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FLOCCULATION OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* SUSPENSION AND ITS EFFICACY AGAINST MOSQUITO LARVAE

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Bacillus thuringiensis ssp. *israelensis* (Bti) is increasingly used as an ecologically friendly anti-mosquito agent. The bacterium cells undergo fermentation in dilute suspensions; before practical use, therefore it is necessary to concentrate the suspensions. Aggregation by polymers is a powerful tool with which to regulate the stability of suspensions. Typically, polymers at low concentrations destabilize and at high concentrations stabilize colloidal systems. Bti suspensions can be flocculated efficiently by either cationic or anionic polyelectrolytes. Cationic polyelectolytes were found to be the most efficient flocculants for bacterial suspensions. It was shown that the degree of toxicity of the flocculated Bti suspensions for biting mosquito larvae was in the same range than in non-flocculated suspension.

Keywords: Flocculation – cellular suspension – polyelectrolytes – sedimentation – toxicity

INTRODUCTION

Bacillus thuringiensis ssp. *israelensis* (Bti) bacteria are successfully applied for the elimination of mosquito larvae [7, 8, 16, 18]. This is due to the fact that their spores contain a crystalline protein (δ-endotoxin), which after entering the digesting system, starts to activate and kill the mosquito larvae [9, 14]. We have developed a method for the production and spore formation of dilute ($1–3 \times 10^7$ cells/ml) Bti suspensions and determined the conditions of this process [12, 15, 19]. Formulation of this suspension and its use for practical purposes are possible only with relatively concentrated systems.

Many problems relating to the concentration and thickening of microorganism suspensions can be solved by using water-soluble polymers/polyelectrolytes. This is due to the high efficiency (addition of polymers in ppm amounts can drastically change the degree of cell aggregation), relatively low cost and simplicity of the flocculation process. Flocculation by polymers can be used for the concentration of cells

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either as an independent (main) method or as an auxiliary means of enhancing conventional phase separation processes such as sedimentation, filtration, centrifugation or flotation [5, 6]. The laws, kinetics and mechanisms of the flocculation of dispersions by polymers have been described in a number of papers and books. In particular, the application of high-molecular substances in biotechnology has been described and discussed in detail by one of the authors [1, 2].

It has been shown that the main laws and mechanisms of flocculation for cellular suspensions are the same as those for the flocculation of inorganic dispersions. At the same time, the former process is more complicated because of the effects of the bacterial metabolism products, the components of the culture medium and the changing structure of the cell walls in response to the cell surface flocculant interactions and the course of the flocculation process [3–6].

To concentrate large volumes of Bti suspensions by a fast and simple method, we recommend the use of water-soluble polymers. To this end, we have now studied in detail the effects of anionic and cationic polyelectrolytes and of anionic and cationic surfactants on the kinetics and degree of aggregation of cell suspensions as a function of the added reagent, the cell content, the concentration of the polymer solution, the mode of mixing and other variables.

MATERIALS AND METHODS

We studied the possibility of concentrating a dilute suspension of Bti by means of different cationic and anionic polyelectrolytes.

Bacillus thuringiensis *ssp.* israelensis *strain*

Bti is a rod-shaped aerobe microorganism [20]. The length of the vegetative cells is 3–5 μm and their width is 0.8–1.2 μm. These bacteria form spores under unfavourable conditions. The ovoid spore is 2–3 μm in diameter. In our experiments, we used only Bti spores. The Bti was isolated from a commercial product (VECTOBAC 12 AS, Valent BioScience Co., Libertyville, IL, USA) and was fermented for 72 h in T_{20} nutrient solution in a shaking thermostat (T = 28 °C; rpm = 195). The living cell number was determined on diluted plating bouillon-agar [13]. The living cell number was 1.5×10^7 cells/ml.

T_{20} nutrient solution was prepared by boiling 50 dkg/l pork bones for 30 minutes, followed by sterilization.

