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Production of Bacterial Inoculants by Direct Fermentation on Nutrient-Supplemented Vermiculite

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When supplemented with a nutrient source and moisture, sterile finely ground vermiculite can be used to directly ferment bacterial cultures to prepare bacterial inoculants. All tested bacterial species, including *Rhizobium japonicum*, *R. phaseoli*, *R. meliloti*, *R. leguminosarum*, *Bacillus megaterium*, and several *Pseudomonas* strains, grew at least 10,000-fold in 1 week at room temperature. The final product was stable and had no special storage or handling requirements. Due to the unique properties of vermiculite, direct fermentation of bacteria on nutrient-supplemented vermiculite offers a reliable process for manufacturing bacterial inoculants.

The most established use of bacterial inoculants is the practice of inoculating legumes with cultures of *Rhizobium* spp. Of the many inoculant forms available, the most popular formulation is moist, finely powdered peat which is coated on the seed. The peat inoculant is not difficult to produce, usually maintains a high concentration of viable bacteria, and is easy to apply. The conventional means to produce the inoculant involves inoculating neutralized, nonsterile peat with a bacterial suspension of 10^7 cells per g of peat, which reaches a final population density of approximately 10^8 to 10^9 bacteria per g of product (28). This requires large fermentors and curing facilities. Somasegaran and Halliday (31) showed that peat inoculants with high numbers of viable *Rhizobium* spp. also can be produced by inoculating with 10^4 cells per g of sterile peat. The *Rhizobium* spp. subsequently multiplied in the peat to a peak population density of 10^8 to 10^9 bacteria per g without serious competition from contaminants. Others have also documented the improved inoculant quality when the carrier materials were presterilized or heated before the addition of the bacteria (34–37).

If the *Rhizobium* strains are competitive and effective, the peat carrier ultimately controls the quality of the final product (3). Different batches of peat and peat from various sources differ greatly in composition, structure, pH, and microbial populations. Some peat has been known to contain inhibitors to *Rhizobium* strains (3). Since peat is organic, complete sterilization by steam or by gamma irradiation is difficult (22) and undesirable because high temperatures and high dosage of irradiation cause the peat to produce toxic by-products (20, 22) and to undergo structural and compositional changes which are unfavorable for subsequent growth and survival of *Rhizobium* spp. (37). Because of these limitations, and since many parts of the world have no natural deposits or reliable supplies of peat, many alternate carriers, including vermiculite, coal (charcoal) dust, filtered mud, mineral soil, compost, bentonite, lucerne powder, sugar cane bagasse, wheat and rice straw, corn cob, pulverized lignite, and kaolinite, have been explored (1, 2, 4–10, 12–16, 18, 21, 23, 24, 29, 32–34, 38, 41, 42, 44). The acceptance of these carriers varies geographically and is greatly influenced by the availability of raw materials and fermentation facilities. This report demonstrates the successful production of reliable bacterial inoculants with the use of vermiculite not only as a carrier, but also as a medium support for the direct fermentation of bacterial cultures.

Vermiculite is a hydrated magnesium aluminum iron silicate exfoliated at extremely high temperatures (700 to 1,000°C). The exfoliation process kills microorganisms. Its inorganic and preexpanded nature allows it to be sterilized easily by the common sterilization processes without the risk of producing toxic by-products or causing further structural changes. It is relatively inexpensive and is widely available (19). The multilamellate structure of vermiculite provides superior aeration and space for microbial proliferation. Our results demonstrate that good-quality inoculants can be produced consistently in vermiculite with many bacterial species without the need of expensive fermentation and incubation facilities. These properties, in conjunction with its anticrusting (11), moisture-holding, and plant growth-promoting (17, 25, 26) abilities, make vermiculite especially attractive for the production of inoculants for agricultural and horticultural uses.

MATERIALS AND METHODS

Bacterial strains. *Rhizobium japonicum* T1344, S258, K567, T363, and T489; *R. phaseoli* 899 (F. Bliss, University of Wisconsin-Madison), USDA 2667, and USDA 2668 (D. Weber, U.S. Department of Agriculture, Beltsville, Md.); *R. meliloti* 102F51; and *R. legminosarum* 128C5 (J. Handelsman, University of Wisconsin-Madison) were cultured in a yeast extract-mannitol broth (43). *Pseudomonas putida* A12

TABLE 1. Production of *R. japonicum* inoculants by direct fermentation on vermiculite, using various amounts of nutrient supplement at two different incubation temperatures[a]

Sample no.	Temp (°C)	ml of nutrient/ g of vermiculite	No. of bacteria/ ml after 7 days, 10^8 (mean ± SD)
1	23	1.0	1.6 ± 0.2
2	23	1.5	2.4 ± 1.0
3	23	2.0	3.1 ± 0.4
4	30	1.0	3.7 ± 0.6
5	30	1.5	3.2 ± 0.0
6	30	2.0	3.3 ± 0.4

[a] Initial inoculum was 3.4×10^5 bacteria per g of vermiculite.

