Production of *Clostridium bifermentans* Spores as Inoculum for Bioremediation of Nitroaromatic Contaminants†

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Spores of *Clostridium bifermentans* KMR-1 were produced for use as a microbial inoculum for bioremediation and were preserved in both liquid and dry forms. All spore formulations showed good viability and ability to biodegrade the target compound, 2,4,6-trinitrotoluene (TNT), after 4 months of storage. For low-cost bulk spore production, several medium compositions, based on soy peptone, corn steep liquor, and meat peptone, were tested and yielded 10^7 spores per ml. A medium pH above 7.0, a low glucose concentration, and a sufficient concentration of protein favored the sporulation of *C. bifermentans* KMR-1.

Nitroaromatic compounds are widely used as pesticides, explosives, and precursors for dyes, pharmaceuticals, and plastics (12), but their toxicity, mutagenicity, and persistence make them a serious environmental problem. The explosive 2,4,6-trinitrotoluene (TNT), for example, is a common contaminant of soils and groundwater at many military and production sites in Germany and the United States (17, 19). Despite the toxicity of nitroaromatic compounds, many microorganisms are able to transform or degrade them. Such organisms are candidates for use in bioremediation (4, 5, 17, 20). Significant progress has recently been made in studies of aerobic and anaerobic biodegradation of nitroaromatic compounds, making bioremediation with TNT-degrading microorganisms a feasible method for the restoration of sites contaminated with these compounds. Relatively expensive physical (e.g., incineration) or chemical (e.g., solvent extractions) treatments (17, 26) may be replaced in the future by effective and cost-saving bioremediation technologies (31–33).

Previously we developed an ex situ soil bioremediation process (the SABRE [Simplot anaerobic biological remediation ex-situ] process; J. R. Simplot Co., Boise, Idaho) incorporating an anaerobic consortium known to contain clostridia (16), and we used it at the field scale (15, 25, 26, 31) to successfully clean up both dinoseb (2-sec-butyl-4,6-dinitrophenol)- and TNT-contaminated soils (6, 15, 25, 26, 31, 32). This process might be enhanced by the use of clostridial inocula to speed reduction of aromatic nitro groups.

Several clostridial strains were isolated (5a, 24, 28) from an anaerobic digester fed munition compounds including TNT, 1,3,5-triaza-1,3,5-trinitrocyclohexane (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) as its sole carbon and energy source (7) and from an aquifer contaminated with the herbicide dinoseb (10). Spores of such strains are expected to be useful as inoculants for bioremediation of TNT-contaminated soil and water. One isolate is *Clostridium bifermentans* KMR-1, which is able to degrade the munition compounds TNT and RDX (24), as well as dinoseb (11).

In order to produce clostridial spores of KMR-1 for use in bioremediation, knowledge of nutritional requirements for both growth and sporulation of this strain is required. Investi-

gations of the nutritional requirements of *C. bifermentans* strains were carried out by Holland and Cox (14) and Smith and Douglas (29). Holland and Cox (14) studied several *C. bifermentans* strains which were able to grow, but rarely sporulate, in a synthetic medium consisting of amino acids, vitamins, and inorganic salts with glucose as the primary energy source. However, nutritional requirements differ from one clostridial species to another (2, 21, 23), as well as from one strain to another (14). In general, clostridia are more fastidious in their nutritional requirements than are species of the aerobic endospore-forming genus *Bacillus*. Therefore, clostridia are mostly cultivated in complex media (13), and the use of complex media for the production of clostridial spores is very common (9, 22). In addition to the kind of medium, parameters such as pH and the concentrations of protein and sugar play important roles in sporulation. Low glucose concentrations and a neutral to alkaline pH favor sporulation of several *Clostridium* strains (9, 22).

Work presented here involved the development of a process for the production of *C. bifermentans* KMR-1 spores for use as a commercial inoculant. The first goal was to demonstrate efficient spore production at a 10-liter scale, including the downstream processing for harvesting spores. Then, several spore storage (preservation) methods were tested for their suitability for the production of industrially useful spore formulations. Storage experiments were run over a period of several months, and suitability was estimated by monitoring spore viability and the ability of spores to germinate in the presence of TNT and ultimately to degrade it. Finally, a medium development program to produce an inexpensive spore production process was completed. Growth and sporulation studies with a synthetic and a complex medium were compared to allow design of an industrial medium for spore production.

