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FEMS Microbiology Ecology 16 (1995) 135–142

FEMS MICROBIOLOGY ECOLOGY

Evaluation of methods for extraction of bacteria from soil

Viggo Lindahl *, Lars R. Bakken

Department of Biotechnological Sciences, Agricultural University of Norway, P.O. Box 5040, N-1432 Ås, Norway

Received 25 April 1994; revised 13 October 1994; accepted 13 October 1994

Abstract

Several methods for dispersion of soil were tested for possible use in procedures for extraction of bacteria. Physical cell damage on cells and efficiency in extraction of indigenous cells from soil, were investigated. Cell damage by the dispersion methods was investigated by measuring the physical cell integrity and viability of pure cultures of *Escherichia coli* and *Bacillus subtilis*, as well as soil bacteria extracted from soil, when dispersed in slurries of γ-sterilized soil. Separation of bacteria and soil particles on the basis of buoyant density was conducted with the nonionic density gradient medium Nycodenz. When slurries of γ-sterilized soil with added pure cultured cells were centrifuged ($10\,000 \times g$) over cushions of Nycodenz (1.3 g ml^{-1}), practically all the added cells were recovered in a layer on top of the cushion. This proves that a reversible attachment and cosedimentation is not an important phenomenon in this procedure. The efficiency of the different dispersion methods for the extraction of indigenous soil bacteria, was assessed after separation of dislodged and attached soil bacteria. This separation was done either on the basis of sedimentation rate by low speed centrifugation, or buoyant density by Nycodenz density gradient centrifugation. The physical dispersion by ultrasonic treatment and chemical dispersion by the use of a chelating agent together with a detergent, were inferior to physical dispersion either by Waring blender (for large volumes) or a rotating rubber pestle treatment (for smaller volumes). The physical dispersion did not appear to be destructive to the cells tested.

Keywords: Soil dispersion; Bacterial extraction; Cell survival

1. Introduction

Application of many techniques for the study of soil bacteria has been hampered by the presence of humic material and clay. Nonspecific adsorption of antibody to soil particles [1] has not only made immunofluorescence microscopical enumeration difficult, but also the use of immunomagnetic beads for extraction of species populations from soil slurries. Size fractionation and flow cytometric investigations of soil bacteria [2,3] is only possible after separation of cells from soil, and physiological studies of indigenous soil bacteria has been feasible with bacteria extracted from soil [4,5]. Separation of cells from soil prior to extraction of their DNA will reduce problems with contaminating eukaryotic and extracellular DNA present in soil, as well as humic particles and clay interfering with enzymatic reactions like PCR amplification [6].

* Corresponding author. Tel: +47 64 94 77 44; Fax: +47 64 94 77 50; E-mail: viggo.lindahl@biotek.nlh.no

SSDI 0168-6496(94)00077-8

Two steps are common to all methods for extraction of bacteria from soil. Dispersion of soil, followed by separation of cells and soil particles by centrifugation according to diameter, buoyant density or both. Maximum dispersion of soil is crucial for releasing bacteria entrapped within soil aggregates, and important for procedures aimed at dissociating microorganisms from the soil surfaces. Dispersion of soil has been conducted by physical or chemical dispersion techniques, or combinations of these. Physical dispersion techniques frequently used are homogenization of the sample in a Waring blender, shaking the sample with glass beads, or disruption of aggregates by mild ultrasonic treatment. Chemical dispersion agents are often used in combination with mechanical methods, and several substances have been used, like Chelating agents [7,8] sodium charged ion exchange resins [9,10] and detergents [9].

The establishment of the strong binding between soil particulates and bacteria is probably a gradual process, involving a variety of binding mechanisms [11]. Because of the strong binding between cells and soil particulates (including clay), severe cell damage may be the result of breaking these bindings. A successful strategy for extraction of cells will therefore be a choice between cell survival and extraction efficiency, depending on the purpose of extraction. Both factors must be investigated before deciding what method to use.

The aim of this study was to compare the most promising methods for soil dispersion with regard to extraction efficiency and cell survival, for the development of a useful and efficient procedure for extraction of bacteria from soil. Possible cell damage by the various dispersion techniques was investigated by dispersion of pure cultures of *Escherichia coli*, *Bacillus subtilis* and indigenous soil bacteria (previously released and purified) in slurries of γ-sterilized soil. Extracting cells from soil, and then reintroducing them into a suspension of γ-sterilized soil, enables a direct measurement of cell survival (as cfu in the slurry) and reattachment of soil bacteria (as recovery in cell extraction procedures). This can not be done by studying the cfu and cell extraction directly from fresh soil, for obvious reasons (confounding of dislodging/dispersal and cell damage/reattachment).

