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# NORTH DAKOTA RESEARCH REPORT

# Application of Coatings to Vegetative Cells of Bacillus popilliae With the Wurster Air Suspension Coating Process

K.J. McMahon-and L.J. Parmer

McMahon is professor and chairman and Parmer is a laboratory assistant, Department of Bacteriology.



AGRICULTURAL EXPERIMENT STATION NORTH DAKOTA STATE UNIVERSITY FARGO, NORTH DAKOTA 58105 The use of microbial pathogens is one of several possible alternatives to chemical agents for pest control. Although the use of pathogens is still relatively limited in comparison with that of chemical pesticides, this research is growing in intensity (3). One pathogen was registered for use about 1950, another in 1960, five more between 1970 and 1979 and a number of others are under investigation (3).

Bacillus popilliae, the cause of milky disease in the Scarabaeidae, was the first insect pathogen to be registered as a microbial control agent. Although *B. popilliae* has been used mainly for control of the Japanese beetle, studies have shown that milky disease bacteria may have promise for suppression of several different larvae. Dutky (11) reported that second instar larvae of *Phyllophaga anxia*, *P. fusca* and *P. rugosa* were susceptible to milky disease, and Jarvis (15) found *P. anxia* susceptible to the spores of *B. popilliae* in a commercial product when the product was mixed with soil.

Sixty-seven species of the genus *Phyllophaga* have been reported in the North Central region of the U.S., and 13 of these have been reported as pests of sod, corn and other agricultural crops (19). The pests *P. anxia, P. fusca, P. rugosa* and *P. tristis* have been reported in North Dakota (19). The larvae, known as white grubs, damage lawns, pastures and row crops, particularly corn in southeastern North Dakota (D. McBride, personal communication).

For a microbial insecticide to be commercially economical, it must be stabilized to preserve viability and virulence. Stability for at least 18 months under ambient storage conditions has been suggested as being desirable (7). If a product is to be applied at a specific time, stability for three to six months may be acceptable. The short shelf-life of some pathogens may prevent their use in the field.

Spores of *B. popilliae* produced in vivo possess stability and virulence, and production and distribution of these spores has resulted in one of the classic examples of microbial control. These spores, however, are produced in living larvae (14), and preparations have been expensive and in short supply (17). Spores produced in vitro have poor infectivity and survival (4). Attempts to develop a method for economical production of infective spores in vitro have had only limited success, and in view of the cessation of the extensive USDA research effort on the subject (2), it appears that in vitro production of virulent spores cannot be expected soon.

Large numbers of vegetative cells of *B. popilliae* can be produced in vitro. Our objective has been to stabilize these cells so they will remain viable and virulent. We have reported that lyophilized, vegetative cells suspended in pellets of tung oil polymer coated with paraffin wax remained viable under field conditions for at least one week (6), and when the pellets were coated with a mixture of paraffin wax and rubber cement, cells remained viable for at least one month (13). Klein (personal communication) found that vegetative cells extracted from pellets gave up to 93 percent milky larvae when injected. Larvae readily fed on pellets placed in soil, but results from feeding were inconclusive.

To prepare the tung oil polymer, tri-n-octylaluminum was used to polymerize raw tung oil. It was handled under nitrogen and injected beneath the surface of the oil. Because this kind of coating could not be easily applied with an available commercial process, the purpose of the present study was to determine if vegetative cells would remain viable after encapsulation with a hydrogenated, vegetable oil or acetylated glyceride that could be applied with a process that has been used to coat pharmaceuticals, agricultural chemicals and food ingredients. Portions of this work were previously presented (L.J. Parmer, S.K. Priebe and K.J. McMahon, Proc. N.D. Acad. Sci., 1984, 38:80).

### MATERIALS AND METHODS

**Organism and medium.** *B. popilliae* NRRL B-2309 was the organism used. The medium contained 1.5 percent tryptone (Difco), 0.5 percent yeast extract (Difco), 0.6 percent  $K_2HPO_4$  and 0.2 percent glucose (sterilized separately). The pH of the medium was 7.0 to 7.3. For plate counts, the medium was solidified with 1.5 percent agar (Difco).

The culture was maintained by daily transfer of a 5 percent inoculum of broth culture. Flask cultures were incubated at 25 to 30°C on a rotary shaker operating at 200 rev/min.