Bacterial strain and culture maintenance. *C. bifermentans* KMR-1 (5a, 24) isolated from an anaerobic digester fed munition compounds as its sole carbon and energy source (7) was maintained as 20-ml cultures in oxygen-free brain heart infusion (BHI, 37 g/liter; Difco), prepared anoxically as described by Balch et al. (1). Cultures were transferred by syringe every 2 weeks into fresh medium. After incubation for 24 h at 37°C, microscopic examination showed complete sporulation.

Spore counts. For spore counts, samples were heat shocked for 15 min at 70°C prior to plating on prereduced BHI agar plates, prepared by supplementing BHI (37 g/liter) with 2% Bacto Agar (Difco) and incubating the mixture for 24 to 48 h

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TABLE 1. Viability of KMR-1 spores for different applied storage methods

Storage method and medium (temp) ^a	Log spores at mo:			
	0 ^b	1	3	4
Liquid				
Distilled H ₂ O (4°C)	12.34	9.06	8.92	8.98
Distilled H ₂ O (RT)	12.88	8.94	8.93	8.75
50 mM KPi (4°C)	12.78	9.36	9.09	9.09
50 mM KPi (RT)	11.97	9.51	9.05	8.68
Dry				
Skim milk (ls) (RT)	9.70/9.43	9.00	8.97	8.14
Skim milk (ss) (RT)	9.65/9.24	9.04	8.79	9.18
Peat moss (RT)	6.33	5.95	3.70	4.23

^a RT, room temperature; Pi, phosphate; ls, large-scale; ss, small-scale.

^b Initial count. Values with slashes represent counts before lyophilization/after lyophilization.

at 34°C in an anaerobic chamber (a 10% hydrogen–90% nitrogen atmosphere). The quantity of viable spores per milliliter was determined in duplicate as the number of CFU observed.

Large-scale spore production. KMR-1 spores were produced at 37°C under sparging with nitrogen in a BioFlo III fermentor (New Brunswick Scientific Co., Inc.) containing 10 liters of BHI, with a 5% (vol/vol) inoculum to minimize the fermentation time and enhance sporulation (22). Sporulation of the fermentation culture began after 4 h and was complete after ≈22.5 h. The spore yield of the fermentation culture was ≈10¹¹ spores per ml, which was greater by 10³ than the yield of a stationary KMR-1 culture grown on BHI in a Balch tube. The improved sporulation in the fermentation culture, compared to that in a stationary KMR-1 culture, was probably a result of better mixing through sparging and stirring (22). After 22.5 h the broth was harvested, and spores were concentrated approximately 4.2-fold to a density of 1.03 × 10¹² spores per ml by recirculation through a lab-scale ultrafiltration system (model DC10L; Amicon) with a hollow-fiber cartridge (H5 P100-43; Amicon). The concentrated spore suspension was then washed three times with sterile distilled water, giving a final density of 2.9 × 10¹² spores per ml.

Storage experiments. KMR-1 spores produced in the fermentor were preserved both dry and as a liquid spore formulation. For dry storage, spores were either air dried on peat moss or freeze-dried in 10% skim milk and vacuum sealed in sterile plastic bags (large-scale freeze-drying) or in sterile glass ampoules (small-scale freeze-drying). As a liquid spore formulation, spores were crimp sealed in sterile serum bottles, either in sterile distilled water or in potassium phosphate buffer at pH 7.0. Preserved KMR-1 spores were kept at room temperature in the dark. In addition, portions of aqueous spore preparations were stored at 4°C. The viability of KMR-1 spores was checked frequently (Table 1), and after 4 months of storage their ability to germinate and degrade TNT was tested. Stored spores also were examined for aerobic microbial contaminants, since aseptic conditions could not be maintained during processing.