The efficiency in dislodgement of indigenous soil bacteria by the various techniques was assessed by slow speed centrifugation as well as high speed density gradient centrifugation with the nonionic density medium Nycodenz (1.3 g ml^{-1}) so as to separate according to buoyant density. ATP was also measured to see if any of the extraction routines altered the metabolic state of the cells.

2. Materials and methods

2.1. Testing of bacterial survival and reattachment in slurries of γ-sterilized soil

Escherichia coli DH5α and *Bacillus subtilis* BD224 [12] were grown in Luria broth (LB) [13] with aeration at 37°C and harvested either in logarithmic or stationary phase of growth. Indigenous soil bacteria were extracted from an agricultural clay loam soil by dispersion of the soil in distilled water by treatment in the Waring blender (3×1 min), and purified in density gradients of Metrizamide as described by Bakken [14].

Cell survival was tested by dispersing stationary phase cells in 0.1 g ml^{-1} suspensions of γ-sterilized (32 kGy) soil and filter sterilized distilled water (cell density around 2×10^9 cells ml^{-1}). The γ-sterilized soil was agricultural clay loam soil [14], which was also used in fresh (unsterilized) condition (gravimetric moisture content 20% W/W) for the dispersion and extraction experiments. The γ-sterilized soil had been stored at room temperature for 2 years.

The numbers of colony forming units (cfu) in the suspensions of *E. coli* and *B. subtilis* were determined on Luria agar plates [13]. The cfu in the suspensions of indigenous soil bacteria were determined on a low nutrient soil extract agar as described by Bakken and Olsen [15]. The total numbers of structurally intact cells was determined by fluorescence microscopical counting after staining with acridine orange, as described by Hobbie et al. [16]. This was possible with *E. coli*

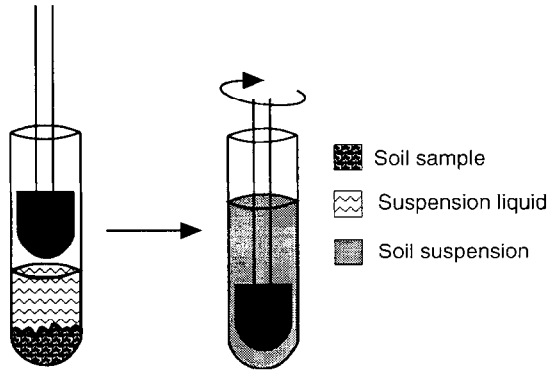

Fig. 1. Rotating pestle method for dispersion of soil. The rubber pestle (0.5 mm smaller diam. than the tube) is rotated by a drill gun (2600 rpm) [11].

and *B. subtilis*, since it was easy to discriminate between these cells and the fluorescing cells in the γ-sterilized soil ($< 5 \times 10^8$ cells g^{-1} dw).

2.2. Physical dispersion

Ultrasonic treatment was conducted with a Sonifier® model B-15 (Branson, Danbury, CT), using a disrupter horn with a tip diameter of 13 mm, on 20 ml suspensions (2 g soil) kept in centrifuge tubes on ice during the treatment. Energy outputs of 40W (approximately 20 μm amplitude) and 55W (40 μm amplitude) were used.

Waring blender (Waring, New Hartford, CT) treatments were conducted on 200 ml of suspensions (20 g soil). The blender was run at full speed in 1 min periods with 5 min cooling on ice in between.

The rotating pestle (RP) method was developed as an alternative to the Waring blender for dispersion of small volumes (Fig. 1) [11]. A drill gun equipped with a 30 cm (0.7 cm diam.) rod, with a rounded rubber pestle at the end was used. The rubber pestle was designed to fit standard test tubes with a clearing of about 0.5–1 mm. Five ml of suspension (0.5 g soil) was added, and treatment was conducted by pumping the pestle up and down while rotating (2.600 rpm) for 1 min periods. The lower end of the tube was kept on crushed ice during the treatment.

Shaking was conducted on a reciprocal shaker (125 cycles min^{-1}) for 2 hours at 4°C. Efficient dispersion of the soil slurry was ensured by keeping the flasks (100 ml Pyrex flasks) in a horizontal position oriented along the movement axis of the shaker.

