Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Comparative analysis of environmental DNA extraction and purification methods from different humic acid-rich soils

F.M. Lakay[1,2], A. Botha[1] and B.A. Prior[1]

1 Department of Microbiology, University of Stellenbosch, Matieland, South Africa
2 CSIR Biosciences, Modderfontein, South Africa

Keywords
environmental, extraction, humic acid, purification, soil.

Correspondence
F.M. Lakay, CSIR Biosciences, Private Bag X2, Building S3, Pinelands site, Modderfontein, 1645, South Africa. E-mail: FLakay@csir.co.za

2005/1264: received 24 October 2005, revised 30 January 2006 and accepted 30 March 2006

doi:10.1111/j.1365-2672.2006.03052.x

Abstract

Aim: To establish a rapid, improved soil environmental DNA extraction and purification protocol.
Methods and Results: Three different soil DNA isolation and four purification strategies were compared on different soil samples with variable rates of success. Bead beating extraction gave significantly higher DNA yields than microwave-based and liquid nitrogen grinding DNA extraction methods. The inclusion of soil washing prior to cell lysis decreased the amount of purification steps required. Although these soil types differed, polyvinylpolypyrrolidone (PVPP)-sepharose 2B column elution was sufficient for all three samples, yielding DNA pure enough for successful application in molecular studies. One soil sample retained 80% of the initial DNA after successful purification.
Conclusions: Optimization of a purification protocol confirmed that only a combination of previously described methods proved sufficient in yielding pure environmental DNA from humic-rich soils. Total processing time for DNA extraction and subsequent purification from multiple samples was considerably more rapid than the previously described methods.
Significance and Impact of the Study: This study developed a new optimized soil DNA extraction and purification protocol that is suitable for different environmental sources that are rich in humic acid content.

Introduction

The proportion of culturable cells using standard techniques is estimated to be less than 1% of the total population. It is believed that a gram of soil may contain up to 10 billion micro-organisms of possibly thousands of different species (Knietsch *et al.* 2003). Soil, considered as a complex environment, appears to be a major reservoir of microbial genetic diversity. Its complexity results from multiple interacting parameters that include pH, water content, soil structure, climatic variations and biotic activity. In general, micro-organisms are heterogeneously distributed throughout the aggregates of soil particles (Robe *et al.* 2003).

Application of molecular techniques in environmental microbiology provides new insights into microbial communities. However, the efficient extraction and purification of whole community nucleic acids remains a challenge (Miller 2001). DNA isolation from soils results in the co-extraction of humic acids, which interfere with DNA detection and measurement (Zhou *et al.* 1996). Humic impurities from soil are resistant to separation from DNA, because of their chemical properties (Saano and Lindstrom 1995). Most common contaminants include humic acids, polysaccharides, or urea that exhibit similar solubility properties to DNA. Consequently, they are not completely removed during classical extraction protocols, such as phenol–chloroform, and detergent and protease treatments, thereby remaining as contaminants in the final DNA preparations (Moreira 1998). Furthermore, highly organic soils containing humic acids with phenolic groups covalently bind to DNA or proteins (Tsai and Olson 1992). Therefore, the primary aim of any purification protocol for soil-extracted DNA should be to

remove phenolic-containing compounds, such as humic substances (Young *et al.* 1993).

Numerous studies have been done to ascertain the most suitable DNA extraction and purification protocol from soil (e.g. Wikström *et al.* 1996; Jackson *et al.* 1997; Cullen and Hirsch 1998; Bürgmann *et al.* 2001; Miller 2001; Stach *et al.* 2001; Kauffmann *et al.* 2004), but comparison of the results showed a number of inconsistencies. Extraction methods ranged from liquid nitrogen grinding (Volossiouk *et al.* 1995), microwave-based rupture (Orsini and Romano-Spica 2001), enzymatic lysis (Zhou *et al.* 1996; Stach *et al.* 2001), to bead beating lysis (Miller *et al.* 1999; Bürgmann *et al.* 2001). Several purification methods have also been evaluated to remove humic acid from soil DNA. Some of these included the use of hexadecyltrimethylammonium bromide (CTAB) (Zhou *et al.* 1996), caesium chloride density gradients (Holben *et al.* 1988), polyvinylpolypyrrolidone (PVPP) (Frostegård *et al.* 1999), various gel filtration resins (Jackson *et al.* 1997; Miller 2001) and ion exchange and size-exclusion chromatography (Kuske *et al.* 1998; Hurt *et al.* 2001). However, Braid *et al.* (2003) found that the use of PVPP and CTAB might be unreliable for the removal of inhibitors from a variety of soils. Therefore, the effective purification of nucleic acids from all soil or sediment types requires many different purification strategies. If the target gene or organism constitutes only a small fraction of the microbial community, additional purification steps should be balanced against potential loss of target nucleic acid (Chandler *et al.* 1997).

The objective of this study was to develop an improved environmental DNA extraction and purification protocol from humic acid-rich soils by comparing the ability of various methods previously described. Ultimately, a suitable protocol was developed by incorporating the properties of both PVPP and sepharose 2B column purifications, as well as a preliminary soil washing step prior to DNA extraction. Environmental DNA was of sufficient purity for molecular-based applications.