Polyelectrolytes

To concentrate the Bti suspension, we used cationic and anionic polyelectrolytes. The cationic polyelectrolytes applied were SNFH 528 (SNF S.A., France), Percol 1697

(CIBA) and DS (Laboratory of Colloid Chemistry of the University of Wageningen, The Netherlands), while the anionic polyelectrolytes were SNFH 149, SNFH 57 (SNF S.A., France), Magnafloc 351 and Magnafloc 1011 (CIBA). The applied polyelectrolytes were copolymers with polyacrylamide; they were used as dilute (0.01–0.1%) aqueous solutions. These polymers are harmless and decompose quickly in nature.

The method of analysis for dilute Bti suspensions

Flocculation experiments were performed under dynamic conditions in a flowing cell, with a PDA-2000 dispersion analyser (Rank Brothers, UK) that permits determination of the size of forming aggregates. 2–5 ml of polyelectrolyte solution was quickly added to 50 ml of the suspension with a given concentration during stirring in a beaker with a magnetic stirrer (50 rpm), and the signal characterizing the degree of flocculation was immediately recorded. For this purpose, the beaker was connected by a transparent flexible tube (inner diameter 2 mm) to the measuring part of the unit. A small (about 1 mm^3) volume of a suspension flowing through the transparent tube is illuminated by a narrow light beam directed to the flow from a luminous diode light source, with the aid of a wave guide. The magnitude of the light flux passing through the suspension is continuously registered by the sensitive photodetector, which is capable of converting a light signal to an electrical one. The light signal consists of a large permanent flux *(dc)* characterizing the average intensity of the transmitted light, and hence the suspension turbidity, and a fluctuation flux *(ac)* resulting from chance variations in the number of particles in the volume of the illuminated zone. It has been shown [4] that the root-mean-square of the fluctuating signal *(rms)* is a characteristic of the number-averaged concentration and size of the suspended particles (cells). The values of *rms* are significantly increased at the onset of aggregation, and decreased at the end of the aggregation. The output signals register the values of moderate intensities of the transmitted light, the root-mean-square fluctuations of the transmittance, and their ratio ($R = rms/dc$). From this point on, the flocculation degree is characterized by the value of *R*. The most sensitive and reliable interval of *R* measurements is between 1 and 2.5.

Rate of sedimentation

The Bti content of the T_{20Bti} suspension in sedimentation experiments proved to be 1.4×10^7 n/ml. As flocculants, 0.1% aqueous solutions of the polymers SNFH 528, SNFH 57 and SNFH 149 were used. We studied the sedimentation rate of 25 ml of Bti suspension (containing 1×10^7 cells/ml Bti cells) mixed with increasing volumes (0, 0.5, 1, 2, 4, 6 and 10 ml) of 0.1% polymer solution in a total volume made up to 35 ml with water. As control, we examined the spontaneous sedimentation in a mixture of 25 ml of Bti suspension and 10 ml of distilled water. During the experiment, we measured the optical density (OD) of the supernatant of the mixture 1, 2 and 5 days after addition of the polymer solution. Additionally, the numbers of living Bti cells were determined in the supernatants of the 5-day samples by the method of diluted plaiting [13]. During the OD measurements, distilled water was applied as blank.

Toxicity test of mosquito larvae

The toxicity tests on the mosquito larvae were performed by determining the inhibition of mobility [21]. We determined the minimum Bti concentration necessary to kill practically all mosquito larvae (24 h-EC100i), and also the initial efficient preventing concentration (24 h-EC50i), i.e. the concentration sufficient to kill 50% of the larvae. The breeding water served as medium for the larvae. For each Bti concentration, we used 25–45 individuals of 3rd instant larvae of *Culex pipiens* L. [8, 17] in 100 ml of local breeding water, with and without the addition of flocculant; the total volume of the system was 250 ml. To water containing the larvae, we added increasing amounts of the Bti product. The tests were performed at 16/8 photoperiods at room temperature. A space without toxic vapour was ensured. After a 24 hour exposure, the number of killed individuals was counted and expressed as a percentage of the initial number of larvae. The retardation of the mobility of the larvae corresponding to a given concentration of the flocculant was likewise determined.