2.3. Chemical dispersion

The chemical dispersion agent tested was the Chelex solution as recommended by Herron and Wellington [10]; final concentrations (ml^{-1}) being 1 mg sodium deoxycholate, 25 mg polyethylene glycol (PEG 6000) and 200 mg Chelex 100 (Bio-Rad, Richmond, CA). Treatment was conducted by shaking on a reciprocal shaker (125 cycles min^{-1}) for 2 h at 4°C.

2.4. Comparison of dispersion methods

When comparing the methods for dislodging indigenous cells from a fresh (unsterilized) clay loam soil, three suspensions of 0.111 g soil ml^{-1} (i.e. 1:9 dilution) in prefiltered (0.2 μm pore size) distilled water were first subjected to the physical dispersion methods (sonication, waring blender and rotating pestle), while one sample was kept untreated. Subsamples were then mixed 9:1 with either distilled water or stock solutions (10 × working concentrations) of the Chelex solution, and shaken on a reciprocal shaker (125 cycles min^{-1}) for 2 h at 4°C. Disruption efficiency of large aggregates was estimated by allowing coarse particles to settle for 0.5 min. Two different procedures, slow speed centrifugation and Nycodenz density gradient centrifugation, were investigated for the separation of dislodged cells from soil particles. The dislodgement efficiency was estimated as the cell yield in the supernatants as % of the number of cells in the soil suspension, by cell counts (AODC) of the dispersed soil and the supernatants containing extracted indigenous soil bacteria.

2.5. Centrifugation procedures

Two principally different procedures were used to separate soil particles from dislodged cells.

Low speed centrifugation ($1000 \times g$, 10 min at 4°C, Sorvall HB-4 swing out rotor, 25 ml per tube in 50 ml tubes) was used to separate soil particles from soil bacteria on the basis of sedimentation rate [4]. High speed centrifugation with a density gradient medium, Nycodenz (Nycomed Pharma AS, Oslo, Norway) [17] was used to separate on the basis of buoyant density [11]. Nycodenz is a water soluble, nonionic and nontoxic derivative of benzoic acid. Two variants of the density based separation was conducted, both based on separation of cells floating in, and particles sedimenting through, a Nycodenz solution with a density of 1.3 g ml^{-1}. When trying to extract the cells that had been mixed with a suspension of γ-sterilized soil, a 2 ml suspension was layered on top of a 7 ml cushion of Nycodenz with a density of 1.3 g ml^{-1}, which was then centrifuged at $10{,}000 \times g$ (at tip) for 20 min in a Sorval HB-4 swing out rotor. (The supernatant obtained after centrifugation included both the Nycodenz cushion and the supernatant above the cushion after centrifugation). When comparing the efficiency of dispersion methods for dislodging indigenous cells from the clay loam soil, 0.15 ml of the soil suspension was mixed with 1.35 ml Nycodenz solution (1.33 g ml^{-1}) in Eppendorf tubes for a final density of 1.3 g ml^{-1} in the whole soil suspension, and centrifuged $9000 \times g$ for 6 min.

2.6. ATP measurements

ATP was extracted from the soil suspensions by addition of 0.5 ml 1 M Trichloroacetic acid (TCA), 1 M Na_2HPO_4 and 0.5 M paraquat, pH 1.2, to 0.5 ml of soil suspension [18]. ATP was measured in 1 ml volume on 1:250 dilutions of the extracts, using the ATP Monitoring Reagent (Bio-Orbit, Turku, Finland) and the 1253 Luminometer (Bio-Orbit, Turku) as described by the supplier.

3. Results and discussion

3.1. Damage of cells by ultrasonic treatment

Cells in the stationary phase of growth were less susceptible to sonication than cells in the logarithmic phase of growth (Table 1). This is probably due to induction of the bacterial starvation response in the cells in stationary phase of growth, which often results in improved tolerance to various external stress factors [19]. Most of the indigenous soil bacteria will be in a dormant or non-growing state and thus have increased resistance to external stress factors. The actively growing cells will be more susceptible. This must be taken into consideration when deciding what en-

Table 1
Susceptibility of pure cultures to ultrasonic treatment showing the percentage [a] of structurally intact cells remaining after ultrasonication, as determined by fluorescence microscopy (AODC) and plate counting. Comparing cells treated in both logarithmic and stationary phase of growth