Lyophilization. For a typical lyophilization run, a total of 7 liters of medium was inoculated. Cells were harvested by centrifugation after 12 hours of growth and resuspended in a solution of 5.0 percent monosodium glutamate plus 0.5 percent gum tragacanth. Some samples (4.0 ml) of the suspended cells were placed in 10-ml screw cap vials fitted with split rubber stoppers (Virtis Co., Gardiner, N.Y.). Other samples (20 ml) were placed in 500-ml flasks. Cells were frozen by rotating the vials or flasks in acetone maintained at -70 to -80°C. Vials or flasks were attached to a New Brunswick freeze dryer (model no. B-67). After a four to five hour drying period, vials were sealed under vacuum by forcing rubber stoppers into place, removed from the dryer, sealed with plastic sealing bands and stored at 4°C. With flasks, the vacuum was released, dry flakes were pulverized and the dried cells were placed in a desiccator wih CaSO₄ at 4°C.

It was necessary to prepare approximately 1 pint (0.47 liter) of dried cells each time coating was to be applied.

Viable counts. Prior to encapsulation, samples (0.1 g) were diluted in 0.1 percent tryptone, and 0.1-ml volumes were spread on the surfaces of five plates of standard medium for each dilution chosen. Dilutions were prepared in triplicate. Plates were incubated for five days at 25 to 30°C, and counts were recorded as averages of the replicate counts.

After encapsulation, duplicate samples (0.1 g) were each added to 9.9 ml of 0.1 percent tryptone in a Sorvall Omni-Mixer (Dupont Co., Newton, Conn.) and ground at speed setting five for one minute or added to the tryptone in a mortar and ground with a pestle. The latter method gave higher counts. After grinding, samples were plated as described for uncoated cells.

**Encapsulation of lyophilized cells.** Cells were encapsulated by H. Hall, Coating Place Inc., Verona, Wis. using the Wurster Air Suspension Coating Process (12). This process applies and dries encapsulating materials onto particles supported by an upward moving airstream resulting in facilitated contact between the particles being coated and the drying air. The movement of particles within the coating chamber is controlled by the size and distribution of perforations in a plate which produce a cyclic flow pattern into which the coating material is atomized. The moving particles cycle past a nozzle every four to six seconds, receiving an increment of coating on each pass. The particles exhibit uniform build-up of coating as the run progresses.

One coating used was Kaorich (Durkee Industrial Foods, Cleveland, Ohio). Kaorich is a hydrogenated, vegetable oil that is used in the food industry to add solids to shortening systems to improve physical stability and product life. Molten Koarich at a temperature of 51 to 54°C was sprayed into the processing chamber. Upon contact with the cells the temperature dropped to approximately 21°C, and processing continued at that temperature until the ratio of Kaorich to cells was 70:30 (wt/wt).

Kaorich-coated cells stored five months at 0 percent relative humidity (RH) and 4°C were overcoated with a mixture of Myvacet-940 (Eastman Chemical Products, Inc., Kingsport, Tenn.), an acetylated glyceride, and ethyl cellulose latex. With water as a carrier, this overcoat was sprayed into the processing chamber at ambient temperature, and the processing temperature was 22 to 23°C. The final ratio of the overcoat to Kaorich-coated cells was 20:80 (wt/wt).

Cells from a second lyophilized preparation were coated with a 90:10 mixture of KLX (similar to Kaorich) and Myvacet-945 (similar to 940) until the ratio of coating to cells was 30:70 (wt/wt). Then KLX alone was continued until the final ratio of total coating to cells was 71:29 (wt/wt). Cells were coated using a hot melt system, and processing was done at 13 to 19°C. The coated material was separated according to particle size into three preparations. The largest particles were approximately 3 mm in diameter.

Storage of encapsulated cells. After coating, cells were placed in glass or plastic jars and stored in desiccators with CaSO<sub>4</sub> at 4°C. To determine survival at ambient temperature and different levels of RH, cells were placed in Petri dishes and stored in desiccators. Saturated salt solutions were used to obtain RH levels. Salts used were as follows: MgCl<sub>2</sub>, 33 percent; K<sub>2</sub>CO<sub>3</sub>, 42 percent and Ca(NO<sub>3</sub>)<sub>2</sub>, 50 percent. For storage in soil, 20 g of Kaorich-coated cells was added to 50 g of autoclaved soil.

## RESULTS

Encapsulation of vegetative cells of *B. popilliae* with a hydrogenated, vegetable oil or a mixture of the oil and an acetylated glyceride using the Wurster Air Suspension Coating Process resulted in a product that was dry and granular and contained viable cells. Within one week after encapsulation, Kaorich-coated cells were plated, and the viable cell count was  $2.0 \times 10^{7}$ /g of material (70 percent coating and 30 percent cells). The count before coating was  $5.3 \times 10^{19}$ /g of material (100 percent cells). A similar drop in viable cell count occurred when cells were encapsulated with a mixture of KLX and Myvacet-945. A further drop in viable cells were overcoated with a mixture of Myvacet-940 and ethyl cellulose latex.