After 1 month of storage the liquid-stored spores showed a 1,000-fold loss of viability (Table 1), which may have been caused by two *Pseudomonas* spp. observed as contaminants. The contaminants probably produced anoxic conditions in the spore preparations by consuming oxygen, promoting the germination of KMR-1 spores. Microscopic examination confirmed extensive germination. Oxygen-sensitive germinated spores would be killed by subsequent aerobic spore count

treatments. The aerobic contaminants in liquid spore preparations seemed to have a negative effect only at the beginning of the 4-month storage period, since spore viability then became stable at ≈10⁹ spores per ml for the next few months.

Freeze-dried spore preparations in 10% skim milk were relatively stable over the entire 4-month storage period, remaining at ≈10⁹ spores per ml. The 10-fold lower spore count of the large-scale freeze-dried preparation was due to a variation in the weight of spore preparations in the storage bags prior to rehydration for counting.

The viability of spores air dried on peat moss fluctuated, probably as a result of differences in spore distribution on peat moss and variations in the efficiency of spore removal from peat moss during counting. After 4 months of storage, ≈10⁴ spores per ml were counted, compared to an initial count of ≈10⁶ spores per ml.

Analyses. For degradation experiments, TNT and its metabolites (4-amino-2,6-dinitrotoluene [4-ADNT] and 2,4-diamino-6-nitrotoluene [2,4-DANT]) were determined by high-performance liquid chromatography (HPLC). HPLC analyses were carried out at 42°C on a Hewlett-Packard model 1090 HPLC apparatus equipped with a diode array detector and a 5-μm C₁₈ reverse-phase column (100 by 4.6 mm; Hewlett-Packard). The initial solvent composition of 10% acetonitrile–90% water was maintained for the first 2 min of the run. Over the next 10 min the gradient was increased to 100% acetonitrile and maintained for 3 min; then it was returned to 10% acetonitrile–90% water over 1 min. After a run time of 16 min, a post-run was carried out for 2 min at the last solvent composition. The injection volume was generally 10 μl. The detector was set at 235 nm.

Extractions. In degradation experiments in which peat moss was used, it was extracted first with acetonitrile at the end of the incubation time and then in ethyl acetate–water (1:1). The recovery of TNT and metabolites was 93%.

Degradation experiments. Degradation experiments were carried out with spore samples stored for 4 months. KMR-1 spore samples, stored at room temperature in liquid preparations (sterile potassium phosphate buffer, pH 7.0, or sterile distilled water) or dry preparations (freeze-dried in 10% skim milk [large-scale process] or air dried on peat moss) were tested for their ability to germinate and degrade TNT. Degradation experiments were performed in triplicate in 50 ml of BHI (serum bottles) supplemented with 50 ppm of TNT, with an inoculum size of 5% of preserved spore formulations. All tested spore samples were heated for 15 min at 70°C in a water bath and cooled on ice to room temperature before TNT was added. The prepared spore samples were then incubated at 37°C for 52 h. Two sterile uninoculated controls containing 50 ppm of TNT were prepared in triplicate, one with and one without sterile peat moss (5%, wt/vol). Samples for HPLC analyses were frequently removed aseptically from inoculated serum bottles and uninoculated controls by syringe.

Degradation experiments with TNT showed that KMR-1 spores are a relatively stable biodegradation inoculant. All tested spore samples, which had been stored at room temperature, germinated (observed microscopically) in the presence of TNT and degraded TNT, even after a 4-month storage period. The degradation profiles of freeze-dried KMR-1 spores and air-dried KMR-1 spores on peat moss are shown in Fig. 1 and 2. In serum bottles inoculated with freeze-dried spores (Fig. 1) neither TNT nor 4-ADNT was detectable at the end of the incubation period, whereas approximately 7 and 10 ppm of 4-ADNT remained in serum bottles inoculated with KMR-1 spores stored in distilled water and potassium phosphate buffer (pH 7.0), respectively (data not shown). Growth

FIG. 1. TNT degradation by dry-stored KMR-1 spores (freeze-dried in 10% skim milk) after 4 months of storage at room temperature. Error bars, standard deviations of three replicate cultures. Symbols: ●, ○, and ■, TNT, 4-ADNT, and OD_{600} , respectively, in uninoculated controls; ▲, ▼, and □, TNT, 4-ADNT, and OD_{600} , respectively, in serum bottles inoculated with spores.

