].

Blastospores provide the lowest resistance against stress factors, compared to mycelium and aerial conidia [158]. A concentration of 0.1 $g \cdot mL^{-1}$ ethanol (reached in a bead containing 0.1 $U \cdot g^{-1}$ amyloglucosidase already after seven days) already led to a reduction of germinating blastospores of more than 50% as demonstrated in **chapter 6.3**. The constant increase of the ethanol amount inside the bead will inhibit further growth in the long run, even for lower amyloglucosidase concentrations, causing the conidiation on the bead surface to be insufficient for an infection of ticks.

Previous approaches towards an attract-and-kill strategy for tick control are versatile. One approach was the combination of attraction-aggregation-attachment pheromones with an acaricide incorporated into a plastic strip attached to an animal's tail for the control of ticks on livestock [131]. Even though this kind of strategy reduces infestation of animals, much effort for the attachment of the strip needs to be raised, resulting in high costs and is still largely dependent on the use of synthetic chemical acaricides. Traps, acting in the field are usually based on a combination of an artificial attractant (such as CO_2, released from dry ice or other semiochemicals) combined with an adhesive surface [88; 267]. Adhesive surfaces are disadvantageous in that, on the one hand, they also fix non-target organisms and, on the other hand, their characteristics (such as stickiness) are dependent on environmental factors. First tests within the project 'BIOZEC' could furthermore demonstrate that the choice of a suitable adhesive substance which safely fixes ticks and does not harm the environment is difficult (personal communication Dr. Hans Dautel, IS Insect Services, Berlin). Furthermore, the disposal effort of such a trap has to be considered, whereby a purely biological control agent is decomposed in the long run by natural processes of soil organisms. Only a few studies made use of entomopathogenic fungi as kill components, such as reported by Maranga *et al.* [268]. The limitation of these approaches, besides the synthetic attract component, is mainly the stability of the applied biomass. By using a suitable formulation for the fungus, its activity can be prolonged and characteristics, such as virulence, can be improved.

The co-encapsulation is still desirable in view of the advantages mentioned even though it seems not feasible at this point, in view of the insight gained in this thesis. The interaction of the two co-encapsulated microorganisms lead to a reduced efficiency, more precisely of CO_2 release and conidiation on the surface, due to occurring by-products, such as ethanol. The co-formulation has to overcome several limitations, such as the low conidiation and CO_2 release, to become applicable in the field. Several possibilities in further research are conceivable. A reduction of the bead diameter to ensure a better supply of oxygen could be considered to reduce

anaerobic growth [257], whereby this impairs water retention within the bead, another important factor for the CO_2 production as pointed out in **chapter 6.1**. Possibly there is also the possibility to absorb the formed ethanol with another supplemented substance. Exchanging starch with other nutrients that allows better utilization of an EPF to reduce the required supplementation of enzymes and thus the glucose release inside the bead might be a different solution. Another option is a more detailed strain selection regarding compatibility to drying processes, utilization of starch and co-encapsulation with *S. cerevisiae*, as already mentioned for **chapter 6.1** and **6.2**. However, a co-application of both formulations instead of a co-formulation of the microorganisms presented in this work could be the next ambitious step. An infection is presumable when both formulations are applied in a close area. An independent application of the single formulations combined with other artificial attractants (independent of growth processes or environmental factors) or kill components (such as substances with botanical origin (Benelli, 2016 #827)) is also conceivable.

The high potential of both, the Attract and Kill formulation, for the control of *I. ricinus* was demonstrated in this work. The significant attraction as well as a 100% mortality of *I. ricinus* nymphs was achieved under laboratory conditions, a promising development starting point for further research. In a broader perspective, the application of both formulations is transferable to other tick species, not only in Germany but also worldwide, such as from the genera *Hyalomma* spp., *Rhipicephalus* spp. and *Amblyomma* spp., as the most important livestock ticks in Latin America, Africa, Australia and Asia [269] and the blacklegged tick *I. scapularis* as an increasing public health concern in the USA [270; 271]. For the species *R. sanguineus* and *D. reticulatus* the virulent effect of the Kill formulation was already demonstrated. Gaining further knowledge about attractants for ticks, other than CO_2, to optimize the Attract formulation is another main concern of further research [95]. Since CO_2 acts as a host attractant for nearly all bloodsucking arthropod pests, an adaption to other above ground pests, like mosquitoes and biting flies, is also conceivable [272-274]. A subsequent incorporation of other EPF, adapted to the corresponding pest, such as *B. bassiana*, is practicable [44].