	Amplitude (μm)	Treatment time (s)	Logarithmic phase		Stationary phase	
			AODC	cfu	AODC	cfu
E. coli	–	0	100% ± 5	100% ± 16	100% ± 9	100% ± 16
	20	15	42% ± 3	42% ± 7	72% 7	91% ± 12
		30	48% ± 3	36% ± 26	73% ± 8	70% ± 30
		45	38% ± 4	14% ± 6	71% 6	75% ± 13
	40	30	35% ± 4	N.D.	75% 5	89% ± 8
B. subtilis	–	0	100% ± 8	100% ± 18	100% ± 5	N.D.
	20	15	53% ± 6	62% ± 16	84% ± 8	N.D.
		30	57% ± 5	59% ± 12	75% ± 10	N.D.
		45	55% ± 8	73% ± 21	59% ± 7	N.D.
	40	30	47% ± 5	N.D.	53% ± 6	N.D.

[a] All values are means ± standard errors.
[b] N.D. = not detected.

ergy levels to use for such treatment of soil samples. Testing of experimental parameters on pure cultures before using ultrasonic energy for dispersion of soil gives an indication of the energy level to use. From our results with *E. coli* and *B. subtilis*, it was decided to use an amplitude of 20 μm for 30 s for dispersal of soil. This is considered a rather conservative choice, since indigenous soil bacteria are likely to be much less susceptible to the treatment than *E. coli* [2].

One problem using energy output from ultrasonic equipment, is that the actual output will deviate from the readout on the instrument, depending on the age of the converter (converting electric energy to mechanical vibrations), due to changes in the converters crystal structure. It is therefore not possible, by standard methods and equipment, to calculate the correct energy levels used in ultrasonic treatment. The energy levels used in such experiments must be considered as approximate values.

3.2. Damage of cells by Waring blender and rotating pestle treatment

A logarithmic decrease in the number of structurally intact cells as counted with AODC was experienced when cells of *B. subtilis* and *E. coli* in stationary phase of growth, were exposed to Waring blender treatment in a slurry of γ-sterilized soil (0.1 g ml^{-1}) (Fig. 2). The decay rate for *E. coli* was 2% min^{-1} treatment time, and for *B. subtilis* 0.05% min^{-1} (regression estimate indicated by the line in Fig. 2). It must be kept in mind though, that the decay rate as determined by AODC may be lower than what would be found if survival was determined as cfu by plate counting.

The survival of cells, counted as cfu after homogenization with the rotating pestle method produced a similar pattern as that with the Waring blender (Table 2). The apparent increase in numbers of viable cells for the *B. subtilis* cells after 2 min treatment is probably due to disruption of the chain structure. The results for indigenous soil bacteria could indicate that approximately 10% of the cells are very sensitive and that the remaining 90% are practically unaffected. However, an error in the estimated number of cells in the original suspension may be an alternative explanation.

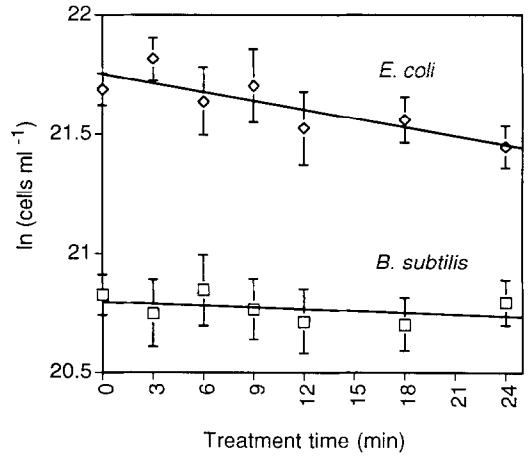

Fig. 2. The number of structurally intact cells remaining after physical destruction by Waring blender treatment of pure cultures added to sterilized soil, as determined by fluorescence microscopy (AODC).

It seems reasonable to conclude that the two physical dispersion methods have similar effects per minute of treatment. A treatment of 3 min seems a reasonable minimum, probably destroying only a minority of the indigenous cells. For fragile organisms, obvious candidates being large Gram-negative rods, both dispersion methods could be disastrous; this apparently being confirmed by the observation that the viability of *Pseudomonas cepacia* was reduced by dispersion in a Waring blender (C. Jacobsen KVL, Copenhagen, Denmark, personal communication). These results underscores the need to carefully

Table 2
Survival of cells added to sterilized soil depending on time of dispersal by the rotating pestle method, determined by plate counting as percentage [a] of the total number in the original suspension

	Treatment time			
	0 min	1 min	2 min	3 min
E. coli	100% ± 1	103% ± 4	93% ± 14	81% ± 4
B. subtilis	100% ± 10	79% ± 15	110% ± 7	91% ± 3
Soil bacteria	100% ± 6	87% ± 19	92% ± 6	91% ± 1