Materials and methods

Microbial strains and culture conditions

The euascomycetous yeast, *Aureobasidium pullulans* NRRL Y-2311-1, was obtained from the Agricultural Research Service (ARS) culture collection, located at the National Center for Agricultural Utilization Research (Peoria, IL, USA) and cultivated in YPD medium (containing per litre: 10 g of yeast extract, 20 g of peptone and 20 g of glucose) at 30°C for 3 days. The bacterium *Bacillus pumilus* PLS was obtained as described previously (La Grange *et al.* 1997) and cultivated in Luria-Bertani (LB) medium (containing per litre: 10 g of tryptone, 5 g of yeast extract and 10 g of sodium chloride) at 37°C for 3 days.

Source and properties of environmental samples

Different soil types (Table 1) were obtained locally (Stellenbosch area), sieved to pass through a 2-mm sieve and stored at −20°C until further use. Sand 1 was collected from around the campus gardens (nutrient poor environment), while the loamy sands came from shady areas in either the local nature reserve (Jonkershoek, Stellenbosch) or botanical gardens (central Stellenbosch) (nutrient-rich areas). The poor, sandy soil was included as crude negative control. Soil types were classified using the Munsell soil colour charts (Anonymous 2000).

Soil content analyses

Bacterial enumeration of soil samples was conducted with 4′,6-diamidino-2-phenylindole (DAPI) staining (Miller *et al.* 1999). Chemical analysis of soil samples included the following: pH according to the method of McClean (1982); plant available (extractable) phosphorous in a Bray-2 extract (Thomas and Peaslee 1973), manganese in a di-ammonium ethylenediaminetetraacetic acid (EDTA) extract (Beyers and Coetzer 1971); exchangeable cations in a 1 mol l^{-1} ammonium acetate extract (Doll and Lucas

Table 1 Soil content analyses (mean ± range of duplicate values)

Sample	Clay (%)	Cells (10^9) g^{-1}*	pH	C†	N‡	CEC§	HA¶
Sand	4·5 ± 0·5	0·40 ± 0·08	6·2	3·34 ± 0·15	0·20 ± 0·02	121·6 ± 0·0	15 ± 4
Loamy sand 1	7·5 ± 0·5	13·6 ± 4·00	6·3	9·55 ± 0·60	0·62 ± 0·03	426·5 ± 0·1	25 ± 2
Loamy sand 2	7·5 ± 0·5	3·20 ± 0·00	6·2	10·85 ± 0·10	0·68 ± 0·02	182·8 ± 0·1	24 ± 0

*, Cell counts were done with 4′,6-diamidino-2-phenylindole (DAPI) staining according to Miller *et al.* (1999).
†, C – organic carbon content (%).
‡, C – organic nitrogen content (%).
§, CEC (cation exchange capacity) given in μmol g^{-1}.
¶, HA – humic acid content (mg g^{-1}).

1973), organic carbon content with the Walkey-Black method (Nelson and Sommers 1982) and organic nitrogen through digestion in a nitrogen analyser (Model FP-528; LECO Corp., St Joseph, MI; Bremner 1965). Soil humic acid content was determined according to Swift (1996).

Screening of environmental DNA extraction and purification protocols from soil

Individual environmental samples (sand 1, and loamy sands 1 and 2) and sand containing the control organisms ($1{\cdot}6 \times 10^9$ cells g^{-1} each) were screened for optimal DNA extractability and purification. The three different DNA extraction methods tested were: (i) optimized bead beating (Jackson *et al.* 1997; Miller *et al.* 1999; La Montagne *et al.* 2002), (ii) microwave-based (Orsini and Romano-Spica 2001) and (3) liquid nitrogen grinding (Volossiouk *et al.* 1995). Purification methods included the use of one of the following: microspin column (Amersham, Piscataway, NJ, USA), overnight Tris-EDTA wash, PVPP, or a combination of PVPP and sepharose 2B gel filtration.

The DNA content was determined from gel images relative to a molecular weight marker of known concentration with UN-SCAN IT software (Silk Scientific, Orem, UT, USA). DNA purity or the decrease in humic acid content was measured by two different detection methods, to determine the accuracy and reproducibility of each method. Humic acid concentration was calculated from standard curves done in triplicate with serial dilutions (0·1–100 ng μl^{-1}) of commercial humic acid (Fluka). Absorbancy readings at 340 nm were measured with a microtitre plate reader (Model 2001; Anthos Labtec Instruments, Salzburg, Austria), while fluorescence (excitation at 460/40 and emission at 560/40) (Kuske *et al.* 1998; Howeler *et al.* 2003) was measured with a microplate fluorescence reader (Model FLx800; Bio-Tek Instruments, Winooski, VT, USA), using a sensitivity value of 75. All DNA concentrations were determined in duplicate and values are given as the mean ± range. Although the humic acid content of extracted DNA was determined with both fluorescence and spectrophotometric readings, more accurate results were obtained with spectrophotometric readings at 340 nm. Therefore, only the A_{340} readings were used for subsequent calculations.