RESULTS

Kinetics of flocculation

In sheared dispersions, the particles/cells probably undergo several collisions before they acquire the amount of polymer necessary for aggregation. This might be the cause of an initial lag in the flocculation time dependences [4] observed for silica and clay mineral particles containing cationic polyelectrolytes. The lag in our case was very short: after a contact time of 0.5–1 min, we observed a fast increase in the degree of flocculation, R. This means that, during the relatively fast polymer adsorption in a stirred suspension, the collision efficiency increases considerably, boosting the flocculation rate. Subsequently, the flocculation rate becomes constant, and after a contact time of 7–8 min decreases in collision efficiency and flocculation rate are observed. The latter can be explained by the polymer adsorption continuing, leading to a high surface coverage and the start of re-stabilization. Accordingly, we measured the efficiency of flocculation within 2–3 min of the polymer solution coming into contact with the cells (Fig. 1).

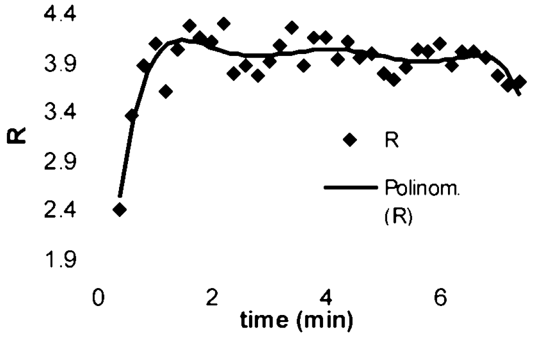

Fig. 1. Kinetics of flocculation of Bti cell suspension (7.5×10^5 cells/ml) by cationic polymer SNFH 528 (0.01%). R denotes the flocculation degree

Nature of the flocculant

We studied the effects of different anionic and cationic polyelectrolytes on the degree of aggregation of Bti cells in a flow system, using a PDA-2000 dispersion analyser. According to the literature data [1, 2], the most efficient reagents for the flocculation of cellular suspensions are cationic polyelectrolytes, and the main objects of our study were therefore positively charged polymers (Fig. 2). It is seen that, with increasing amount of polymers added to the dispersion, the degree of aggregation first increases,

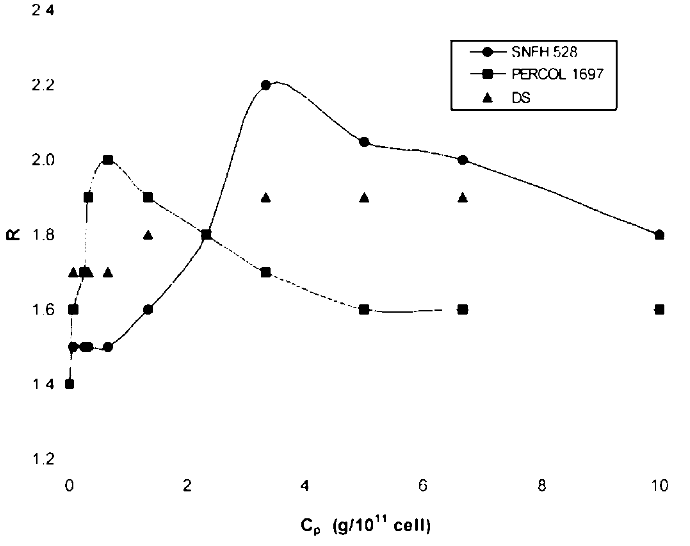

Fig. 2. Dependence of the flocculation degree (R) of bacterial cells on the content of 0.01% solutions of cationic polyelectrolytes in a Bti suspension of 3×10^5 cells/ml (dilution ×50). C_p denotes the surface coverage of the Bti cells by the macromolecules

