Release of *Trichoderma viride* Spores from Microcapsules Simultaneously Loaded with Chemical and Biological Agents

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Summary

Recent studies of bioactive agents, simultaneous encapsulation in chitosan/alginate microcapsules revealed that encapsulation in the same compartment does not inhibit activity either of *Trichoderma viride* spores nor copper cations. The objective of this work was to investigate the influence of formulation variables (concentration of copper cations, chitosan layer and microcapsule size) on *T. viride* spores release. Results showed that the increase in copper cation concentration promoted, but the increase in microcapsule size and presence of the chitosan layer on microcapsule surface reduced *T. viride* spores release. Fitting to simple Korsmeyer–Peppas empirical model elucidated the underlying mechanism of release. Fickian diffusion controlled release from microcapsules without chitosan layer and smaller microcapsules with chitosan layer, whereas anomalous diffusion mechanism (a combination of the diffusion and erosion mechanisms) was found to be the rate-controlling mechanism from larger microcapsules with chitosan layer. The investigation showed that proper selection of formulation variables helps in designing microcapsules with the desirable release of *T. viride* for plant protection and nutrition.

Key words

sodium alginate, chitosan, copper ions, *Trichoderma viride*, controlled release

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Introduction

The encapsulation of active agents has been developed in recent years as a new potential tool for ecological and sustainable plant production (Hack et al., 2012). The benefits conferred by microencapsulation include slow release of a bioactive ingredient, more efficient exploitation of the chemicals used, greater safety for the user, and better protection of the environment (Rodham, 2000). Biopolymer based microcapsules with a single active agent have extensive applications in agriculture and became one of standard formulation techniques. Nevertheless, our knowledge of biological and chemical agents simultaneous encapsulation is still rather limited.

Recently, we have demonstrated the possibility of simultaneous encapsulation of copper cations and *T. viride* spores (abbreviation, *Tv*) in chitosan/alginate microcapsules (Vinceković et al., 2016). Due to benefits for crop protection and nutrition as well as high compatibility, *T. viride* and copper ions were taken as a suitable couple of chemical and biological agents. Experiments performed on *T. viride* spores survival revealed that during storage at the room temperature the encapsulation of *T. viride* in the same compartment with the copper cations does not inhibit their activity. Characterization of prepared microcapsules showed high copper cation encapsulation efficiency and loading capacity as well as good release behavior from microcapsules.

Controlled release, that is successful delivery of active agents at the right place and the right time, is an attainable and desirable characteristic for all bioactive agents' delivery systems. To obtain the well-designed microcapsules efficient for simultaneous encapsulation and prolonged release, it is important to optimize parameters during microcapsule preparation. Consequently, the aim of the present work was to study the influence of copper cation concentration, chitosan layer and microcapsule size on *T. viride* spores release from alginate microcapsules with the intention of delivering active agents to plants at the rate that closely approximates plant demand over an extended period.

Material and methods

Materials

Low viscosity sodium alginate (ALG) (CAS Number: 9005-38-3; Brookfield viscosity 4 - 12 cps (1% in H_2O at 25°C) was purchased from Sigma Aldrich (USA). Low molecular weight chitosan (CS) (CAS RN: 9012-76-4, molecular weight: 100000–300000) was obtained from Acros Organic (USA). A commercially available copper sulfate pentahydrate, $CuSO_4 \cdot 5H_2O$ (Kemika, Croatia) was used as a copper cation donating substance. All other chemicals were of analytical grade and were used as received without further purification. An indigenous isolate of *T. viride* (abbreviation, *STP*) originated from parasited sclerotia of *Sclerotinia sclerotiorum* was used in all experiments (Topolovec-Pintarić et al., 2013). To obtain spore suspensions, the STP was grown in Erlenmeyer flasks containing potato dextrose broth (Vinceković et al., 2016).

Microcapsule preparation

Microcapsules without chitosan layer (ALG/(Cu+*Tv*)) were prepared by ionic gelation, while those with chitosan layer (CS/(ALG/(Cu+*Tv*)) were prepared in two stages (ionic gelation followed by polyelectrolyte complexation) as was previously described (Vinceković et al., 2016). Ionic gelation involves the preparation of alginate core microcapsules simultaneously loaded with copper cations and *T. viride* spores (uncoated microcapsules), and polyelectrolyte complexation includes a coating of alginate core microcapsules by chitosan (coated microcapsules). The concentration of sodium alginate and chitosan, as well as the size of microcapsule were kept constant, while initial copper concentration varied from 5 to 15 mmol dm^{-3}. The microcapsule diameter was determined by using encapsulator nozzle size of 0.45 or 2 mm, respectively (Encapsulator Büchi – B390, BÜCHI Labortechnik AG, Switzerland).