[a] All values are mean ± standard errors.

test the sensitivity of the target organism when deciding the intensity of physical dispersion used. The use of a slurry of γ-sterilized soil for such testing is recommendable, since the viscosity and the presence of particles (particularly for the rotating pestle method) will probably have a (negative) influence on the survival of cells. For the majority of soil bacteria, neither Waring blender nor the rotating pestle seems to do much harm, even after long periods of treatment. Further experimentation was conducted with a treatment time of 3×1 min for both methods, on background of the observed susceptibility of the *E. coli* cells, and the fact that this is the treatment time most often used in other studies, based on Fægri et al. [4].

3.3. Cosedimentation of cells and soil particles

The suspensions of cells in a slurry of γ-sterilized soil which had been dispersed with the rotating pestle method for 3 min (Table 2) were stored for 4 h (4°C), then resuspended and centrifuged on cushions of Nycodenz. The number of cells floating in the cushion as measured by AODC were 96% ± 11 for *E. coli*, 113% ± 14 for *B. subtilis* and 89% ± 14 for soil bacteria. (The γ-sterilized soil contained negligible numbers of cells that were floating in the Nycodenz). This demonstrates that very few of the cells were able to attach to soil particles so as to cosediment through the cushion. Hence the concentration of divalent cations was sufficiently low to ensure efficient repulsion between the negatively charged bacteria and soil colloids. Otherwise, great losses should be expected, considering that the soil particles and the cells were present in the same suspension for a sufficient period of time for attachment to occur (4 h at 4°C). This strongly indicates that a reversible attachment between bacteria and soil colloids, during treatments for dispersion of soil, did not occur.

3.4. Comparison of dispersal methods

The percentage yield of indigenous soil bacteria obtained by sedimentation of coarse particles for 0.5 min, and by the two different centrifugation procedures, low speed centrifugation or Nycodenz density gradient centrifugation, after dispersion of fresh (unsterilized) soil, (Table 4) reflects the efficiency of the dispersion methods, and leaves little doubt that ultrasonication and shaking are inferior to the other two physical dispersion methods. Ultrasonication was so inefficient in disrupting aggregates that substantial soil material was lost during the 0.5 min sedimentation of coarse particles. The use of chemical dispersion agents (the Chelex mixture) did not have a consistent effect on the yield. As to the ATP level in the dispersed soil (Table 3), when expressed on a per cell basis, two patterns emerge: the use of Chelex mixture consistently lowered the ATP content per cell compared to distilled water; and the ultrasonic treatment resulted in much lower levels than the other physical treatments.

The fact that very little reattachment of liberated organisms occurred after mixing with γ-sterilized soil and the lacking effect of cation

Table 3
ATP content [a] and number of cells determined by fluorescence microscopy (AODC) in soil samples after different dispersion treatments

	Cells ml^{-1}	ATP (nM)	ATP $cell^{-1}$ (mol $cell^{-1}$)
Waring blendor + Chelex mixture	4.1×10^8	69 ± 4	1.7×10^{-19}
Waring blendor + Water	4.2×10^8	95 ± 5	2.3×10^{-19}
Drill-gun + Chelex mixture	6.3×10^8	77 ± 1	1.2×10^{-19}
Drill-gun + Water	5.9×10^8	91 ± 4	1.5×10^{-19}
Ultrasonication + Chelex mixture	2.2×10^8	9 ± 4	4.1×10^{-20}
Ultrasonication + water	2.9×10^8	11 ± 1	3.7×10^{-20}
Shaking + Chelex mixture	3.8×10^8	43 ± 7	1.1×10^{-19}
Shaking + water	4.7×10^8	84 ± 4	1.8×10^{-19}

[a] ATP values are means ± standard deviation.

exchanger (Chelex) on the yield of indigenous cells from fresh soil (Table 4) are in general agreement, indicating a reasonable stability of the suspension of dislodged bacteria and soil colloids when soil is dispersed in water.

The chemical dispersion mixture also contained PEG and Na-deoxycholate, that might help in dissolving hydrophobic materials. The lack of a positive effect of these substances in the present experiment does not exclude the possibility that they may have a positive effect on dislodgement of specific types of cells. One should have in mind that any treatment that has a specific binding mechanism as its target may have a positive effect on certain groups, without being detectable as an increase in the overall extraction efficiency as determined in the present study. The consistent negative effect of the chemical dispersion mixture on the total ATP (Table 3) suggests a substantial negative effect on the metabolic status of a fraction of the cells. We suspect that this may be due to Na-deoxycholate affecting the functioning of the cell membranes.