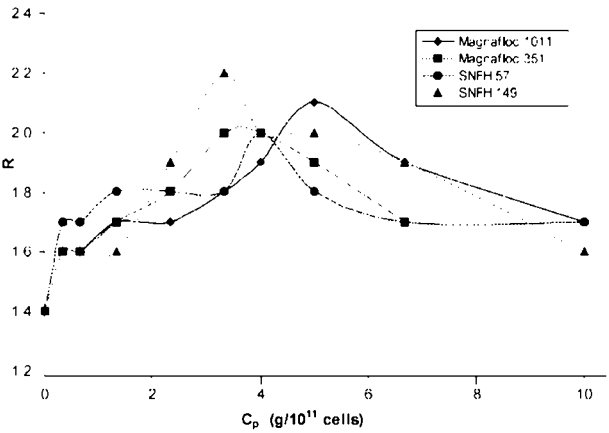

Fig. 3. Dependence of the flocculation degree (R) of bacterial cells on the content of 0.01% solutions of anionic polyelectrolytes in a Bti suspension of 3×10^5 cells/ml (dilution 50×). C_p denotes the surface coverage of the Bti cells by the macromolecules

and then decreases, i.e. classical behaviour is observed: at low contents, high-molecular substances cause the destabilization of dispersions, whereas at high concentrations they act as stabilizers of suspensions. The optimum dosage is several g/10^{11} cells; this value is in line with the flocculation concentration of cationic polyelectrolytes for *E. coli* suspensions [1, 2]. The high affinity of strongly charged Percol 1697 (with a charge density of 14.9 milli-equiv./g polymer [6]) for the cell surface is probably the cause of the increased flocculation activity of this sample, which

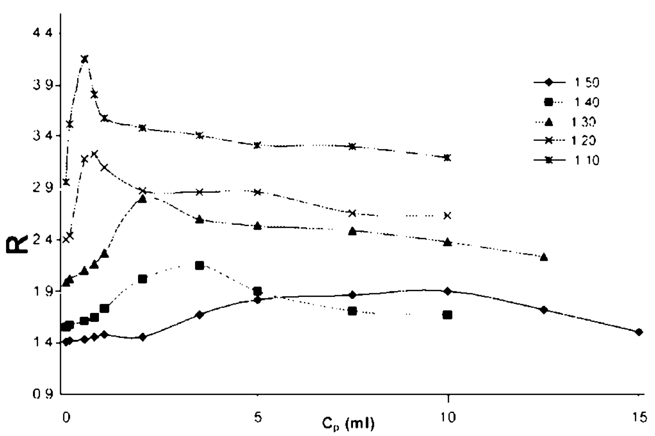

Fig. 4. Flocculation degree (R) of Bti cells vs. cationic polyelectrolyte SNFH 528 dose for suspensions of different concentrations. The concentration of the polymer solution was 0.01%. C_p denotes the surface coverage of the Bti cells by the macromolecules

amounts to 1–2 g/10^{11} cells. At the same time, the zone of destabilization in the presence of this polymer is rather narrow. A more efficient flocculant is the cationic SNFH 528: its addition results in a higher degree of aggregation and a wider zone of destabilization of the suspension. The flocculating activity of the DS sample is the poorest. The effect of a cationic polyelectrolyte on the stability of a microorganism suspension is due to the adsorption of positively charged segments of the flocculant on the cell surface, which has a mosaic structure of negative and positive charges.

It is interesting that anionic polyelectrolytes also efficiently flocculate Bti cells, as may be seen from the data in Fig. 3. The experiments were conducted in the same way as described for the cationic polyelectrolytes. Of the four samples tested (SNFH 149, SNFH 57, Magnafloc 351 and Magnafloc 1011), the SNFH 149 possesses the best flocculation activity. It may be presumed that anionic segments of the polymer are attached to positive functional groups of the cell surface, resulting in the formation of extended adsorbed layers of weakly deformed macromolecules that are able to bond several cells via polymeric bridges.

Let us now consider the main laws of the flocculation of suspensions by polymers.