The final optimized environmental extraction and purification protocol was as follows: sieved soil samples were prewashed twice in a 100 mmol l^{-1} of phosphate buffer (pH 8·0) containing 100 mmol l^{-1} of Na_2EDTA (La Montagne *et al.* 2002). A wet weight of 0·5 g of washed sample was added to a 2-ml screw cap tube and processed according to the method of Miller *et al.* (1999), with the following modifications: after NH_4OAc addition and centrifugation, the supernatant was loaded onto a previously packed PVPP column (La Montagne *et al.* 2002). The DNA in the eluant was precipitated with 0·6 volumes of isopropanol at room temperature for 15 min, with subsequent centrifugation (15 000 ***g***) for 5 min. The DNA pellet was dissolved in a suitable volume and applied to a previously packed sepharose 2B (Sigma, St Louis, MO, USA) column and purified (Jackson *et al.* 1997; Miller 2001). The DNA was of sufficient purity for use in molecular techniques, including PCR and restriction digestions.

PCR inhibition assay

The PCR amplification mixture contained: 1 μl of purified DNA, 2 μmol l^{-1} of each primer, 2·5 mmol l^{-1} of each dNTP, 3 mol l^{-1} of dimethylsulfoxide (DMSO) as polymerase enhancer and 2 U of *Taq* DNA polymerase (Promega, Madison, WI, USA) in a final reaction volume of 50 μl. Environmental DNA was amplified with the following nested program: 94°C for 5 min; five cycles consisting of 94°C for 45 s, 61°C for 30 s and 72°C for 2 min; 25 cycles consisting of 94°C for 45 s, 60°C for 30 s and 72°C for 2 min; followed by a final extension cycle of 72°C for 7 min. Primers used in this study were ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) of the fungal 5·8S rDNA gene (White *et al.* 1990), as well as 16S universal bacterial-specific primers UNIV (5′-CCGGATCCGTCGACGTGCCAGCIGCCGCGGTAA-3′) and rPP2 (5′-CCAAGCTTCTAGACGGITACCTTGTTACGACTT-3′) (Rawlings 1995), corresponding to positions (510–529) to (1492–1513) of the *Escherichia coli* 16S rRNA gene sequence, respectively (Brosius *et al.* 1978). Underlined bases are indicative of added restriction sites.

Statistical analysis

A one-way ANOVA of environmental soil samples, comparing DNA yields *vs* extraction methods, were performed to determine if any significant statistical differences occurred amongst methods. Bonferroni and bootstrap tests were performed with STATISTICA version 7·0 (StatSoft, Inc., Tulsa, OK, USA).

Results

Soil content analyses

The cell counts obtained (Table 1) showed a lower value for the nutrient-poor soil (sand 1) compared with the nutrient-rich soils (loamy sands 1 and 2), which correlates well with data described in literature. Miller *et al.* (1999) reported soil cell counts ranging from 1·9 to

11×10^9, which was similar for the data of Frostegård *et al.* (1999) (1·4 to 10×10^9). Sand 1 and loamy sands 1 and 2 gave an almost neutral pH of 6·2, and was therefore considered as optimal for enhanced bacterial activity. Acidic soils contain a large number of free H^+-ions, leading to reduced bacterial activity (Anonymous 2001; accessed 17 January 2005). The loamy sand 1 soil sample had the highest cation exchange capacity (CEC) value, which is indicative of a high nutrient and water holding capacity (Camberato 2001). The CEC of soil refers to its ability to supply major nutrients, such as K^+, Ca^{2+} and Mg^{2+}. It is related to the amount of soil colloids, clay and organic matter, which are negatively charged, thereby enabling soil to retain cations. Therefore, the CEC of soil is affected by changes in pH and salt content, e.g. certain soils suffer aluminium toxicity below pH 5·0 and soil structure is affected at high salinity (Arias *et al.* 2005).

The two loamy sand samples had higher cell counts, organic carbon and nitrogen, clay content and humic acid content, compared with the normal sand sample. Previous studies focused mainly on humic acid contamination following DNA extractions, instead of determining the humic acid content prior to extraction, as this will give an indication on the level of purification that needs to be exercised. An optimal soil pH ranging between 6·0 and 7·0, combined with a high CEC ratio and humic acid content will ultimately yield the highest microbial activity (Camberato 2001).

Optimized environmental DNA extraction and purification protocol from soil

Different DNA extraction and purification methods were screened for high DNA yields and purity with a limited amount of processing time and materials. The different samples were sterile sand, spiked with either *A. pullulans* or *B. pumilus*, as well as three different soil samples. Crude DNA extracts from all the various samples showed great diversity among different extraction methods (Fig. 1). From the gels, it is clear that the most DNA was obtained with bead beating extraction for all the various samples. This method also showed the most fragmented DNA, compared with the microwave-based method. The liquid nitrogen method gave neglible amounts of DNA for all tested samples and therefore proved insufficient (Tables 2 and 3).

