PROCESS FOR THE ENCAPSULATION OF BIOLOGICAL ACTIVE SUBSTANCES

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The PGSS high pressure spray process, which utilizes the solubility of supercritical fluids in molten substances to generate particles, is well known for the production of fine powders in the micrometer scale and is also used for the encapsulation of active substances. The process was now advanced to meet the requirements of biologically active materials with respect to temperature sensitivity and environmental compatibility.

In this contribution the *Cydia pomonella* granulo virus (CpGV), which is deployed world-wide in apple orchards to control the codling moth, is used as biologically active substance. This CpGV combines ecological and economic aspects due to high selectivity, protection of beneficial organisms and high effectiveness towards the target organism. Disadvantages of the untreated CpGV like strong UV sensitiveness, which results in many applications, and a low uptake, could be avoided by the encapsulation with an organic matrix.

The efficiency of the encapsulated CpGV formulation was already successfully verified in laboratory tests. The pesticide is produced in batch mode. The challenge is now to transfer the process to a flexible, continuously operated plant to meet the requirements of the market, which is currently under way.

INTRODUCTION

Ecologically produced groceries are more and more favoured by many consumers and protect the environment, too. Apples for example are grown extensively in Europe but also world-wide. The most destructive pest in the apple cultivation is the codling moth (*Cydia pomonella* L., Lep.: Tortricidae). After hatching the larva enters the side of the apple then tunnels to the centre where it feeds and develops. The larva leaves excrements and tunnels within the apple, which starts to decay (figure 1, [1]).

The *Cydia pomonella* granulo virus (CpGV), a genus of the baculo viruses (figure 2, [2]), is used world-wide to control the codling moth. The larva has to feed on the virus in the first stage of its developement. This virus combines ecological and also economic aspects due to high selectivity, protection of beneficial organisms and high effectiveness towards the target organism. It is assumed that already one CpGV particle is mortal. Disadvantages of the untreated CpGV like strong UV sensitiveness, which results in frequent reapplications during the growing season, a low uptake and the removal of the water-soluble virus through rainfall from the trees could be avoided by the encapsulation with organic materials [3].

During the last four decades majour efforts have been realized especially to improve the stability of biological pesticides, but the general shortcoming of these preceding encapsulated pesticides is the low lifetime. Smith *et al.* promised improvement by enclosing granuloviruses with a mixture of a polymer and an UV protectant based on lignin. The shell material is dissolved in a highly concentrated acetone solution, pressurized and expanded through a nozzle into a spray tower. Shortly before the nozzle the virus is admixed [4].

Figure 1: Attacked apple with larva

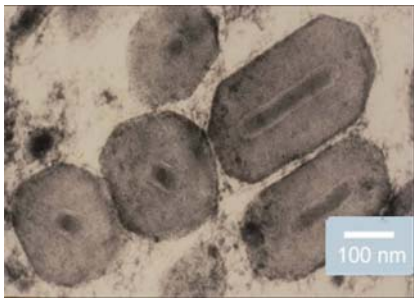

Figure 2: SEM image of the granulo virus

Arthurs *et al.* also evaluated the encapsulation of the granulovirus as UV stabilization. But they report no clear benefit from the spray-dried lignin-based virus capsules [5]. This contribution however shows that the encapsulation of the virus formulation with an organic matrix material, which is based on fat, is very successful.

MATERIALS AND METHODS

The PGSS (Particles from Gas Saturated Solutions) process was chosen to encapsulate the virus formulation for various reasons. Firstly it runs at low temperatures so that the heat sensitive virus proteins are not denatured. Secondly the utilized supercritical fluid, carbon dioxide, is environmentally benign, non-oxidizing and relatively cheap. Thirdly organic solvents can be avoided. The possibilities of the PGSS process have been investigated for more than a decade and have successfully been demonstrated for several solids and liquids. Examples are polyethylene glycols, powder coatings, monoglycerides, citric acid, vitamins, pharmaceuticals, antioxidants, solid fats, green tea, as well as food and food related products. Closed and open solid-liquid composites have also been generated [6-19]. As far as we know nobody has ever used the PGSS process to enclose a virus or virus formulation. The experiments are carried out on the rig, which is displayed in figure 3 and enables the production of powders with different morphology and size distribution.