Characterization of microcapsules loaded with bioactive agents

The size of prepared microcapsules was controlled using a light binocular. Diameters of about 100 microcapsules were measured. Microphotographs were taken with a Leica DFC295 digital camera on a trinocular mount of a Leica MZ16a stereo-microscope (Leica Microsystems Ltd., Switzerland). Spores of *T. viride* (stained with Rhodamine 123; 0.1% w/v) and microcapsules (stained with eosin; 0.01% w/v) were examined by confocal laser scanning microscope (CLSM, TCP SP2, Leica Lasertechnik, Germany).

Information on intermolecular interactions between components in microcapsules was obtained using Fourier transform infrared spectroscopy (FTIR). IR spectra of the samples were recorded with the FTIR Instrument - Cary 660 FTIR (MIR system) spectrometer (Agilent Technologies, USA). Dry samples were crushed with potassium bromide to get pellets. Spectral scanning was done in the range of 400-4000 cm^{-1}.

Loading capacity (LC) is defined as the content of *T. viride* spores per gram of dry microcapsules. Wet microcapsules loaded with bioactive agents were air-dried at the room temperature for several days until all the liquid evaporated. Loading capacity was determined by dissolving of dry microcapsules (4 g) in 100 ml of a mixture of 0.2 M $NaHCO_3$ and 0.06 M $Na_3C_6H_5O_7$ x $2H_2O$) (Li et al., 2007). The concentration of *T. viride* spores (expressed as the number of spores per 1 g of the dry microcapsule) was determined spectrophotometrically (Waghunde et al., 2010). Absorbance was measured by using UV-VIS spectrophotometer at 550 nm (UV-1700, Shimadzu, Japan).

Loading capacity presented as a number of *T. viride* spores per 1 g of dry capsules (NS g^{-1}) was calculated by the equation 1:

$$(LC)_{Tv} = \frac{c_{Tv}}{w_{Tv}} \quad (1),$$

where c_{Tv} is a number of *T. viride* spores and w_{Tv} is a weight of used capsules.

The swelling degree (S_w) was determined on microcapsules dispersed in deionized water. Microcapsules (10 mg) were dispersed in a glass vial containing 10 ml of deionized water and allowed to swell at room temperature during three hours. The wet weight of the swollen microcapsules was determined by blotting them with filter paper to remove moisture adhering to the surface, immediately followed by weighing (Liu et al., 2004). The swelling degree (S_w) was then calculated using equation 2:

$$S_w\% = \frac{w_t - w_0}{w_0} \quad (2),$$

where w_t is the weight of the swollen microcapsules, and w_0 is their initial weight.

The release studies of *T. viride* spores from microcapsules were carried out at room temperature. Microcapsules prepared from 100 ml of the reaction mixture were weighed and dispersed in 100 ml of deionized water and allowed to stand without stirring during experiments. At appropriate time intervals, the dispersion was stirred for 60 sec, aliquots were withdrawn and the spore count was determined spectrophotometrically at 550 nm. Results of release experiments are presented as the fraction of released *T. viride* spores using equation 3:

$$f(Tv) = \frac{R_t}{R_{tot}} \quad (3),$$

where f(*Tv*) represents the fraction of released *T. viride* spores, R_t is the amount of *T. viride* spores released at time t and R_{tot} is the total amount of *T. viride* loaded in capsules.

All experiments were carried out at the room temperature and measurements were replicated three times.

Results and discussion

Identification of molecular interactions

Information on intermolecular interactions between components in the alginate matrix coated with chitosan was obtained using FTIR. FTIR spectra of sodium alginate, chitosan, a mixture of *T. viride* spores and copper cations, and microcapsules (CS/(ALG/(Cu + *Tv*)) prepared at various initial copper concentrations are presented in Fig. 1.