(measured as optical density at 600 nm [OD_{600}]) of spores in both liquid preparations was also less (maximum $OD_{600} = 0.2$; data not shown) than that of freeze-dried spores (Fig. 1). Growth was minimal as a result of the toxicity of TNT and its metabolites. Spores that had been air dried on peat moss removed TNT completely from the aqueous phase (Fig. 2); 1% of TNT was still adsorbed on peat at the end of the incubation period. About 25% of TNT was seen as 4-ADNT, 10% in the aqueous phase and 15% adsorbed on peat. The subsequent metabolite of 4-ADNT, 2,4-DANT, was not detected in any degradation time course.

This stability of bacterial spores is an advantage, because dehydration had no negative effects on the spores, whereas dehydration often causes inactivation of non-spore formers, with a loss of viability and degradation ability (27).

Storage experiments showed that besides dry storage methods for spore formers, such as freeze-drying in skim milk (3, 8) and air drying on sand or soil (here represented by peat moss) (3, 8, 30), spore preservation in liquid is also a possible storage option. Though somewhat variable, the viability and degradation activities for all storage methods were sufficiently stable for use in a commercial process.

Preserved spores should have a high initial viability, maintain good TNT degradation ability, and be relatively easy to store (e.g., at room temperature or 4°C), use, handle, and distribute as inocula with a low specific weight and volume. Furthermore, the spore formulation should be highly stable during storage (shelf life, 1 year at room temperature) and should be inexpensive to produce, store, and transport. The storage methods developed here generally fulfill these needs. Although freeze-drying might be somewhat more expensive than air drying, it can often be subcontracted to a specialty processing company at a relatively low cost. Stored spores should be tested in a lab-scale system as a quality assurance check of each production lot, prior to their use in a full-scale bioremediation process.

Medium experiments. Low-cost mass production of *C. bifermentans* KMR-1 spores requires an industrial medium. Therefore, nutritional requirements and sporulation were investigated in a synthetic medium, as compared to BHI. The synthetic medium (20 ml) of Holland and Cox (14) was inoc-

ulated with a 0.1-ml spore suspension of KMR-1, prepared by washing a stationary-grown and completely sporulated KMR-1 culture (cultivated in 20 ml of BHI) three times with the same volume of sterile distilled water. To eliminate a carryover of nutrients from the inoculum, two transfers were performed before growth was monitored (in duplicate).

Studies of nutritional requirements in the synthetic medium showed that *C. bifermentans* KMR-1 was able to grow and sporulate in a medium consisting of salts, 21 amino acids, and 4 vitamins, with or without a sugar such as glucose, fructose, maltose, mannose, and/or sorbitol. Growth and sporulation of KMR-1 were stimulated when complex supplements such as peptone, tryptone, yeast extract, and BHI were added to a synthetic medium consisting of only 14 amino acids, vitamins, glucose, and inorganic salts. KMR-1 is an example of a *Clostridium* sp. which carries out the Stickland reaction by using amino acids as redox pairs.

All growth and sporulation experiments in BHI with different initial pHs, glucose concentrations, or BHI concentrations were carried out in duplicate, as were spore counts. The modified BHI broth (20 ml) was prepared anoxically as described previously and was usually inoculated with 0.1 ml of a BHI culture of KMR-1. Sporulation studies with the model BHI medium showed several nutritional requirements for sporulation, as indicated by decreasing sporulation with decreasing BHI concentration. In addition, the ratio of protein to glucose seemed to be important for sporulation. High glucose concentrations, up to 1% (wt/wt) glucose, resulted in enhanced growth at first, but at 5% (wt/vol) glucose, a decrease in growth was observed (data not shown). Sporulation correlated with growth but decreased more rapidly (Fig. 3). Two percent (wt/vol) glucose yielded $\approx 10^8$ spores per ml, whereas 5% (wt/vol) glucose yielded only $\approx 10^2$ spores per ml. Spontaneous lysis dominated at higher sugar concentrations. The low spore yield with high glucose concentrations may be an indirect effect of sugar, through the formation of an acidic pH unfavorable to sporulation (Fig. 3).