Effect of the suspension concentration

Figure 4 illustrates that, for the optimum aggregation of more concentrated suspensions, a lower amount of polymer is needed, and the addition of this amount results in a higher degree of flocculation (higher RMS values). With decreasing cell numbers in the system, the degree of flocculation decreases, but the destabilization zone is extended. Hence, the higher the cells content, the lower the polymer concentration that causes efficient aggregation. Similar observations have been reported for the flocculation of polystyrene lattices and other inorganic dispersions with anionic and cationic polyelectrolytes [4, 11]. Below, we present the polyelectrolyte concentrations relating to the same number of cells (10^{11}) that are necessary for maximum flocculation to be attained. On increase of the cell number from 3×10^5 cells/ml to

Table 1
Optimum polymer dosage consumed to reach maximum flocculation for suspensions of different concentrations

Dilution	No. of cells (cells/ml)	No. of cells (cells/50 ml)	Optimum polymer dosage Cationic SNFH 528 (g/10^{11} cells)	Optimum polymer dosage Anionic SNFH 149 (g/10^{11} cells)
100×	1.5×10^5	0.75×10^7	*	2.7
50×	3×10^5	1.5×10^7	3.3	1.3
40×	3.75×10^5	1.87×10^7	1.9	*
30×	5×10^5	2.5×10^7	0.8	*
20×	7.5×10^5	3.75×10^7	0.2	1.3
10×	15×10^5	7.5×10^7	0.07	0.5

* Not measuring.

1.5×10^6 cells/ml, the optimum cationic polymer amount decreases considerably, from 3.3 g/10^{11} to 0.07 g/10^{11} (Table 1).

Table 1 shows the optimum polymer dosage consumed for maximum flocculation to be reached with suspensions of different concentrations. On decrease of the cell number, the degree of flocculation decreased, but the zone of destabilization was extended. This is probably due to the role of the kinetic factor in flocculation: with increasing cell number, the frequency of collisions increases, and there is less time for the macromolecules to spread out on the surface after their contact with it and to adopt the equilibrium conformation. This means that, in more concentrated suspensions, the (more efficient) flocculation is caused by less deformed macromolecules of larger size relative to dilute suspensions, where the polymer molecules have more time to change their conformation on the surface, and to create more contacts with it, with the resulting formation of less extensive adsorbed layers having a weaker flocculating capacity. Similar behaviour was observed with the other anionic polyelectrolytes, e.g. SNFH 149 and SNFH 57.

Effect of the mode of adding the polymer

It is known [1, 6] that the flocculation activity of polymers can be enhanced if the polymer solution is added to the suspension in two portions; such behaviour has been observed with inorganic dispersions. In the present work too, the addition of the same amount of polymer to the Bti suspension in two equal portions caused a much higher degree of aggregation (Fig. 5, upper curve) than the adding the whole amount of flocculant in one step (Fig. 5, lower curve).

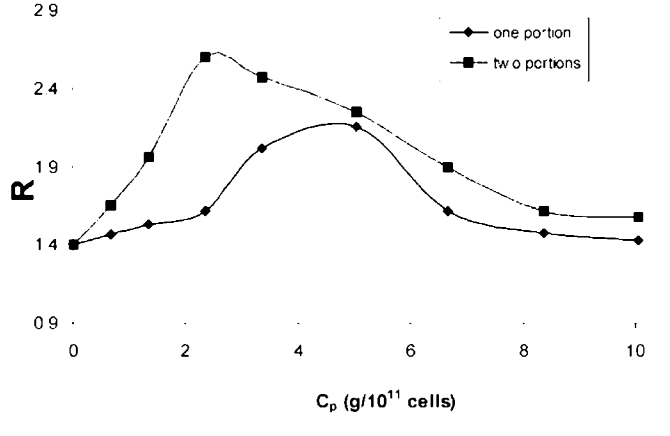

Fig. 5. Effect of mode of addition of the 0.01% cationic polymer SNFH 528 solution to a Bti cell suspension on the degree of flocculation (R). The Bti content of the T_{20Bti} suspension proved to be 3×10^5 cells/ml (dilution 50×). Cp denotes the surface coverage of the Bti cells by the macromolecules. *Lower curve:* Addition of the whole polymer dosage in one portion. *Upper curve:* Addition of the same polymer dose in two equal portions, within an interval of 5 min

In the first case, not only does the degree of flocculation increase, but a lower amount of polymer is consumed to reach this effect. This can be explained by the fact that flocs formed during the addition of the first portion of the polymer undergo further aggregation with the second portion of the added reagent, i.e. the flocculation occurs in two steps. In the first step, only small aggregates are formed, which then combine into much larger, quickly sedimenting secondary flocs.