Characteristic FTIR bands of alginate and chitosan presented in Fig. 1 are in accordance with literature data (Sankalia et al., 2007). The strong and broad absorption band between 3700 and 3000 cm^{-1} recorded in all the spectra belongs to the hydroxyl (O-H) group stretching vibrations which are characteristic of natural polysaccharides. Comparing with alginate spectrum, in chitosan spectrum, this strong and broad band corresponds to the amine (N-H) groups superimposed on the hydroxyl groups. Additional specificity of chitosan spectrum is the bending vibrations at the 1648 and 1582 cm^{-1} corresponding to the carbonyl (C=O) stretching of the secondary amide (amide band I) and to the N-H (N-acetylated residues, amide II band) vibrations, respectively. The bands at 1595 and 1405 cm^{-1} present in sodium alginate corresponds to asymmetric and symmetric stretching peaks of carboxylate (COO^-) groups.

The spectrum of *T. viride* spores with bind copper cations shows very strong and broad -OH and -NH stretching vibrations band, the disappearance of *T. viride* bands at 2921, 2854 and 1545 cm^{-1}, and the absence of small peaks between 1452 and 1200 cm^{-1} (Fig. 1). The disappearance of bands and shifting of peaks towards the lower frequency (from 3321 to 3274 cm^{-1}) or towards higher frequencies (1625 to 1635 cm^{-1}, 1072 to 1087cm^{-1}, and 887 to 981 cm^{-1}) have suggested that at least amine, hydroxyl, carbonyl and amide bonds are the major sites for binding of copper cations.

It has been well known that the (COO^-) groups interact with the amino (NH_3^+) groups of chitosan forming a polyelectrolyte complex. The spectrum of (CS/(ALG/(Cu+*Tv*)) shows the disappearance of peak corresponding to chitosan (at 1582 cm^{-1}; -N-H bending vibration) and shifting of characteristic alginate peaks, from 1595 to 1590 cm^{-1} as well as from 1405 to 1411 cm^{-1} indicating electrostatic interactions between two oppositely charged polyelectrolytes. The disappearance of chitosan distinct peaks is probably due to the very low chitosan concentration compared with alginate. Characteristic peaks of alginate corresponding to carboxylate groups are less intense and shifted to lower and higher frequencies, respectively. Changes in the FTIR spectra of sodium alginate and *T. viride* spores (the disappearance of *T. viride* bands at 3198, 2925, 1595 cm^{-1} and bands attributed to the alginate saccharide structure) revealed interactions, at least with an amine, carboxylate and carbonyl groups (Vinceković et al., 2016).

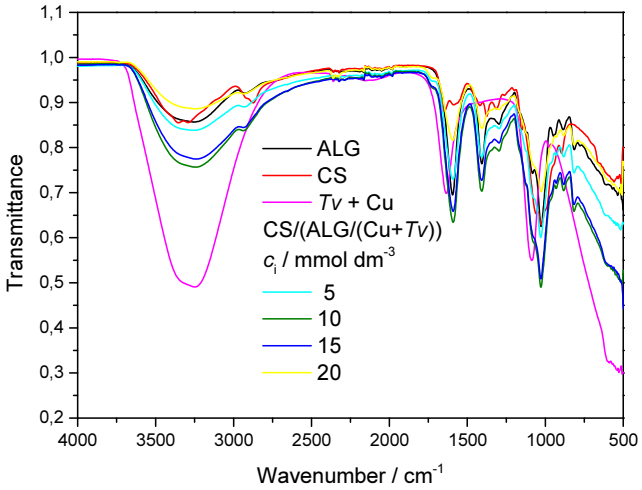

Figure 1. FTIR spectra of alginate (ALG, black line), chitosan (CS, red line), mixture of *T. viride* spores and copper cations (20 mmol dm^{-3}) and coated microcapsules (CS/ALG/(Cu + *Tv*) prepared at various initial copper cation concentrations (c_i/mmol dm^{-3} = 5 (cyan line), 10 (olive line), and 15 (blue line) and 20 (yellow line).