To conclude, the approach of the combination of an Attract-and-Kill formulation for the control of above-ground living pests has high potential which indeed is not exploited yet and still needs further research. The formulation is supposed to contribute to the reduction of acaricides in

livestock and to facilitate the control of ticks in contact areas for people, such as gardens and parks, as well as in risk areas for infection with tick borne diseases. In order to advance research for biological control agents, highly interdisciplinary work is required where bioprocess and formulation technology brings together microbiological and entomological knowledge to obtain an efficient control agent in the end.

8 Appendix

The Project BIOZEC

The present work was part of the ZIM (Central innovation program of the middle class) project "BIOZEC" (No. 2426511CR4) which was funded by the BMWi (German Federal Ministry for Economic Affairs and Energy). Figure A1 shows a detailed flow chart for the project 'BIOZEC'. The development and production of the biocontrol formulations as well as cultivation and a part of the lab-scale efficacy tests were carried out at the University of Applied Sciences Bielefeld, whereas the remaining parts were conducted at the University of Hohenheim (working group of Prof. Dr. U. Mackenstedt) and by IS Insect Services GmbH Berlin.

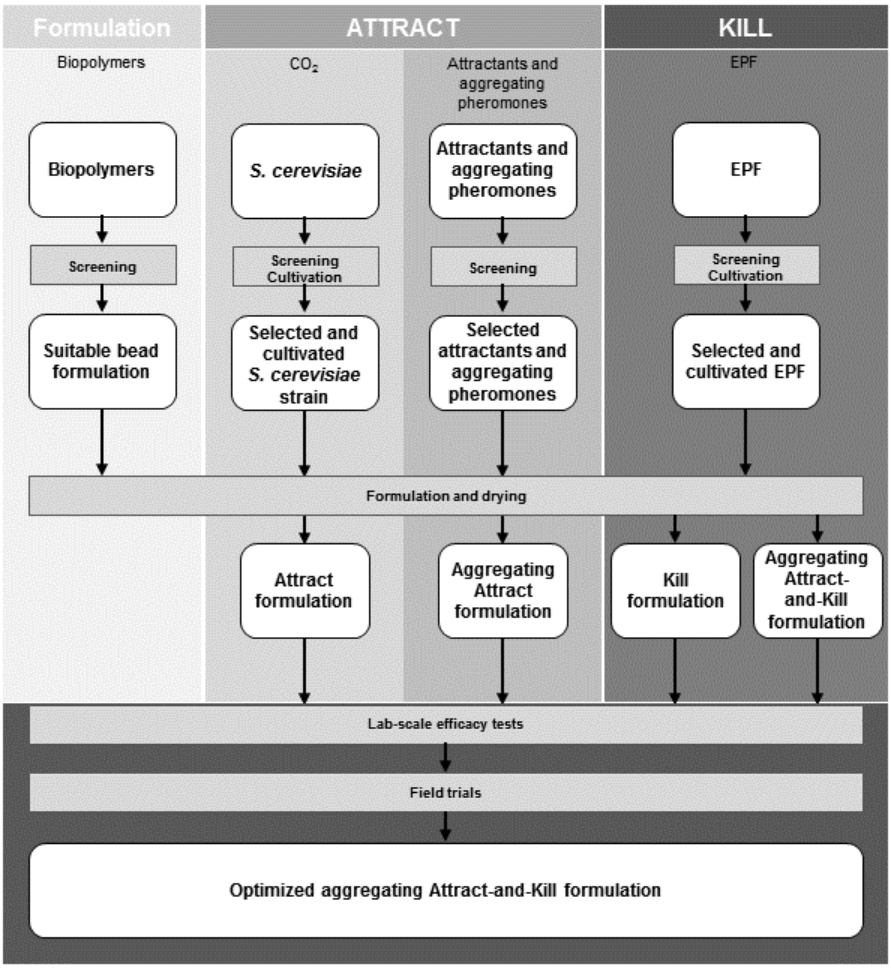


Figure A.1 Flow chart of the ZIM project 'BIOZEC' – Development of a biological tick control agent based on an innovative Attract-and-Kill strategy.

Supplementary material

Screening of five *Metarhizium* spp. isolates

Please note: This experiment was carried out by the working group of Prof. Dr. U. Mackenstedt (University of Hohenheim) under the control of Dr. M. Wassermann who keeps all rights for editing, exploiting and publication of the data presented in figure A.3.

As a previous screening, five *Metarhizium* spp. isolates were examined on their virulence against *I. ricinus* nymphs. For the experiment 20 petri dishes (Ø=50 mm) were prepared for each isolate with a final aerial conidiation of 10^5 conidia·cm^{-2} by adding a conidia solution into the petri dish (in 0.9% NaCl + 0.1% Tween-80) and let it dry overnight. Air holes were pricked in the lid with a hot needle. Then, five *I. ricinus* nymphs were placed in each plate and sealed with Parafilm M (Pechiney Plastic Packaging Inc., IL, USA). The dishes were incubated at 25 °C in a sealed box with saturated $MgSO_4$ solution (relative humidity >90%) in the dark and monitored daily for the first two weeks and afterwards twice a week with a digital microscope (Keyence VHX-1000; Keyence Corporation, Osaka, Japan) to identify infected nymphs, respectively. The pre-screening ended after 97 days.