The choice of method for dislodgement of cells from soil particles depends on the purpose of the cell extraction. If cells are to be investigated as to their metabolic status, growth potential or viability, a straight forward mechanical dispersion by Waring blender in distilled water seems preferable. Investigations that are less demanding as to the metabolic status may tolerate more harsh physical and chemical treatments. The use of a dispersion medium with high pH (pH 8–9) will increase the number of cells recovered above a Nycodenz cushion [11], maybe due to its dissolving effect on the 'humic substances' or by altering the structure of extracellular polymeric materials. Such a high pH obviously jeopardizes the metabolic integrity of some cells, and the impression was that the bacterial fraction obtained was contaminated by humic substances. For extraction purposes where the target is the bacterial genome, it may be justifiable to reinvestigate such 'harsh' methods however, since the genome is unlikely to be seriously harmed by exposure of the cells to a slightly alkaline environment for a limited amount of time.

The effect of a harsher physical treatment is illustrated by a small experiment, where we investigated the effect of extending the physical dispersion in the Waring blender on the yield of indigenous cells after Nycodenz density gradient centrifugation. A 0.1 g ml^{-1} soil slurry in distilled water was treated in the Waring blender for a total effective dispersion time of 18 min (intermittent cooling in ice), and subsamples (10 ml) were taken at intervals. The percent cell yield after high speed centrifugation over cushions of Nycodenz was 24, 30, 32 and 42% after 3, 6, 12 and 18 min of dispersion, respectively (data not shown). The result illustrates the importance of physical treatment to 'tear' bacteria off the soil particles. Obviously, 18 min treatment in a Waring blender is too much for cells as fragile as *E. coli*, since 20–40% of such cells would be destroyed (Fig. 2). However, a harsh treatment like

Table 4
Percentage [a] of indigenous bacteria in soil suspensions [b] after settling of coarse particles for 30 s, and after extracting bacteria by either low speed centrifugation or Nycodenz density gradient centrifugation, as determined by fluorescence microscopy (AODC)

	Settling of coarse particles (30 s)	Low speed centrifugation	Nycodenz density gradient centrifugation
Waring blendor + Chelex mixture	54% ± 9	18% ± 2	17% ± 2
Waring blendor + Water	55% ± 8	13% ± 2	17% ± 2
Drill-gun + Chelex mixture	82% ± 9	17% ± 2	20% ± 4
Drill-gun + Water	77% ± 12	20% ± 3	36% ± 4
Ultrasonication + Chelex mixture	29% ± 5	5% ± 1	4% ± 1
Ultrasonication + water	38% ± 9	3% ± 1	4% ± 3
Shaking + Chelex mixture	N.D.	8% ± 1	11% ± 2
Shaking + water	N.D.	8% ± 1	11% ± 2

[a] All values are means ± standard errors.
[b] Number of bacteria in the primary soil suspension = $7.7 \times 10^8 \pm 6.2 \times 10^7$cells ml^{-1}.

this is obviously a feasible alternative for certain cell types, and possibly less harmful than a chemical treatment such as an alkaline dispersion medium. It should be kept in mind though, that the integrity of the cell membrane (to exclude the density material Nycodenz from entering the cells) is essential for flotation of cells during Nycodenz density gradient centrifugation.

When separating cells from soil particles on the basis of buoyant density by high speed centrifugation with Nycodenz density material, the use of a Nycodenz cushion is equally effective as mixing the soil suspension with Nycodenz to a final density of 1.3 g ml^{-1} (data not shown), but the use of a cushion is often preferable since it minimizes the amount of Nycodenz per ml of soil slurry applied. High speed centrifugation with a Nycodenz density gradient is preferable to the low speed centrifugation for a number of reasons. As the present investigation demonstrates (Table 4), the yield is similar to or even slightly higher than that obtained by slow speed centrifugation, in part attributable to the fact that the method yields practically 100% recovery of cells that are dislodged from soil particles. In contrast, the slow speed centrifugation will only recover a fraction of the dislodged cells, since those positioned at the lower part of the centrifugation tubes will be sedimented. Further, the purity is grossly improved by the density based separation, and the method does not require more skill and labor than the slow speed centrifugation.

Acknowledgements

This work was financially supported by the Research Council of Norway.

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