From spiked sand, DNA yields of 8·11 $\mu g\ g^{-1}$ sand from *A. pullulans* and 18·56 $\mu g\ g^{-1}$ of sand from *B. pumilus* was extracted with bead beating, which is 90% and 97% more than the microwave-based method, respectively (Table 2). Based on the assumption that a single bacterial cell contains an average DNA content of 9 fg (Tsai and Olson 1991), a DNA concentration of 14·4 $\mu g\ g^{-1}$ of sand

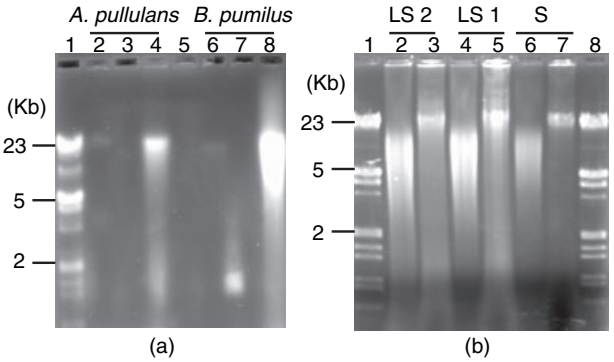

Figure 1 Comparison of DNA extractability with different methods from: (a) control organisms used in spiked sand, and (b) soil samples. Lanes 1A, 1B and 8B: lambda molecular weight marker; lanes 2A, 6A, 3B, 5B and 7B: microwave method; lanes 3A and 7A: liquid nitrogen method; and lanes 4A, 8A, 2B, 4B and 6B: bead beating method. Abbreviations: LS2 – loamy sand 2; LS1 – loamy sand 1; and S – sand. Note: All bead beating crude DNA in (b) are 10-fold dilutions.

was calculated (80% of the actual concentration), which is within the limits of experimental error. A typical yeast cell such as *Saccharomyces cerevisiae* has an average of 20 fg of DNA content (Guthrie and Fink 1991). Using this value for *A. pullulans*, a DNA concentration of 32 $\mu g\ g^{-1}$ of sand was calculated, which is 395% greater than the actual concentration obtained (Table 2). However, the DNA content per cell of *A. pullulans* might be considerably greater than the estimated 20 fg. A single-step microspin purification was sufficient to remove residual humic acid background. The overnight TE wash purification decreased the DNA yield substantially, making it impossible to detect afterwards.

Environmental samples were also subjected to different extraction and purification procedures (Table 3). Bead beating featured as the most prominent extraction method for the three different soil samples, where both the sand 1 and loamy sand 1 samples showed 85%, while loamy sand 2 had 90% higher DNA yields, when compared with the microwave-based method for extraction efficiency. This is consistent with previous studies (Cullen and Hirsch 1998; Miller *et al.* 1999), where bead beating was more superior to other protocols, with respect to yield and total cell lysis, especially for resistant cells like conidia or spores (Bürgmann *et al.* 2001). It is also apparent that the bead beating method extracts more humic substances than the microwave-base method (see crude loamy sands 1 and 2 in Table 3).

Sufficient purification for all three soils occurred only with a combination of PVPP and sepharose 2B. DNA recoveries after purification differed for each sample, where the sand 1 sample showed *c.* 50%; while loamy sands 1 and 2 revealed *c.* 30% and 80% yields, respectively.

Table 2 Extracted DNA and humic acids (HA) of spiked sand samples* after isolation and purification (mean ± range of duplicate values)

Method	Sample	[DNA]† (μg g^{-1})	HA extracted‡ (ng g^{-1})	PCR product§
Bead beating	*Crude A*¶	8·11 ± 0·99	1·19 ± 0·36	–
	Microspin A	7·85 ± 1·35	BDL**	+
	Crude B¶	18·56 ± 2·17	0·05 ± 0·00	–
	Microspin B	16·75 ± 1·60	BDL	+
Microwave-based	*Crude A*	0·95 ± 0·09	0·27 ± 0·05	–
	Microspin A	0·81 ± 0·25	BDL	+
	Crude B	0·57 ± 0·15	0·12 ± 0·05	+
	Microspin B	0·45 ± 0·08	BDL	+
Liquid nitrogen grinding	*Crude A*	BDL	0·82 ± 0·06	+
	Microspin A	BDL	BDL	+
	Crude B	0·09 ± 0·09	0·38 ± 0·16	+
	Microspin B	BDL	BDL	+

*, Sterile, acid-washed sand containing either bacterium or yeast added at $1{\cdot}6 \times 10^9$ cells g^{-1}.
†, [DNA] in μg g^{-1} of soil was determined by band density, relative to standard with UN-SCAN-IT software.
‡, HA content in ng g^{-1} of soil was calculated from a HA standard of known concentration.
§, PCR amplification was done with either ITS (fungal) or 16S (bacterial) primers.
¶, Samples listed with the letters A or B denotes *Aureobasidium pullulans* or *Bacillus pumilus*, respectively.
**, BDL – below detection limit [less than 0·01 μg g^{-1} (DNA) or 0·01 ng g^{-1} (HA)].

Therefore, comparison of purification methods indicated that only a combination of two or more methods render sufficiently pure DNA from humic acid-rich environments, such as soil.