The presence of copper cations in alginate matrix causes the most significant changes in the alginate functional groups region: hydroxyl (OH), ether (COC) and carboxylate (COO^-). It can be seen from the Fig. 1 that depending on the copper cation concentration, the intensities of main alginate peaks changed and shifted to lower or somewhat higher wavenumbers. The absorption band around 3400 cm^{-1} is much stronger at lower than those at higher copper cation concentration. It can be seen from the Fig. 1 that intensities first increase and begin to decrease between 10 and 15 mmol dm^{-3} of copper cation concentration implying changes in intensities of hydrogen bonding and copper cation interactions with *T. viride*. The intensity of hydrogen bonding is the weakest at the highest copper cation concentration, even weaker than in pure alginate. Similar increasing/decreasing effect of copper cation concentration can be seen at the asymmetric and symmetric COO^- stretching indicating complex interactions between all components in the microcapsule.

Release of *Trichoderma viride* spores from microcapsules

Various mechanisms such as surface wetting, penetration of water into microcapsule, glassy-to-rubbery-phase transitions of polymers, swelling, diffusion of loaded agents through the microcapsule matrix and surface layer, desorption from the surface, disintegration, dissolution or erosion of microcapsule structure, or their combination, may be included in the release of an active

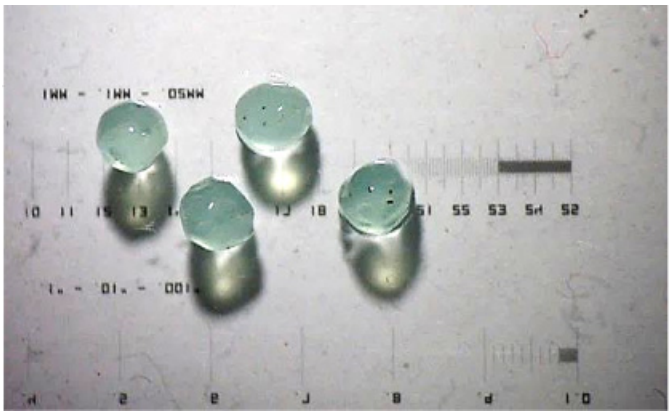

Figure 2. Microphotograph of spherical microcapsules loaded with copper and *T. viride* spores.

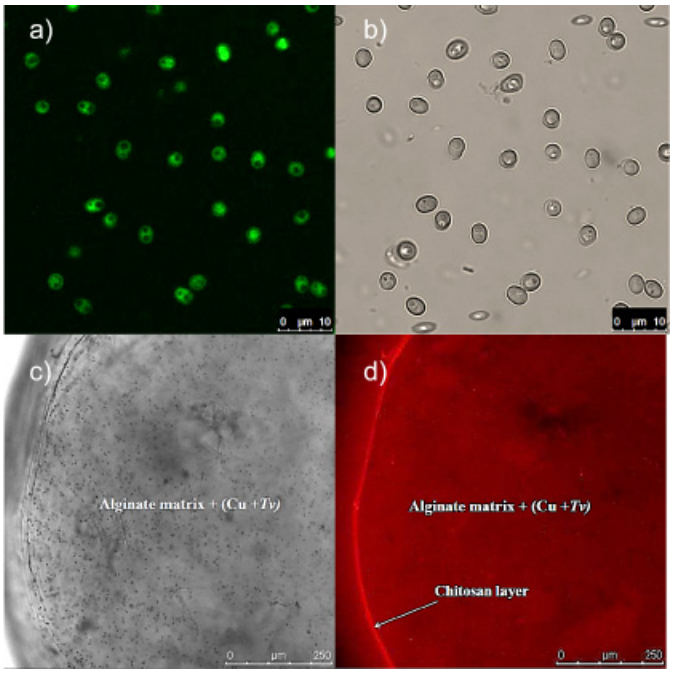

Figure 3. Confocal laser scanning images of *T. viride* spores in (a) fluorescence (stained with Rhodamine 123) and (b) transmitted mode, (c) *T. viride* spores distributed through the alginate matrix in transmitted mode and (d) part of microcapsule stained with eosin. Bars are indicated.