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TABLE 2. Stability of *R. japonicum* inoculants prepared by direct fermentation on nutrient-supplemented vermiculite at room temperature[a]

Sample no.	No. of bacteria/g, 10^8	
	After 7 days	After 30 days
1	1.7	13
2	3.1	13
3	3.2	16
4	3.2	10

[a] Initial inoculum was 1.4×10^4 bacteria per g of vermiculite. Nutrient supplement was 1.5 ml/g of vermiculite.

(R. Baker, University of Colorado, Boulder), *Pseudomonas* sp. strains 2-79 (NRRL B-15132) and 13-79 (NRRL B-15134; Northern Regional Research Laboratory, Peoria, Ill.), *Bacillus* sp. strain BLA6A-2, and *Bacillus megaterium* BLA6A-4 were cultured in nutrient broth (Difco Laboratories, Detroit, Mich.).

Inoculant production. Exfoliated vermiculite (Terra-Lite; W. R. Grace and Co., New York, N.Y.) was ground in a Wiley mill. Vermiculite particles (45/80 mesh; U.S. Standard Sieves) were autoclaved with 1.5 ml of culture medium per g of dry vermiculite in small containers. Containers used for sterilization and subsequent bacterial growth included disposable polypropylene specimen cups (4-oz [113.4-g] Fisher brand and 16-oz [453.6-g] Nalgene; Fisher Scientific Co., Itasca, Ill.). Alternatively, vermiculite was autoclaved in bulk in polypropylene biohazard bags (Bio-check; American Scientific Products, McGaw Park, Ill.) and dispensed into small sterile polyethylene bags (NASCO Whirl-pak; Fisher Scientific). A total of 10^2 to 10^7 bacteria was added per g of vermiculite. The inoculant was stored at room temperature (22 to 25°C). A 0.1- to 1-g portion of the inoculant was removed from the container periodically for bacterial population and purity determinations by suspending the vermiculite in 10 ml of nutrient broth or sterile distilled water. The suspended samples were serially diluted and plated on nutrient agar plates.

Seed coating. Bacterial inoculants were applied to soybean (Corsoy 79, Williams 82, and Centennial) at a rate of 6 g (wet weight) per pound (1 lb = 453.592 g) of seed. Corn seeds (CFS hybrids 4004, 6007, 5504, and 7801) were coated with 4 g of inoculant per pound of seed with Pelgel (Nitragin, Milwaukee, Wis.) or xanthan gum (Sigma Chemical Co., St. Louis, Mo.) as a sticker. The coated seeds were allowed to dry for several hours, and then 10 seeds were suspended in 10 ml of nutrient broth or sterile distilled water. The samples were serially diluted and then plated to determine the bacterial population.

TABLE 3. Production of various *Rhizobium* inoculants by direct fermentation on nutrient-supplemented vermiculite[a]

Strain	No. of bacteria/g		Days stored
	Initial inoculum	Final product	
R. japonicum			
K567	7.0×10^3	4.2×10^8	7
S258	6.4×10^3	5.8×10^8	7
T1344	1.1×10^4	9.1×10^8	7
R. phaseoli			
899	2.2×10^3	1.7×10^9	3
USDA 2667	2.2×10^2	1.1×10^9	11
USDA 2668	5.2×10^2	4.5×10^9	11
R. meliloti			
102F51	1.7×10^3	1.9×10^9	63
R. leguminosarum			
128C5	5.0×10^2	5.4×10^8	63

[a] Nutrient supplement was 1.5 ml/g of vermiculite. Samples were stored at room temperature.

TABLE 4. Production of miscellaneous bacterial inoculants by direct fermentation on nutrient-supplemented vermiculite[a]

Strain	No. of bacteria/g		Days stored
	Initial inoculum	Final product	
B. megaterium			
BLA6A-4	8.0×10^4	2.5×10^9	12
Bacillus sp.			
BLA6A-2	8.0×10^4	5.1×10^9	12
Pseudomonas sp.			
2-79	2.5×10^3	2.0×10^8	63
13-79	2.4×10^3	2.0×10^8	63
P. putida			
A12	3.6×10^2	6.2×10^8	63

[a] Nutrient supplement was 1.5 ml/g of vermiculite. Samples were stored at room temperature.