A one-way ANOVA of DNA yields *vs* extraction methods were performed to determine if there were significant differences amongst methods ($n = 6$; 95% confidence). The null hypothesis of no differences were rejected with $F = 65$ ($P = 0$), indicating a significant difference amongst methods. The Bonferroni test showed significant difference between bead beating when compared with the other two methods ($P = 0$). However, the residuals were not normally distributed as confirmed by the normal probability test. Therefore, a bootstrap test was done to confirm that bead beating differed significantly from the other two methods ($P = 0{\cdot}003$).

Discussion

Several environmental DNA isolation and purification strategies have previously been investigated with variable rates of success (Cullen and Hirsch 1998; Miller *et al.* 1999; Tien *et al.* 1999). In this study, several approaches have been investigated to determine an improved DNA extraction and purification protocol from different humic acid-rich soils. According to Zhou *et al.* (1996), every type of environmental sample, because of its own nature, requires optimization of the extraction methods used. In addition, the efficiency of a soil microbial DNA extraction depends on soil quality, particularly on its clay and organic matter contents because micro-organisms can interact with soil colloids, such as clay-organic aggregates (Roose-Amsaleg *et al.* 2001).

Higher cell counts are directly proportional to high organic carbon and nitrogen, clay content and humic acid content. Therefore, soil characteristics that need to be taken into account for optimal DNA extractions include pH, organic matter, clay and silt content (Fortin *et al.* 2004), as these will inevitably also indicate high microbial activity.

Humic substances can compete with nucleic acids for adsorption sites during purification with minicolumns (Harry *et al.* 1999). They are three-dimensional structures that have the ability to bind other compounds to their reactive functional groups and absorb water, ions and organic molecules (Stevenson 1976). Therefore, almost all natural organic compounds can become bound or absorbed to humic substances (Fortin *et al.* 2004). Because humic acids have physicochemical properties that are similar to nucleic acids, they are generally co-extracted with environmental DNA. It is very difficult to remove humic acids and phenolic compounds in soil or sediment, and therefore only a combination of purification methods can yield DNA of sufficient purity for successful molecular applications (Harry *et al.* 1999; Roose-Amsaleg *et al.* 2001). Successful PCR amplification is generally used as an indicator of suitable soil DNA purity, as indicated by literature (e.g. Cullen and Hirsch 1998; Moreira 1998; Bürgmann *et al.* 2001). However, a more suitable purity marker would have to be successful

Table 3 Extracted DNA and humic acids (HA) of environmental samples* after isolation and purification (mean ± range of duplicate values)

Method	Sample	[DNA]† (μg g^{-1})	HA extracted‡ (ng g^{-1})	PCR product§
Bead beating	*Crude (sand)*	58·7 ± 6·68	200·0 ± 16·6	–
	Microspin purified	31·4 ± 3·61	33·1 ± 0·70	–
	TE wash purified	1·27 ± 0·22	BDL¶	–
	PVPP	30·6 ± 3·51	30·1 ± 0·24	–
	PVPP and sepharose 2B	29·1 ± 3·42	0·10 ± 0·02	+
	Crude (loamy sand 1)	95·6 ± 10·8	540·2 ± 23·3	–
	Microspin purified	36·7 ± 4·45	10·3 ± 1·13	–
	TE wash purified	4·60 ± 0·66	BDL	–
	PVPP	45·3 ± 5·42	31·0 ± 0·20	–
	PVPP and sepharose 2B	28·2 ± 1·19	BDL	+
	Crude (loamy sand 2)	63·5 ± 7·22	351·6 ± 25·5	–
	Microspin purified	53·9 ± 6·22	24·4 ± 0·67	–
	TE wash purified	7·28 ± 1·06	BDL	–
	PVPP	59·2 ± 8·67	59·2 ± 5·03	–
	PVPP and sepharose 2B	49·3 ± 6·20	BDL	+
Microwave-based	*Crude (sand)*	8·57 ± 0·96	353·1 ± 38·8	–
	Microspin purified	6·75 ± 0·84	23·9 ± 0·50	–
	TE wash purified	BDL	BDL	–
	PVPP	6·31 ± 0·78	81·9 ± 3·86	–
	PVPP and sepharose 2B	0·01 ± 0·00	1·55 ± 0·07	+
	Crude (loamy sand 1)	13·7 ± 1·53	300·5 ± 10·7	–
	Microspin purified	5·15 ± 0·63	11·9 ± 0·13	–
	TE wash purified	0·04 ± 0·06	BDL	–
	PVPP	5·50 ± 0·67	71·6 ± 14·1	–
	PVPP and sepharose 2B	0·21 ± 0·08	0·50 ± 0·09	+
	Crude (loamy sand 2)	7·21 ± 0·80	244·0 ± 10·4	–
	Microspin purified	7·11 ± 0·89	58·8 ± 0·59	–
	TE wash purified	BDL	BDL	–
	PVPP	6·78 ± 1·01	59·3 ± 0·41	–
	PVPP and sepharose 2B	0·55 ± 0·11	BDL	+
Liquid nitrogen grinding	*Crude (sand)*	2·40 ± 1·47	0·92 ± 0·26	–
	Microspin purified	BDL	BDL	–
	TE wash purified	ND**	ND	ND
	PVPP	BDL	BDL	ND
	PVPP and sepharose 2B	BDL	BDL	ND
	Crude (loamy sand 1)	0·25 ± 0·12	2·95 ± 0·22	–
	Microspin purified	BDL	BDL	–
	TE wash purified	ND	ND	ND
	PVPP	BDL	0·95 ± 0·15	ND
	PVPP and sepharose 2B	BDL	BDL	ND
	Crude (loamy sand 2)	0·45 ± 0·10	13·9 ± 3·25	–
	Microspin purified	BDL	1·44 ± 0·15	–
	TE wash purified	ND	ND	ND
	PVPP	BDL	1·61 ± 0·13	ND
	PVPP and sepharose 2B	BDL	BDL	ND