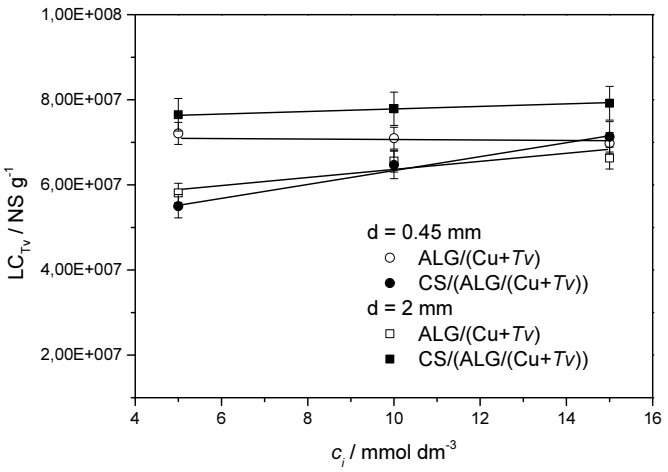

Figure 4. Variation of *T. viride* spores loading capacity (LC_{Tv}/NS g^{-1}) of uncoated (ALG/(Cu+*Tv*)) (open signs) and coated (CS(ALG/(Cu+*Tv*)) (full signs) microcapsules with initial copper cation concentration (c_i / mmol dm^{-3}). Microcapsule diameters are denoted. The error bars indicate the standard deviation of the means.

agent from biopolymeric microcapsules. The mechanism controlling the active agent release primarily depends on the characteristics of microcapsule material and the type of active agents. It was shown that the most important rate-controlling release mechanisms from hydrophilic microcapsules are diffusion, swelling and erosion (Siepmann and Siepmann, 2012). Knowledge of mechanism involved in the release of an active agent is important for the optimal development of formulations based on biopolymeric materials. A common way of achieving the desired release rate is the use of cationic and anionic biopolymeric physical mixture that is the "shell-core" structure (Li et al., 2014). Coating of alginate microcapsules with chitosan reduces the porosity of the alginate matrix (Huguet et al., 1996) and could improve the possibilities of extended release by affecting the rate-controlling mechanisms of release. Having in mind all factors involved in the release and the combinatorial effect of different microcapsule composition, the concentration of crosslinking cation and microcapsule size, we prepared uncoated and chitosan-coated alginate microcapsules of diameters 0.45 and 2 mm with various concentrations of copper cations. All prepared microcapsules were colored due to the presence of copper cation (Fig. 2).

Figs. 3a,3b present *T. viride* spores that were used for encapsulation. Staining with fluorophore revealed *T. viride* spores are spherical. After loading, they were almost homogeneously distributed inside the alginate matrix (Fig. 3c). Thin chitosan layer on the surface of coated microcapsule became visible by staining with eosin, which bounds to the amino groups of chitosan (Fig. 3d). The thickness of coating layer was approximately 11 μm.

Encapsulation efficiency determined by the method of Xue et al. (Xue et al., 2004) exhibited almost 100% of the efficiency of *T. viride* spores encapsulation. Results on *T. viride* spores loading capacity expressed as the number of spores per 1 g of dry microcapsules are presented in Fig. 4. The increase in copper cation concentration slightly increased *T. viride* spores loading capacity, except those uncoated small microcapsules that had almost constant loading capacity.

When dispersed in deionized water microcapsules swelled thus influencing the release of active agents from them. The effect of increasing copper cation concentration on swelling degree is presented in Figs. 5a,b. Chitosan-coated microcapsules exhibited a higher swelling degree when compared with the uncoated one due to high swelling and water uptake capabilities of chitosan (Silva et al., 2004). It can be clearly seen that the increase in copper cation