Kaorich-coated cells were still viable after one year of storage at 0 percent RH. The number of viable cells decreased 2 logs during the first two months of storage and then stabilized. The data for coated cells stored at 4°C and ambient temperature are shown in Fig. 1. Survival was similar for cells stored at the two temperatures. Viability was maintained during a year of storage in dry soil at 4°C. Immediately after addition of coated cells to soil, the viable count was 1.0 x  $10^{\circ}/g$  of soil. After one year of storage, the count was  $1.0 \times 10^{\circ}/g$  of soil (Fig. 2).

Survival of Kaorich-coated cells stored at 11 and 22 percent RH and ambient temperature was similar to that of cells stored at 0 percent RH (data not shown). Cells survived at least three months when held at 33 percent RH and ambient temperature. The viable count after three months of storage was 4.5 x 10<sup>3</sup>/g of material. It was not possible to determine if viable cells could be recovered beyond this time because the quantity of the preparation that was still available was insufficient for further testing.

Viability decreased rapidly when Kaorich-coated cells were stored at 42 percent or higher RH. Cells were recovered after storage for one month at 42 per-





Figure 1. Effect of temperature on survival of Kaorichcoated vegetative cells of *B. popilliae* stored at 0% RH.

cent RH and ambient temperature and after three weeks at 50 percent RH (Fig. 3). Some cells survived storage in water for 48 hours at 4°C.

Viability was affected very little by the overcoating of Kaorich-encapsulated cells with a mixture of Myvacet-940 and ethyl cellulose latex. Survival in the presence of moisture was not increased.

Preliminary survival studies on cells encapsulated with the mixture of KLX and Myvacet-945 indicate that this coating provides about the same protection from moisture as that provided by Kaorich. Data collected after two months of storage are similar for coated particles of different sizes up to 3 mm in diameter.

# DISCUSSION

Although the Wurster Air Suspension Coating Process has been used for encapsulation of pharmaceuticals, agricultural chemicals and food ingredients, to our knowledge, this is the first report of its use for encapsulation of bacteria.

Figure 2. Survival of Kaorich-coated vegetative cells of *B.* popilliae stored in sterile soil at 0% RH and 4°C.



Figure 3. Effect of RH on survival of Kaorich-coated vegetative cells of *B. popilliae* stored at ambient temperature.

A reduction in viability occurred during processing, but 2.0 x 10<sup>7</sup> viable cells per g of material (only 30 percent cells by weight) were recovered. Although the number of viable cells decreased 2 logs during storage at 0 percent RH, a sufficient number of viable cells might still be available in 0.1 g or less of the material to initiate infection. According to St. Julian et al. (20), the injection of 300 to 1000 viable, vegetative cells of *B. popilliae* into the hemolymph of larvae causes 50 to 80 percent of the larvae to become grossly infected.

To be effective in the field, a product must cause infection in larvae after feeding. Many years ago, Beard (1) suggested that larvae could become milky after feeding on vegetative cells, but adequate proof for infection by this route is still lacking (17). Vegetative cells have been shown penetrating the gut wall and initiating infection in the European chafer, *Amphimallon majalis* (16, 21).

Lyophilized, vegetative cells to be encapsulated were infective when injected into beetle larvae, but it has not been determined if they were still virulent after encapsulation. Klein (17) has suggested that it may be necessary to have additional components such as spore or parasporal material with vegetative cells to aid infection.

Results of the present study indicate that the Wurster Process may be used for encapsulation of bacteria, but the coatings used did not provide a product with an adequate shelf-life when moisture was present. We plan to try other coating materials. If one can be found that preserves viability of vegetative cells of *B. popilliae*, it may be useful for extending the shelf-life of other potential pest pathogens.

#### SUMMARY

Lyophilized, vegetative cells of *B. popilliae* were encapsulated with a hydrogenated, vegetable oil or a mixture of an acetylated glyceride and ethyl cellulose latex. Coatings were applied with the Wurster Air Suspension Coating Process. The product was dry and granular and contained viable cells. Viability was maintained for at least one year when the product was stored at 0 percent RH. Viability decreased rapidly when encapsulated cells were stored at 42 percent or higher RH.

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