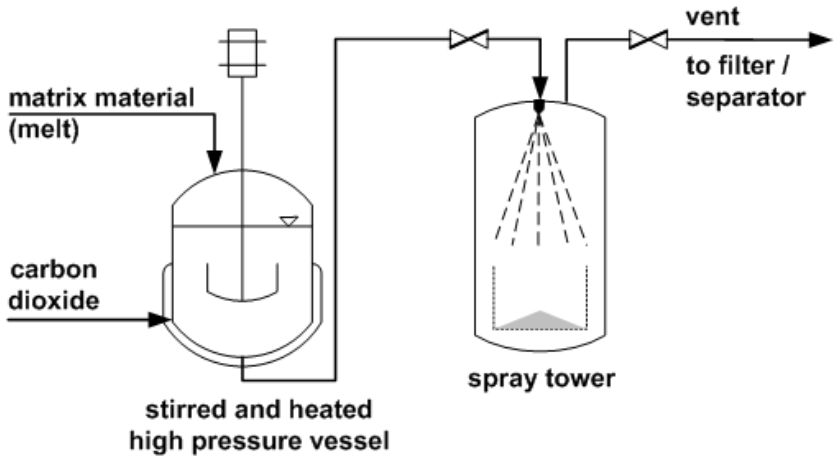

Figure 3: Flow diagram of the rig, which was used to encapsulate the virus formulation

All employed materials must fulfill the following: They have to be biocompatible, nontoxic and should maintain the properties of the virus [20]. These requirements are met by the substances used. At first the matrix material, a commercially obtainable fat, is melted and heated to the designated temperature (below 60°C) and admixed with the CpGV formulation

and an also commercially available lecithin-based surfactant. The amount of each component is chosen such that it is possible to get approximately one virus per capsule [3]. The mixture is then poured into the tempered autoclave. After the autoclave is closed, carbon dioxide pressure is applied and the supercritical liquid is allowed to dissolve into the melt for at most 15min. The content of the high pressure vessel is stirred thoroughly to assure optimal mixing. A thermocouple monitors the critical parameter temperature within the formulation. Then the mixture is expanded to ambient conditions through a nozzle into a spray tower, where small droplets are formed due to the large pressure decline and the expansion of the gas. The Joule-Thomson effect causes a strong temperature reduction and hence the solidification of the matrix material. The particles are collected at the bottom of the tower. Carbon dioxide is vented.

The collected capsules are tested for biological activity in bioassays. But before the particles are suspended in water and sprayed onto Petri dishes, which contain the culture medium and one larva, they are thoroughly rinsed with water, to make sure that all viruses on the surface are gone [20]. So when a larva dies because of the virus, which can be clearly seen by the white colour (figure 1), it must have eaten and digested the capsule. During a bioassay several sets of 30 Petri dishes each are sprayed to get results for the different issues, but also to validate the cleaning procedure of the particles.

SEM pictures show the morphology. Spherical particles are favourable because they give free flowing powder [20]. Distorted particles tend to clog the nozzles that the farmers use to spray the virus formulation. Spheres can be obtained if the difference between melting and spraying temperature is large enough [21]. The size distribution of the powder was measured by laser diffraction.

RESULTS

The aim is to show that the virulence of the CpGV is not reduced by the encapsulation process. The figures display representative results of a biological test in the laboratory. On the x-axis the duration of the experiment in days is plotted, on the y-axis the number of inserted larvae can be seen. The key right to the diagrams explains the colour code:

Dead	-	Death by the virus
Withered	-	Death due to denial of nutrition
Migrated	-	Escaped from the Petri dish owing to air inlets
Living	-	Living larvae

Figure 4 depicts a set that was sprayed with water used to wash the powder. The water shows no virulence, as can be seen, since no larvae die because of the virus. The number of the migrated larvae stays constant. No deaths due to denial of nutrition. Figure 4 verifies the quality of the washing step.

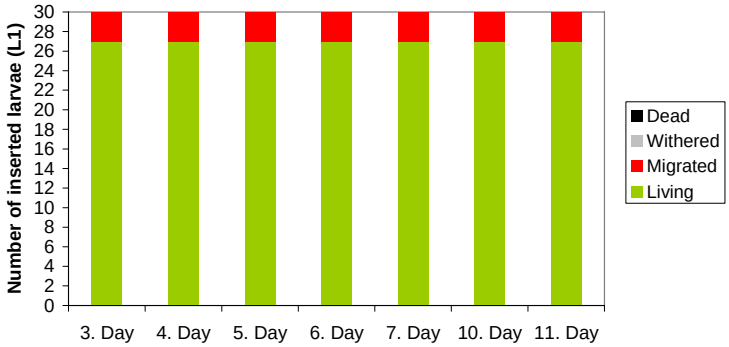

Figure 4: Result of the bioassay: sprayed water used to wash these particles (L1: first instar of the larva)

Figure 5 shows the result of a set, where the washed powder was investigated. The number of dead larvae increases steadily. The number of withered and migrated rises only on the first two days and then remains the same.