Effect of the polymer solution concentration

In many cases, the polymer solution concentration also affects the degree of aggregation [6]. We studied this problem via the flocculation of the cellular suspensions with 0.1% and 0.01% solutions of the cationic polyelectrolyte SNFH 528. The more concentrated polymer solution caused better flocculation than that with the less concentrated one for 50 times and 20 times diluted suspensions (Fig. 6).

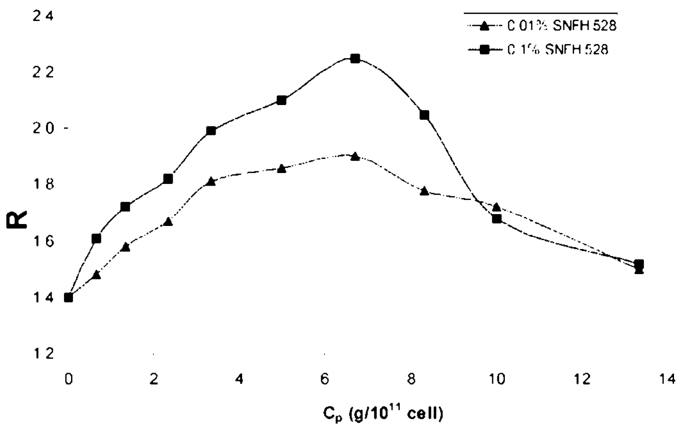

Fig. 6. Effect of the cationic polyelectrolyte SNFH 528 concentration on the efficiency of flocculation of a Bti cell suspension. The Bti content of the T_{20Bti} suspension proved to be 3×10^5 cells/ml (dilution 50×). C_p denotes the surface coverage of the Bti cells by the macromolecules. R denotes the flocculation degree

Especially strong effects were observed for suspensions with higher contents of cells. The reason for this behaviour is not yet clear. It may be assumed that, in concentrated polymer solutions, higher-order associates, i.e. supermolecular structures, are formed, the adsorption of which on the cell surface determines the degree of aggregation. It may also be conceived that, in (more) concentrated solutions, the polyelectrolyte molecules are coiled to a greater extent (due to the screening of charges) and their unfolding on the surface takes more time, so that access to their charge prior to interaction with the cell surface charges is hampered, and therefore

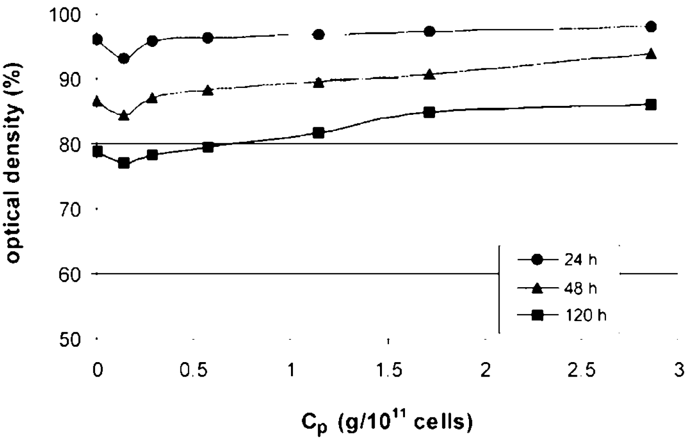

Fig. 7. The relationship between the rate of sedimentation of Bti cells (OD%) and the volume of added anionic polymer SNFH57; 24, 48 and 120 h after addition of the reagent. The initial number of Bti cells in the test tube was 1×10^7 cells/ml. C_p denotes the surface coverage of the Bti cells by the macromolecules

the (more efficient) flocculation in this case is achieved by less deformed adsorbed macromolecules.