*, Soil containing different cell counts g^{-1} (see Table 1).
†, [DNA] in μg g^{-1} of soil was determined by band density, relative to standard with UN-SCAN-IT software.
‡, HA content in ng g^{-1} of soil was calculated from a HA standard of known concentration.
§, PCR amplification was done with 16S (bacterial) primers.
¶BDL – below detection limit [less than 0·01 μg g^{-1} (DNA) or 0·01 ng g^{-1} (HA)].
**, ND – not determined.
PVPP, polyvinylpolypyrrolidone.

Table 4 Environmental DNA yields: estimated *vs* actual from bead beating extraction (see Table 3)

Sample	Estimated (μg g^{-1})*	Actual (μg g^{-1})†	Bacterial DNA content (fg cell^{-1})	Yield (%)‡	PCR product§
Sand	3·60	58·7	146·8	1630	+
Loamy sand 1	122·4	95·6	7·03	78	+
Loamy sand 2	28·8	63·5	19·8	220	+

*, Based on assumption that one cell contains an average of 9 fg of DNA (according to Miller *et al.* 1999).
Cell counts were done with 4′,6-diamidino-2-phenylindole (DAPI) staining according to Miller *et al.* (1999).
†, [crude DNA] × total volume × total sample weight.
‡, Crude DNA obtained relative to expected DNA.
§, PCR amplification was done with 16S (bacterial) primers.

cloning of such DNA, as the PCR protocol can be sufficiently optimized by addition of enhancers, such as DMSO and bovine serum albumin (BSA), as well as the use of high-activity *Taq* polymerases. Therefore, a detailed knowledge of the contaminant inhibitory effect from environmental DNA on molecular methods is essential when deciding on a suitable extraction and purification strategy (Robe *et al.* 2003).

The expected DNA content of environmental samples was calculated, based on the assumption that a bacterial cell contains on average of 9 fg of DNA (Tsai and Olson 1991) and compared with the obtained values (Table 4). The actual DNA extraction efficiencies of sand 1 and loamy sand 2 samples were considerably greater than that estimated from DAPI staining direct cell counts (Table 1). According to Miller *et al.* (1999), extraction efficiencies greater than 100% are indicative of eukaryotic, plant and animal DNA. Therefore, nonbacterial DNA pools also need to be quantified in order to get a more accurate assessment of the environmental (soil) diversity (Miller *et al.* 1999). However, there is still no available means for the quantification of total DNA content in soil (Bürgmann *et al.* 2001). Stach *et al.* (2001) also cautions that a greater DNA quantity recovered does not always equal a greater species richness, demonstrating that there is always the possibility that extracted DNA might be from mainly easily lysed cell types (Stach *et al.* 2001). Notwithstanding this, the complete cell lysis from environmental samples is still unattainable (Miller *et al.* 1999).

In conclusion, several environmental DNA isolation and purification strategies have previously been investigated with variable rates of success (Cullen and Hirsch 1998; Miller *et al.* 1999; Tien *et al.* 1999). The present study established bead beating as the most suitable DNA extraction protocol, confirming the observation by Howeler *et al.* (2003) that 95% of native cells are lysed via this method (Howeler *et al.* 2003). Sample washing prior to cell lysis was included in the optimized extraction protocol as it reduced extracellular DNA and soluble organic contaminants (La Montagne *et al.* 2002). It also decreased the amount of purification steps required, as a single PVPP-sepharose 2B column elution was sufficient to retain 80% DNA for the loamy sand 2 soil. Cullen and Hirsch (1998) followed a similar purification approach, except that they used sephadex G-75 as purification matrix instead of sepharose 2B used in the current study. According to Miller (2001), sepharose resins are more efficient than sephadex resins at purifying humic acids away from DNA in soil and sediment extracts, with sepharose 2B as the most efficient purification resin (Miller 2001). In addition, this study also employed an initial soil-washing step, thereby differentiating itself for purification of DNA isolated from humic acid-rich environments.

Purified environmental DNA was successfully used in generating a clone library that contains *c.* 5×10^5 CFU ml^{-1} (results not shown). Therefore, it can be deduced that the optimized extraction and purification protocol designed, will render DNA of sufficient purity from almost any soil type, including compost- and plant litter-containing environments. The purification protocol was also successfully applied to DNA obtained from kudu rumen fluid contents and dung beetle larvae viscera, yielding *E. coli* clones after $CaCl_2$-transformation (unpublished results).