RESULTS AND DISCUSSION

Particle sizes in the range of 45 to 80 mesh provide the best moisture-holding capacity and enable the final inoculant product to adhere uniformly to the seed surface. For fermentation of *R. japonicum*, no significant effects are apparent by varying the incubation temperatures from 23 to 30°C or by varying the nutrient/carrier ratio from 1 to 2 (Table 1). At a ratio of 2, the final product is wet, and unless the product is planned for prolonged storage (e.g., more than 6 months), a ratio of 1.5 provides the best moisture content for routine handling. For all tested bacterial strains, incubation at elevated temperature is not necessary since fermentation at room temperature eventually produces final products with population densities of 10^8 to 10^9 bacteria per g (see Tables 2 to 5) after 12 days. Even with the slow-growing *R. japonicum* strains, fermentation at room temperature on vermiculite requires no more than 7 days (Tables 2 and 3) for the bacterial population density to reach 10^8 per g. This is comparable to the growth of these organisms in either shake flasks or aerated carboys at 30°C. For faster growing strains,

TABLE 5. Production consistency of various *R. japonicum* inoculants by direct fermentation on nutrient-supplemented vermiculite[a]

R. japonicum strain	No. of samples tested	No. of bacteria/g at 4 wk, 10^8 (mean ± SD)	No. of samples with $>10^9$ bacteria/g
K567	16	10.0 ± 4.0	5
S258	30	9.8 ± 3.5	16
T1344	9	13.0 ± 4.7	7
T363	9	14.0 ± 1.0	5
T489	9	14.0 ± 3.0	9

[a] Initial inoculum was 10^3 to 10^4 bacteria per g of vermiculite. Samples were stored at room temperature. None of the samples contained $<10^8$ bacteria per g of final product after 4 weeks.

TABLE 6. Comparison of properties of vermiculite and peat related to production of bacterial inoculants

Property	Vermiculite	Peat
pH	Approximately neutral	Usually requires neutralization before use as carrier
Buffering capacity	Good	Little
Toxicity	Inorganic; will not produce organic toxic by-products or undergo structural changes upon sterilization	Organic; known to occasionally contain inhibitor(s) to bacterial strains; may produce toxic substances and undergo compositional and structural changes upon sterilization
Contamination	Exfoliated at extremely high temp which kill microorganisms; its mineral nature does not support microbial growth	Usually contains unknown microbial contaminants able to grow on organic compounds
Physical structure	Multilamellate; provides good aeration, quick temp equilibration and space for microbial growth during fermentation	Not layered; structure may change at high temp or upon exposure to strong gamma irradiation
Seed sticking	Flaky; good sticking properties for seed coating	Powder or granular; often requires sticker to adhere to seeds

such as *R. phaseoli* 899 (Table 3), fermentation on vermiculite at room temperature requires no more than 3 days to achieve a multiplication factor of 10^6.

Bacterial inoculants produced by the procedure described above are very stable and require no special storage. Table 2 demonstrates the stability of *R. japonicum* inoculants stored at room temperature for 1 month. The bacterial population density in the four separate batches remained at $>10^9$ bacteria per g. Provided no significant moisture loss occurs through leakage from the container, the number of viable bacteria in the inoculants is usually between 10^8 and 10^9 per g of final product after storage at room temperature for 1 year.

The direct fermentation procedure and the ability of the final inoculant product to maintain a high population density are not limited to *R. japonicum*. Tables 3, 4, and 5 demonstrate the applicability of the procedure and the consistent results that can be obtained with a wide range of microbes. More than 30 different bacterial inoculants have been produced by direct fermentation on nutrient-supplemented vermiculite, and they maintain at least 10^8 bacteria per g after storage at room temperature for up to 28 days. Over a period of 3 months, we have prepared and stored at room temperature 73 different batches of *Rhizobium* inoculants, using five different strains. The consistent quality of some of these inoculants is demonstrated in Table 5.

The vermiculite-based inoculant has good seed-sticking properties. Without the use of any polymer-based sticker, 10^4 to 10^5 rhizobia per seed can easily be adhered to soybean seeds. The number of viable rhizobia on the seeds does not change significantly for at least 1 day if stored at room temperature. Prolonged storage of the coated seeds, however, decreases the number of viable rhizobia to about 10^3 per seed after 7 days. The decrease may be due to the physical detachment of the inoculant from the seeds or to the death of the microbes through desiccation and toxicity from substances released by the seeds. Adherence of the vermiculite-based inoculant to corn seeds is less efficient. This is due to the waxy surface of the corn kernels. However, with the use of commercial seed stickers (such as xanthan gum and Pelgel), adherence of $>10^4$ bacteria per seed can be obtained routinely. Table 6 compares some of the properties of vermiculite and organic peat which are relevant to the production of bacterial inoculants. Along with the desirable properties it possesses as a potting medium (11, 17, 25, 26, 30, 39, 40), vermiculite should be considered a desirable alternative to peat for the production of bacterial inoculants.

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