Rate of sedimentation of flocculated Bti suspension

Addition of the anionic polymers SNFH57 and SNFH149 in amounts up to 0.143 $g/10^{11}$ cells increased the sedimentation velocity of the cells slightly, but higher polymer doses inhibited the sedimentation of the suspension (Fig. 7).

Addition of a cationic polymer to a Bti suspension enhanced the rate of sedimentation as compared with anionic polymers (Fig. 8). The differences between the rates of sedimentation of Bti cells treated with anionic or cationic polyelectrolytes can be explained in that much larger and more quickly sedimenting aggregates are formed in the latter systems. Five days after the addition of the cationic polymer, the optical density of the suspension was lower than 2.2% of the initial value. It should be stressed that the sedimented cells remain living cells that can be used for the formulation of anti-mosquito agents (Fig. 9).

The aggregated Bti spores were formed by anionic and cationic polymers too, as observed with a dispersion analyser. The sedimentation rates of the Bti aggregates were studied. It was found that too little polyelectrolyte made the suspension more sensitive. Too much polyelectrolyte covered the bacterium cells so that there was no contact between the cells. In the flocculation zone, the appropriate volume of polymer caused aggregation. We experienced that the cationic polymers accelerated, while the anionic polymers decelerated the sedimentation of suspensions.

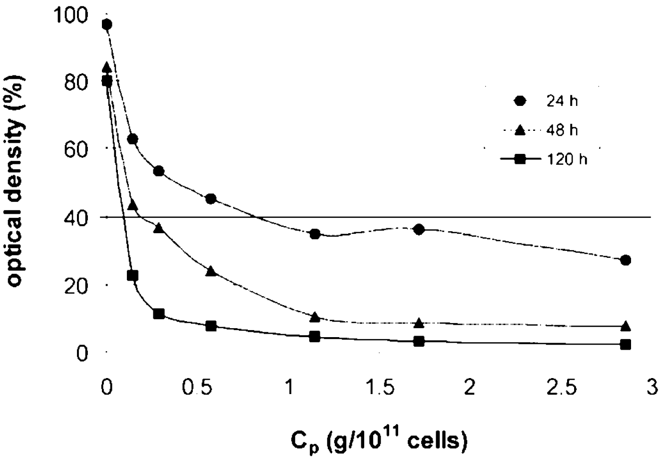

Fig. 8. The relationship between the rate of sedimentation of Bti cells (OD%) and the volume of added cationic polymer SNFH 528; 24, 48 and 120 h after addition of the reagent. The initial number of Bti cells in the test tube was 1×10^7 cells/ml. C_p denotes the surface coverage of the Bti cells by the macromolecules

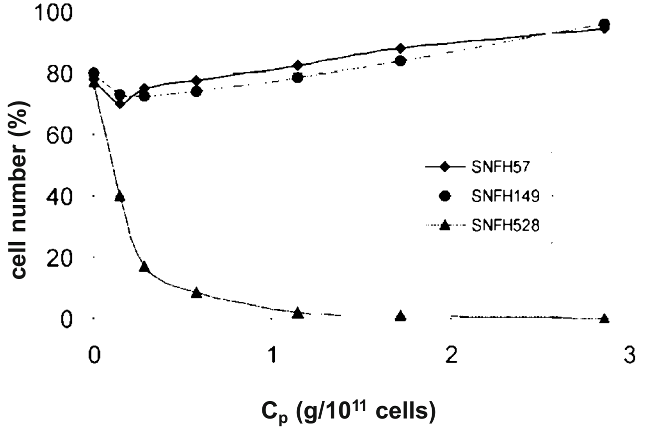

Fig. 9. The relationship between the living cell number (%) in Bti suspensions and the volume of added polymers, 5 days after addition of the reagent. C_p denotes the surface coverage of the Bti cells by the macromolecules

Toxicity of the examined Bti products

Our experiments revealed that 100% toxicity of the Bti cells was reached in a solution of 2.5–3 × 10^3 cells/ml (Fig. 10). Flocculation did not change the toxicity of the cells substantially. In the concentration interval studied, the toxicity values were similar to those of non-flocculated commercial VECTOBAC products. We applied the latter as control system in our experiments. We also observed similar toxicity values for non-flocculated Bti cells incubated in T_{20} nutrient solution. All these bacteria are easily accessible for mosquito larvae.