This study therefore succeeded in establishing an optimized soil DNA extraction and purification protocol that can be applied to various environmental sources that are rich in humic acid content. The total duration of DNA extraction and subsequent purification from 12 × 0·5 g of samples lasted *c.* 2·0–2·5 h.

Acknowledgements

This work was financially supported by the National Research Foundation (NRF). We thank Dr F. Ellis for identification of soil types, Prof. D. Nel for statistical comparisons and BEMLab for soil analysis.

References

Anonymous (2000) *Munsell Soil Color Charts*. New Windsor, NY: Gretag-Macbeth.

Anonymous (2001) *COG Organic Field Crop Handbook*. Canadian Organic Growers, Inc. Website address: http://www.eap.mcgill.ca/MagRack/COG/COGHandbook/COGHandbook_1_3.htm.

Arias, M.E., González-Pérez, J.A., González-Vila, F.J. and Ball, A.S. (2005) Soil health – a new challenge for microbiologists and chemists. *Int Microbiol* **8**, 13–21.

Beyers, C.P. and Coetzer, F.J. (1971) Effect of concentration, pH and time on the properties of di-ammonium EDTA as a multiple soil extractant. *Agrochemophysica* **3**, 49–54.

Braid, M.D., Daniels, L.M., Kitts, C.L. (2003) Removal of PCR inhibitors from soil DNA by chemical flocculation. *J Microbiol Methods* **52**, 389–393.

Bremner, J.M. (1965) Inorganic forms of nitrogen. In *Methods of Soil Analysis*. Part 2 ed. Black, C.A. pp. 1179–1237. Madison, WI: American Society of Agronomy.

Brosius, J., Palmer, J.L., Kennedy, H.P. and Noller, H.F. (1978) Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* **75**, 4801–4805.

Bürgmann, H., Pesaro, M., Widmer, F. and Zeyer, J. (2001) A strategy for optimizing quality and quantity of DNA extracted from soil. *J Microbiol Methods* **45**, 7–20.

Camberato, J.J. (2001) *Cation exchange capacity – everything you want to know and so much more*. Website address: http://virtual.clemson.edu/groups/turfornamental/tmi/fert-lime/Cation%20-exchange%20capacity.pdf.

Chandler, D.P., Schreckhise, R.W., Smith, J.L. and Bolton, H. Jr. (1997) Electroelution to remove humic compounds from soil DNA and RNA extracts. *J Microbiol Methods* **28**, 11–19.

Cullen, D.W. and Hirsch, P.R. (1998) Simple and rapid method for direct extraction of microbial DNA from soil for PCR. *Soil Biol Biochem* **30**, 983–993.

Doll, E.C. and Lucas, R.E. (1973) Testing soils for potassium, calcium and magnesium. In *Soil Testing and Plant Analysis* ed. Walsh, L.M. and Beatons, J.D. pp. 133–152. Madison, WI: Soil Science Society of America.

Fortin, N., Beaumier, D., Lee, K. and Greer, C.W. (2004) Soil washing improves the recovery of total community DNA from polluted and high organic content sediments. *J Microbiol Methods* **56**, 181–191.

Frostegård, Å., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., Jeannin, P., Nesme, X. *et al.* (1999) Quantification of bias related to the extraction of DNA directly from soils. *Appl Environ Microbiol* **65**, 5409–5420.

Guthrie, C. and Fink, G.R. (1991) Guide to yeast genetics and molecular biology. *Methods Enzymol* **194**, 3–21. New York: Academic Press.

Harry, M., Gambier, B., Bourezgui, Y. and Garnier-Sillam, E. (1999) Evaluation of purification procedures for DNA extracted from organic rich samples: interference with humic substances. *Analysis* **27**, 439–442.

Holben, W.E., Jansson, J.K., Chelm, B.K. and Tiedje, J.M. (1988) DNA probe method for the detection of specific microorganisms in the soil bacterial community. *Appl Environ Microbiol* **54**, 703–711.

Howeler, M., Ghiorse, W.C. and Walker, L.P. (2003) A quantitative analysis of DNA extraction and purification from compost. *J Microbiol Methods* **54**, 37–45.

Hurt, R.A., Qiu, X., Wu, L., Roh, Y., Palumbo, A.V., Tiedje, J.M. and Zhou, J. (2001) Simultaneous recovery of RNA and DNA from soils and sediments. *Appl Environ Microbiol* **67**, 4495–4503.

Jackson, C.R., Harper, J.P., Willoughby, D., Roden, E.E. and Churchill, P.F. (1997) A simple, efficient method for the separation of humic substances and DNA from environmental samples. *Appl Environ Microbiol* **63**, 4993–4995.

Kauffmann, I.M., Schmitt, J. and Schmid, R.D. (2004) DNA isolation from soil samples for cloning in different hosts. *Appl Microbiol Biotechnol* **64**, 665–670.

Knietsch, A., Waschkowitz, T., Bowien, S., Henne, A. and Daniel, R. (2003) Metagenomes of complex microbial consortia derived from different soils as sources for novel genes conferring formation of carbonyls from short-chain polyols on *Escherichia coli*. *J Mol Microbiol Biotechnol* **5**, 46–56.