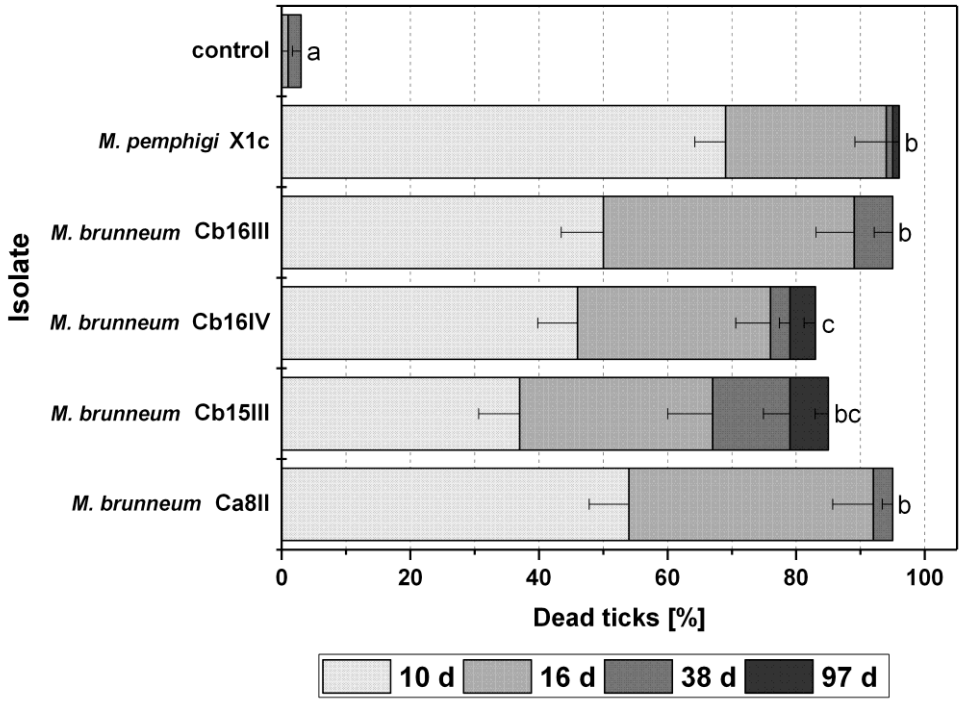
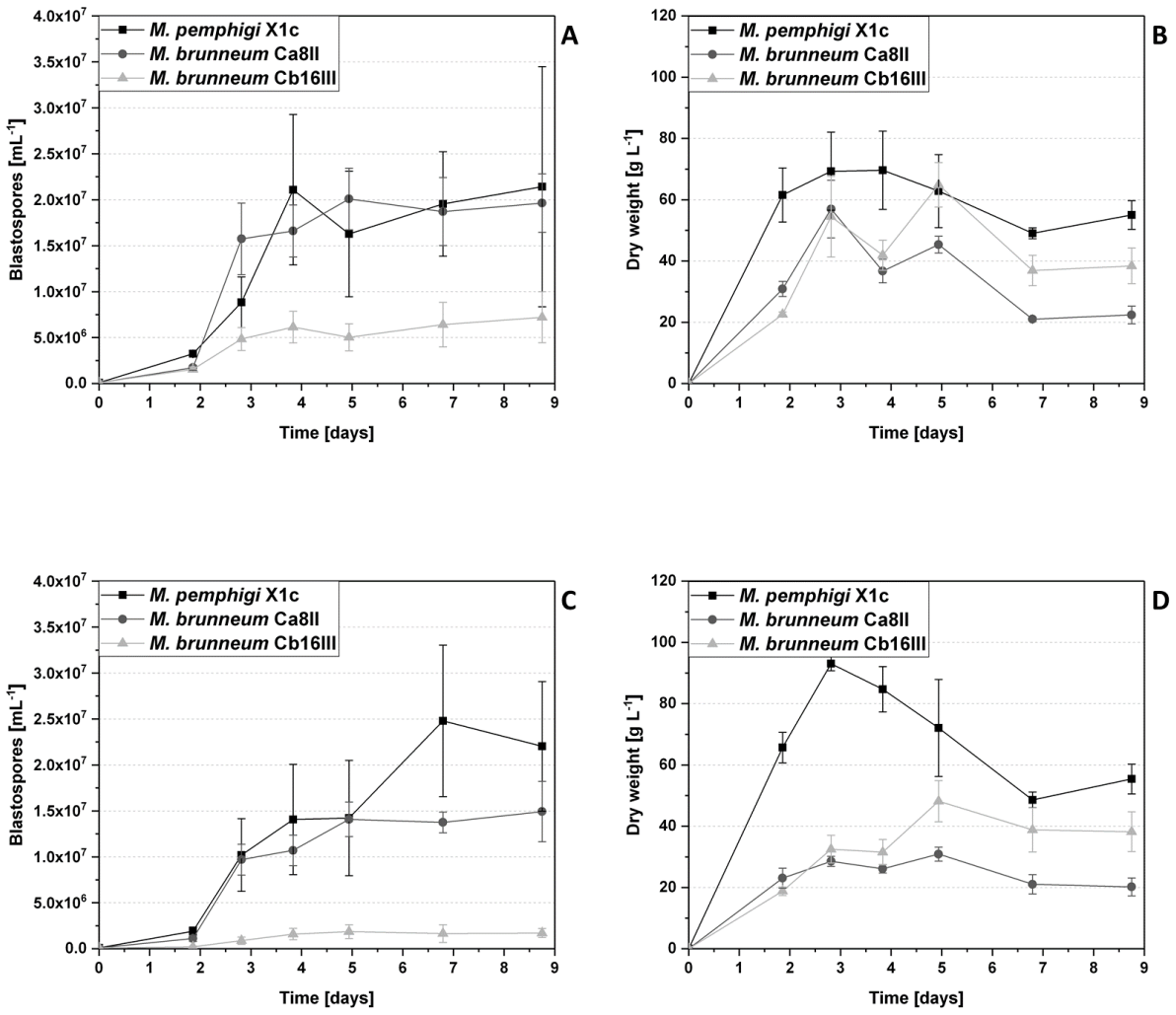