The toxicities of the Bti products treated with polymers proved slightly lower than those of the free-cell products. Polymer-containing Bti are bound via polymer bridges, which explains why the larvae can consume larger numbers of cells in unit time than the number necessary for the lethal effect. The differences between the rates required to reach a certain degree of toxicity of Bti cells flocculated with cationic and anionic polymers were minimal. Nonetheless, the Bti products flocculated with an anionic macromolecule proved more toxic and lethal. We assume that the binding between the negative surface of the Bti cells and anionic polymers is weaker than that for Bti cells flocculated with cationic polymers.

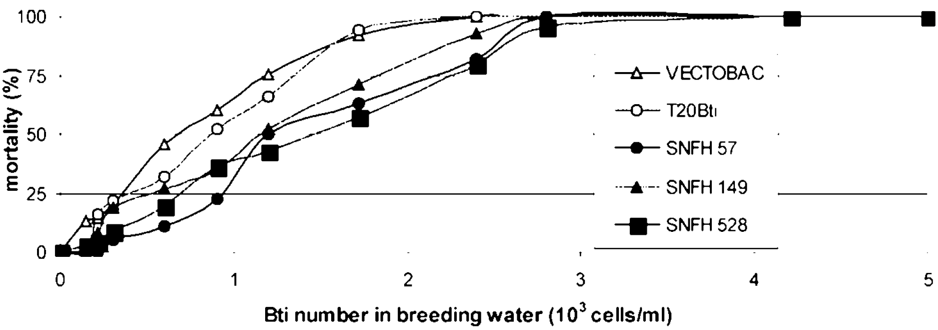

Fig. 10. Comparison of the toxicity for Bti cells in insecticide products. We examined the Bti nutrient solution with polymers, the Bti cells in the fermented T_{20} nutrient solution and the commercial product Vectobac. The mortality rate of the mosquito larvae vs. the Bti content in the breeding water is shown

DISCUSSION

By measurements of the intensity of a scattered laser beam in a flowing system, it was shown that dilute suspensions of Bti (fermented by a microbiological method) can be effectively flocculated and concentrated by using different cationic (SNFH 528, Percol 1697 and DS) or anionic (SNFH 149, SNFH 57, Magnafloc 351 and Magnafloc 1011) polyelectrolytes necessary for its formulation and use as an anti-mosquito agent [1, 2, 8, 9]. The maximum and constant (within 7–8 min) degree of aggregation was reached within 1–2 min after the added macromolecules came into contact with the cells, and the optimum flocculant dosage necessary to reach the maximum degree of flocculation decreases substantially on increase of the suspension concentration; it ranged between several tenths of a mg of polymer/10^6 cells to several mg of polymer/10^6 cells [5, 6]. This feature is explained by the influence of kinetic factors and the adsorbed polymer layer structure on the efficiency of flocculation [10, 11]. Further, addition of the optimum polymer dosage in two equal portions enhanced the flocculation activity of the flocculant as compared with one-step addition, and the more concentrated (0.1%) polyelectrolyte solution exhibited a higher flocculation efficiency than that of the more dilute one (0.01%). The flocculation of cellular suspensions by polymers is a complicated process that can be explained by the adsorption of negatively or positively charged macro-ions on the cell surface with a mosaic structure, having both positive and negative functional groups, taking into account and the kinetics of polymer adsorption and kinetics of aggregation of cells on which polymers are adsorbed as well as the possible charge neutralization and polymer-bridging mechanisms. Cationic polymers were observed to accelerate, and anionic polymers to decelerate the sedimentation of a non-diluted suspension. The degree of toxicity of the flocculated Bti suspensions for biting mosquito larvae was in the same range as that of the non-flocculated suspension, and was comparable with the effect of commercial Vectobac. Flocculation is an effective method for the industrial production of domestically fermented Bti preparations.

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