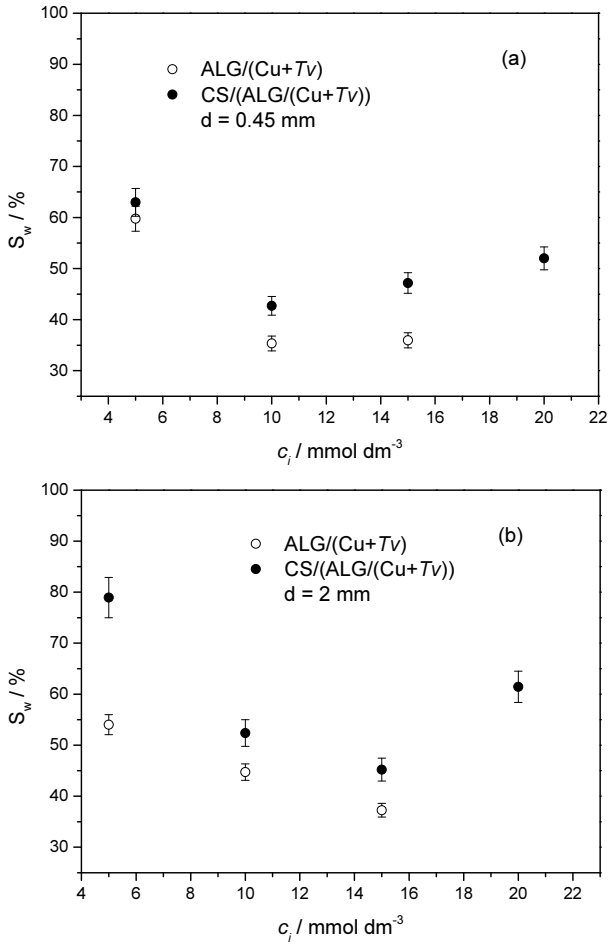

Figure 5. Variation of swelling degree (S_w) with initial copper cation concentration (c_i) of uncoated (ALG/(Cu+*Tv*)) and coated (CS(ALG/(Cu+*Tv*)) microcapsules. Microcapsule diameters are (a) 0.45 and (b) 2 mm. The error bars indicate the standard deviation of the means.

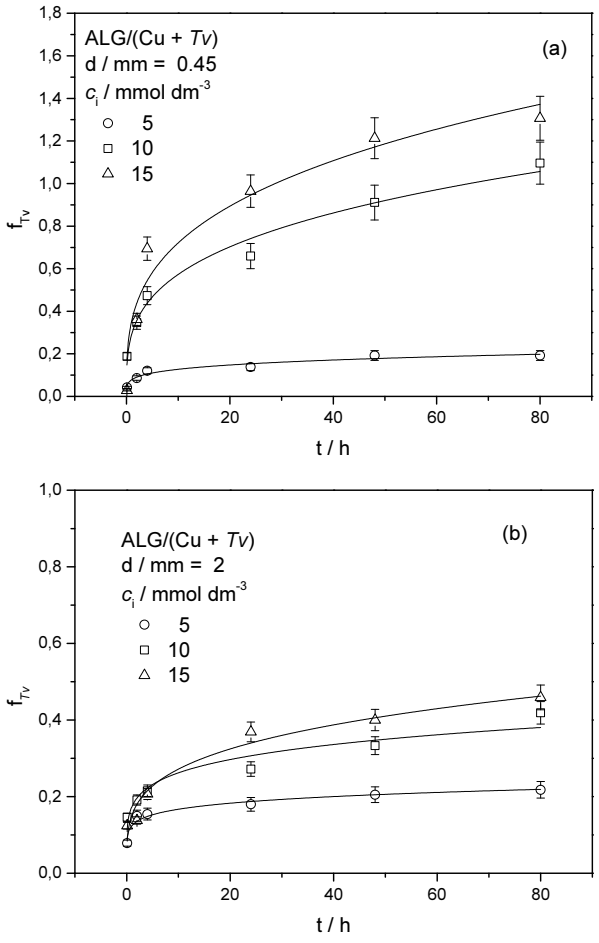

Figure 6. Fraction of released *T. viride* spores (f(*Tv*)) with initial copper cation concentration (c_i) from uncoated (ALG/(Cu+*Tv*)) microcapsules. Microcapsule diameters are (a) 0.45 and (b) 2 mm. The error bars indicate the standard deviation of the means.

concentration decreased swelling of uncoated microcapsules, however, coated microcapsules exhibited first a decrease and at the higher concentration an increase in swelling degree. The differences in the swelling behavior can be attributed to the differences in microcapsule structure. The concentration of crosslinking cation affects the size of cavities inside alginate bead that accommodate water (Roy et al., 2009). As the extent of crosslinking increases, the water uptake decreases (Bajpai and Sharma, 2004).

Release profiles of *T. viride* spores were determined for uncoated (Figs. 6a,b) and coated microcapsules (Fig. 7a,b). It can be seen that the increase in copper cation concentration enhanced, but the chitosan layer on microcapsule surface diminished *T. viride* spores release. Additionally, results revealed that the microcapsule size also affects *T. viride* spores release. Small and uncoated microcapsules released the highest amount of loaded active agent. The higher amount of *T. viride* spores detected in water than concentration loaded in uncoated microcapsule (Fig. 6a) indicated germination of *T. viride* and formation of germ tube biomass in the surrounding medium. Actually, copper cations released from microcapsule promote germination in water. The increasing amount of *T. viride* spores in the dispersing medium is closely related to two processes, one is the release from microcapsules and the other is germination.