Figure A.2 Preliminary screening of five different *Metarhizium* spp. isolates for virulence of their aerial conidia against *I. ricinus* nymphs during incubation at 25°C for 97 days. Different letters behind bars indicate significant differences according RM-ANOVA with Games-Howell post hoc test at $p<0.05$ (n=20; mean ± se).

Cultivation at different initial pH values

In addition to the results, presented in figure 6.9, the *Metarhizium* spp. isolates were cultivated with an initial pH value of 4.5 and 7.5, as presented in figure A.3.



FigureA.3 Blastospore concentration during liquid cultivation of three *Metarhizium* spp. isolates in shaking flasks at 25 °C and different initial media pH value 4.5 (A, B) and 7.5 (C, D) for nine days. A, C: Blastospores, B, D: total dry weight. Different letters in the legend indicate significant differences according to RM-ANOVA with Games-Howell post hoc test at $p<0.05$ (blastospores: n=7, dry weight: n=5; mean ± sd).

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- Süddeutscher Zeckenkongress Hohenheim 14.03.2018 Hohenheim, Germany: *Auswahl eines entomopathogenen Nutzpilzes zur Bekämpfung von* Ixodes ricinus *nach Virulenz und biotechnischen Kriterien*.
- 28th Annual Meeting of the German Society for Parasitology 23.03.2018 Berlin, Germany:
  Selection of an entomopathogenic fungus infective for ticks based on biotechnological criteria and virulence.
- 22. Jahrestagung des AK Biologischer Pflanzenschutz 22.03.2019 Bielefeld, Germany: *Formulierung von* Metarhizium *– Blastosporen zur biologischen Schädlingsbekämpfung*.

Posters at conferences

- 10th Annual Biological control Industry Meeting ABIM, 19-21.10.2015, Basel, Switzerland:
  Fermentation and Formulation of Biologicals and Chemicals.
- Society for Invertebrate Pathology (SIP) 24.-28.07.2016 Tours, Frankreich: *Development of a biological tick control agent based on an innovative Attract-and-Kill strategy (BIOZEC).*
- Society for Invertebrate Pathology (SIP) 13.-17.08.2017 San Diego, USA: *Development of a biological tick control agent based on an innovative attract-and-kill strategy (BioZec).*
- 4. Süddeutscher Zeckenkongress Hohenheim 14.03.2018 Hohenheim, Germany: *Verkapselung und Trocknung von* Metarhizium *spp. Blastosporen in Calcium-Alginat-Kapseln mit verschiedenen Additiven.*
- 28th Annual Meeting of the German Society for Parasitology 23.03.2018 Berlin, Germany:
  Development of a biological tick trap based on ATTRACT-and-KILL strategy – Screening of attractants.

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Declaration - *Erklärung*

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Hiermit erkläre ich, dass

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- *Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Vermittlungstätigkeiten oder für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.*
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Sissy-Christin Lorenz

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