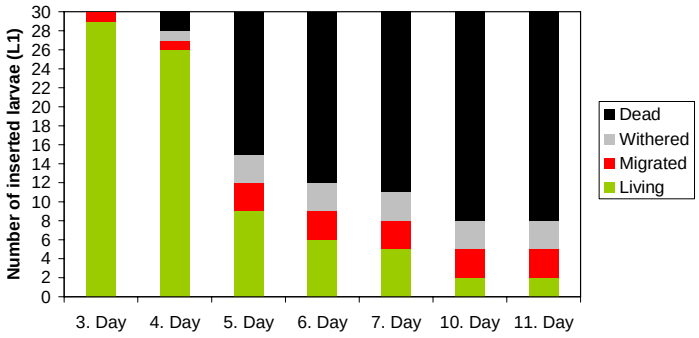

Figure 5: Result of the bioassay: sprayed, washed particles (L1: first instar of the larva)

The quintessence of these figures is that the water-soluble virus is actually encapsulated and biologically active. Although compressed carbon dioxide is said to have a strong germ-killing effect [11], which is for example used to sterilize medical instruments by the physical destruction of the virus during depressurization of a high pressure vessel because of dissolved CO_2 [22]. The probably very low pH value within the autoclave [23] does not cause a decrease of the virulence either. pH values of as low as 4 are tolerable for the CpGV, only high values induce degradation [24].

Figure 6 shows a SEM image of the capsules. Almost perfect spheres are obtained, which avoid clogging of the nozzles during the spraying of the bioassays, because interlocking of particles is less probable. The process parameters must be chosen such that the production of distorted paricles is inhibited.

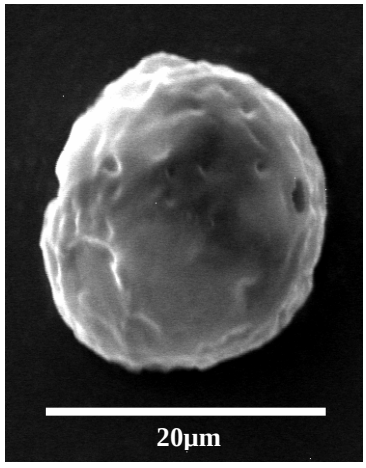

Figure 6: SEM image of a produced particle with encapsulated virus

A representative particle size distribution by weight is displayed in figure 7. Only powder with a certain distribution guarantees smooth spraying hence a uniform coverage of the Petri dishes. On the x-axis the particle size can be read, on the y-axis the corresponding percentage of particles with a certain diameter is plotted. Therefore it can be stated that there are almost

no particles larger than 70µm and most of the particles have a diameter of about 17µm. This result is important because according to experience particles of such a distribution do not cause plugging of the nozzle, during bioassays. The nozzle diameter is about 250µm.

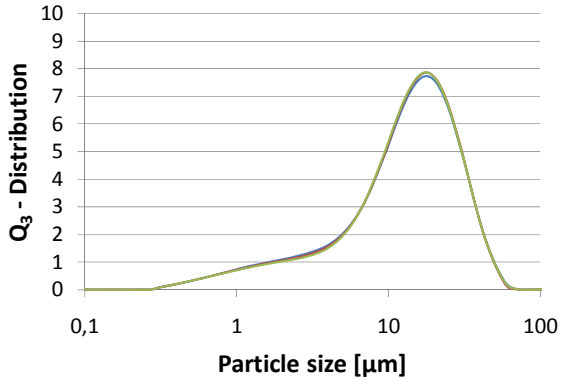

Figure 7: Particle size distribution of the powder by volume (Q_3); maximum at approx. 17 µm; largest particles about 70 µm

CONCLUSION

The results of the bioassays state clearly that it is possible to encapsulate the CpGV with the PGSS process without the loss of virulence. The enclosed virus is at least as good as the non-encapsulated virus formulation.

The SEM pictures of the capsules, the particle size distribution as well as the bioassays show that the morphology of the powder is suitable for spraying, which is very important for an agricultural application. The spherical particles do not stick together, when suspended in water and hence cause no clogging of the nozzle.

The produced powder with the encapsulated virus shows a very promising result. The challenge is now to increase UV protection, which is already given to a certain degree by the fat and to transfer the process to industrial scale. Currently a continuously operated rig is built. Since other baculoviruses are already used as pesticides, this process could be applied to improve the protection of further crop such as cotton or walnut. So this work shows the great potential that lies within the encapsulation of viruses.

ACKNOWLEDGMENTS

The authors gratefully acknowledge funding of this project by the German Federal Ministry for Economy and Technology and by the European Union. We also like to thank Ms. Bibow and Mr. Roßmann for their invaluable work. Dr. Schwab and Dr. Scheurer also gratefully acknowledge funding of this work by the loan programm “Flügge” from the Free State of Bavaria. Finally Dr. Schwab, Dr. Scheurer and Mr. Pemsel want to express their gratitude to their mentor Prof. Schlücker.

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