All curves for *T. viride* spores release from uncoated microcapsules showed rapid initial release followed by slower release obeying power law equation (Figs. 6a,b), whereas from coated microcapsules showed an initial lag time (Figs. 7a,b). To identify the kinetics and type of mechanism involved in *T. viride* spores release a semi-empirical Korsmeyer–Peppas model (Korsmeyer et al., 1983) was applied. According to Korsmeyer–Peppas, the release exponent n can be characterized by three different mechanisms: Fickian diffusion, and two anomalous (non Fickian diffusion or Type II transport). Values of $n < 0.43$ indicate that the release is controlled by classical Fickian diffusion, and $n > 0.85$ indicate that release is controlled by Type II transport, involving polymer swelling and relaxation of the polymeric matrix, whereas values of n between 0.43 and 0.85 show that the anomalous transport kinetics are determined by a combination of the diffusion mechanism and Type II transport.

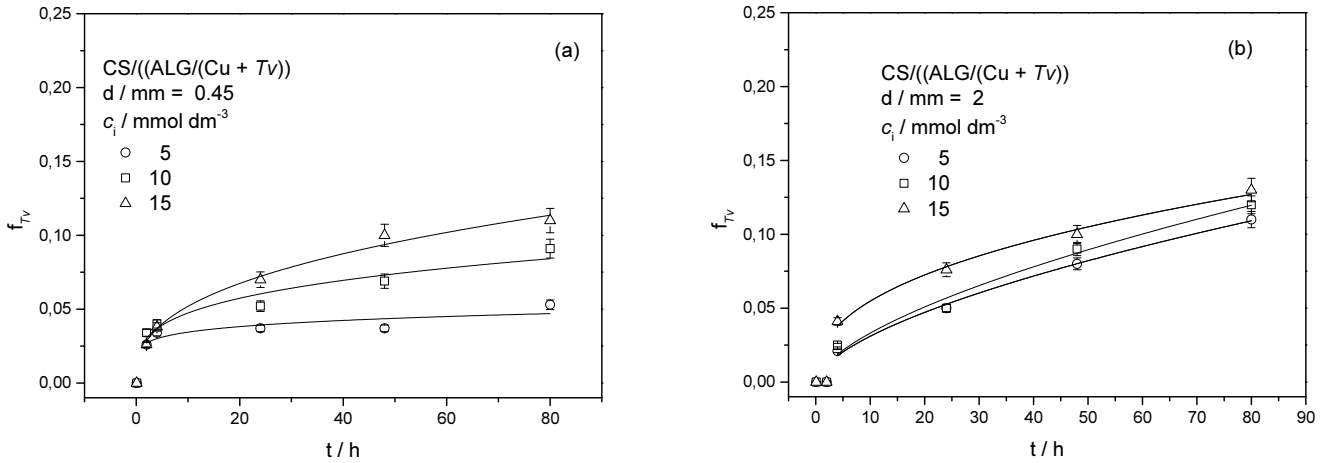

Figure 7. Fraction of released *T. viride* spores (f(*Tv*)) with initial copper cation concentration (c_i) from coated (CS(ALG/(Cu+*Tv*)) microcapsules. Microcapsule diameters are (a) 0.45 and (b) 2 mm. The error bars indicate the standard deviation of the means.

Table 1. The values of the release constant (k) and exponent (n) of *T. viride* spores encapsulated in uncoated (ALG/(Cu + *Tv*)) and coated (CS/(ALG/(Cu+*Tv*))) microcapsules.

c_i/mmol dm^{-3}	0.45 mm			2 mm		
	k/h^{-n}	n	R^2	k/h^{-n}	n	R^2
ALG/(Cu+ *Tv*)						
5	0.078	0.215	0.95	0.164	0.130	0.95
10	0.291	0.295	0.98	0.214	0.118	0.98
15	0.354	0.309	0.95	0.273	0.197	0.95
CS/(ALG/(Cu + *Tv*)						
5	0.124	0.119	0.95	0.005	0.699	0.99
10	0.184	0.130	0.98	0.004	0.773	0.97
15	0.190	0.256	0.95	0.013	0.511	0.99