Kuske, C.R., Banton, K.L., Adorada, D.L., Stark, P.C., Hill, K.K. and Jackson, P.J. (1998) Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. *Appl Environ Microbiol* **64**, 2463–2472.

La Grange, D.C., Pretorius, I.S. and Van Zyl, W.H. (1997) Cloning of the *Bacillus pumilus* β-xylosidase gene (*xynB*) and its expression in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **47**, 262–266.

La Montagne, M.G., Michel, F.C. Jr., Holden, P.A. and Reddy, C.A. (2002) Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. *J Microbiol Methods* **49**, 255–264.

McClean, E.O. (1982) Soil pH and lime requirement. In *Methods of Soil Analysis*. Part 2 ed. Page, A.L. pp. 570–571. Madison, WI: American Society of Agronomy.

Miller, D.N. (2001) Evaluation of gel filtration resins for the removal of PCR-inhibitory substances from soils and sediments. *J Microbiol Methods* **44**, 49–58.

Miller, D.N., Bryant, J.E., Madsen, E.L. and Ghiorse, W.C. (1999) Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl Environ Microbiol* **65**, 4715–4724.

Moreira, D. (1998) Efficient removal of PCR inhibitors using agarose-embedded DNA preparations. *Nucleic Acids Res* **26**, 3309–3310.

Nelson, D.W. and Sommers, L.E. (1982) Total carbon, organic carbon and organic matter. In *Methods of Soil Analysis*.

Part 2 ed. Page, A.L. p. 199. Madison, WI: American Society of Agronomy.

Orsini, M. and Romano-Spica, V. (2001) A microwave-based method for nucleic acid isolation from environmental samples. *Lett Appl Microbiol* **33**, 17–20.

Rawlings, D.E. (1995) Restriction enzyme analysis of 16S rRNA genes for the rapid identification of *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans* strains in leaching environments. In *Biohydrometallurgical Processing*, vol. 2 ed. Jerez, C.A., Vargas, T., Toledo, H. and Wiertz, J.V. pp. 9–17. Santiago, Chile: University of Chile Press.

Robe, P., Nalin, R., Capellano, C., Vogel, T.M. and Simonet, P. (2003) Extraction of DNA from soil. *Eur J Soil Biol* **39**, 183–190.

Roose-Amsaleg, C.L., Garnier-Sillam, E. and Harry, M. (2001) Extraction and purification of microbial DNA from soil and sediment samples. *Appl Soil Ecol* **18**, 47–60.

Saano, A. and Lindstrom, K. (1995) Small-scale extraction of DNA from soil with spun column cleanup. In *Molecular Microbial Ecology Manual* ed. Akkermans, A.D.L., van Elsas, J.D. and De Bruijn, F.J. pp. 1.3.4:1–1.3.4:6. Dordrecht: Kluwer Academic Publishers.

Stach, J.E.M., Bathe, S., Clapp, J.P. and Burns, R.G. (2001) PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. *FEMS Microbiol Ecol* **36**, 139–151.

Stevenson, F.J. (1976) Stability constants of Cu, Pb and Cd complexes with humic acids. *Soil Sci Soc Am J* **40**, 665–672.

Swift, R.S. (1996) Organic matter characterization. In *Methods of Soil Analysis*, Part 3 ed. Sparks, D.L., Page, A.L., Helmke, P.A., Loeppert, R.H., Soltanpour, P.N., Tabatabai, M.A., Johnston, C.T. and Sumner, M.E. pp. 1011–1069. SSSA Book Series no. 5. Madison, WI: SSSA and ASA.

Thomas, G.W. and Peaslee, D.E. (1973) Testing for soil phosphorus. In *Soil Testing and Plant Analysis* ed. Walsh, L.M. and Beatons, J.D. pp. 15, 122. Madison, WI: Soil Science Society of America.

Tien, C.C., Chao, C.C. and Chao, W.L. (1999) Methods for DNA extraction from various soils: a comparison. *J Appl Microbiol* **86**, 937–943.

Tsai, Y. and Olson, B.H. (1991) Rapid method for direct extraction of DNA from soil and sediments. *Appl Environ Microbiol* **57**, 1070–1074.

Tsai, Y. and Olson, B.H. (1992) Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Appl Environ Microbiol* **58**, 2292–2295.

Volossiouk, T., Robb, E.J. and Nazar, R.N. (1995) Direct DNA extraction for PCR-mediated assays of soil organisms. *Appl Environ Microbiol* **61**, 3972–3976.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications* ed. Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. pp. 315–322. New York: Academic Press, Inc.

Wikström, P., Wiklund, A., Andersson, A. and Forsman, M. (1996) DNA recovery and PCR quantification of catechol 2,3-dioxygenase genes from different soil types. *J Biotechnol* **52**, 107–120.

Young, C.C., Burghoff, R.L., Keim, L.G., Minak-Bernero, V., Lute, J.R. and Hinton, S.M. (1993) Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. *Appl Environ Microbiol* **59**, 1972–1974.

Zhou, J., Bruns, M.A. and Tiedje, J.M. (1996) DNA recovery from soils of diverse composition. *Appl Environ Microbiol* **62**, 316–322.