Several complex bulk medium sources were tested as inexpensive alternative sporulation media for large-scale spore production. Appropriate media (pH 7.4; 20 ml) with different complex components (soy peptone with or without Casamino

FIG. 2. TNT degradation by dry-stored KMR-1 spores (air dried on peat moss) after 4 months of storage at room temperature. Error bars, standard deviations of three replicate cultures. Symbols: ● and ○, TNT and 4-ADNT, respectively, in uninoculated peat controls; ▲ and ▼, TNT and 4-ADNT, respectively, in serum bottles inoculated with the spore-plus-peat mixture.

FIG. 3. Sporulation of KMR-1 in BHI with different glucose concentrations (□), different initial pHs (▲), or different BHI concentrations (●). BHI concentrations were adjusted from the standard composition of 37 g of BHI/liter, which was set at 100%. In compositions in which less than 100% BHI was used, salts were adjusted to the 100% value to maintain the buffer capacity; the same adjustment was carried out with glucose.

Acids; meat peptone) were prepared anoxically and contained the following (in grams per liter): soy peptone (10.0 or 20.0) with Casamino Acids (5.0 or 10.0), or soy peptone (5.0 to 50.0), or meat peptone (20.0 to 50.0), glucose (2.0), NaCl (5.0), and Na₂HPO₄ (2.5). Soy peptone, Casamino Acids, and meat peptone were obtained from Marcor Development Corporation. We also tested several medium compositions containing corn steep liquor (CSL) obtained from Sigma as a concentrate with 50% solids. A 20% (wt/vol) suspension was prepared and centrifuged to remove solids. This CSL solution was used as a medium directly (KOH-untreated CSL) or after a further treatment with KOH (KOH-treated CSL) as described by Liggett and Koffler (18). Media with CSL (pH 7.2 ± 0.2) in potassium phosphate buffer (250 mM with KOH-untreated CSL or 25 mM with KOH-treated CSL) contained the following (in grams per liter): KOH-untreated CSL (10.0 to 50.0) or KOH-treated CSL (10.0 to 40.0), and glucose (2.0). In several variations, glucose was replaced by corn syrup (2.0 g/liter).

In general, for all the industrial medium compositions used, two precultures were grown in the appropriate composition before growth was monitored, and spore counts were carried out in duplicate. Industrial media (20 ml) were originally inoculated with 0.1 ml of a BHI culture of KMR-1.

Glucose-supplemented medium compositions with KOH-untreated CSL resulted in a spore yield of about 10⁶ spores/ml; replacement of glucose with corn syrup yielded ≈10⁷ spores/ml. KOH-treated CSL was unfavorable for sporulation, yielding only 10³ to 10⁴ spores/ml. Media with meat and soy peptone produced ≈10⁷ spores/ml, whereas supplementing soy

peptone with Casamino Acids resulted in a 10-fold increase in spore yield (≈10⁸ spores/ml).

Examinations of these various media encouraged the use of several of the complex medium bulk sources, since these provided an acceptable spore yield of ≈10⁷ spores per ml. Acceptable constituents included CSL (\$0.06/kg), soy peptone (\$9.50/kg), and meat peptone (\$21.00/kg). Casamino Acids (\$32.00/kg) were judged unacceptable because of cost. Such particulate-free medium compositions are expected to be compatible with the ultrafiltration unit (downstream processing of spores) and would allow production of high spore densities after concentration by hollow-fiber units.

Although acceptable spore yields and spore storability at reasonable cost were achieved here, continued work on optimization and scale-up of this process could yield even greater economy. Spore quality is very much dependent on the quality of the sporulation medium (34), and further improvements are possible. Bulk spore production processes are clearly achievable for use in bioaugmentation or bioremediation systems.

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