All curves presented in Figs. 6a,b can be described by the equation:

$$f(Tv) = kt^n \quad (4).$$

In accordance with the empirical model, *k* is a kinetic constant characteristic of the active agents/polymer system that considers structural and geometrical aspects, and (*n*) is an exponent that characterizes the transport mechanism of active agent through the microcapsule. Eqn. 4 is based on the assumption that release occurs as soon as the microcapsule is placed in contact with water. The release profiles of coated microcapsules (Figs. 7a,b) were analyzed by modifying Eq. 4 to initial lag time (Costa and Lobo, 2001),

$$f(Tv) = k(t-l)^n \quad (5),$$

where *l* denotes the lag time. The lag time is thought to be equivalent to the time required for the microcapsule to hydrate and reach equilibrium before erosion, and the advance of solvent through the microcapsule occur. The processes involved during the lag phase are penetration of water and filling of the microcapsule surface pore with water, as well as transport of *T. viride* spores through the alginate matrix and the water-filled chitosan pores, and finally diffusion in the surrounding media.

The values of the release constants and exponents are listed in Table 1. As it was expected, the release of *T. viride* spores from uncoated was faster than from coated microcapsules. The release constant *k*, which incorporated the overall solute diffusion coefficient and geometric characteristics of a microcapsule, increased with initial copper cation concentration. The exponent *n*, characteristic of the mechanism controlling the bioactive agent release, increased as a number of copper increases for both microcapsules, with and without chitosan layer. Lower *n* values than 0.43 indicate that the release mechanism involved is controlled by a classical Fickian diffusion, whereas higher *n* values indicate copper cations release followed non-Fickian kinetics (the anomalous transport kinetics determined by a combination of the diffusion mechanism and Type II transport).

The key factors known to influence microcapsule properties are alginate characteristics and concentration as well as a kind and concentration of cross-linking cation (Loh et al., 2012). The rate of alginate gelation is an important factor in controlling microcapsule homogeneity and strength. Slower gelation produces more uniform structures with better mechanical properties. It was demonstrated that the cross-linking cation affects the rate of gelation (Kuo and Ma, 2001). Gelation rate increased with increasing total divalent cation concentration, thus affecting both mechanical and diffusion properties.

We have prepared microcapsules by dropping alginate and *T. viride* spores suspension into a copper cation solution of various

initial concentrations. Upon contact, copper ions binding to carboxyl groups on guluronic blocks of the alginate chains and crosslinking by junctions with the guluronate blocks of adjacent polymer chains form a microcapsule loaded with *T. viride* spores. Faster release of *T. viride* spores at a higher copper concentration may be ascribed to the formation of less uniform microcapsules with poorer mechanical properties. The presence of chitosan layer improved the mechanical properties, but restricted release properties as compared to microcapsule prepared without chitosan layer.

Conclusions

This work presents important information concerning the simultaneous encapsulation of the copper cations and *T. viride* spores into chitosan/alginate microcapsules. Investigation of intermolecular interactions between oppositely charged biopolymers and bioactive agents using FTIR spectroscopy revealed a complex interaction between biopolymers and bioactive agents as well copper cations and *T. viride* functional groups. The release profiles from uncoated microcapsules exhibited rapid initial release followed by slower release. The concentration of copper cations and the presence of chitosan layer influenced the kinetics and mechanism of *T. viride* spores release from microcapsules. The increase in the copper cation concentration promoted, but the chitosan layer on microcapsule surface slowed *T. viride* spores release. Fickian diffusion was found to be the rate-controlling mechanism for *T. viride* spores release from microcapsules without chitosan layer and small microcapsules with chitosan layer, whereas modification of Korsmeyer–Peppas empirical model showed that the release from large coated microcapsules is controlled by a combination of diffusion and the polymer swelling and relaxation. A better understanding of intermolecular interactions between bioactive agents and the delivery system as well as mechanisms controlling the release of active agents enhance the ability to control active agents release behavior and may aid in developing new microcapsules with specifically tailored properties. After initial fast release or lag time, the *T. viride* spores could be constantly delivered to the plant for a prolonged time by choosing the appropriate concentration of cross-linking cation and microcapsule size.

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