An improved extraction procedure of DNA from the "El Halassa" phosphate deposit (Khouibga, Morocco)

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***Abstract*— The direct DNA extraction from Moroccan phosphate can lead to further research proposals that will especially shed light on the microbial diversity in such environment. Since there is no universally applicable DNA extraction method, this study aimed to improve an applicable DNA extraction method to make it more adaptable to the endemic characteristics of the Moroccan phosphate deposit.**

A total of eleven phosphate composite samples were collected from El Halassa phosphate deposit, and three Soil samples were used for the DNA extraction procedures. We performed a comparison between the commonly used protocol and the proposed one in terms of DNA yield and purity. The proposed method is based on a combination of cell dispersion and ultrasonication treatments in the presence of SDS, proteinase K and CTAB.

Our results have shown that the commonly used protocol l was not efficient for the extraction of DNA from the phosphate samples. Only 0,62±0,007 to 5,08 ± 0.007 µg DNA.g-1 was obtained in phosphate deposit versus 36.18±14,2 to 78.81±40,6 µg DNA.g-1 in soil samples. However, the new combined approach has significantly increased DNA yields from phosphate deposit samples ranging from 19,36 ± 3 to 57,19 ± 26,87µg DNA.g-1, making possible its detection by the agarose gel electrophoresis. The improved method produced DNA with quietly similar purity to that produced by commonly used extraction methods from environmental samples. We have also noted the absence of the brownish color of the crude DNA solutions reported by many studies.

This study accomplished its stated goal in extracting DNA from a new complex environment using a combined in situ lysis treatments and can be considered as a robust method more appropriate for complex environment where bacterial cells are not easy to reach.

Keywords: Moroccan Phosphate deposi; Extraction method DNA; Yields; Purity.

I. INTRODUCTION

Direct extraction of nucleic acids from environmental sources is motivated by the widespread use of molecular techniques, allowing the emergence of diverse and innovative studies. DNA, RNA messenger and ancient DNA have been extracted from various environments [1,2,3], such as soil [4,5], Thermal environments [6], marine sediments [7], activated sludge [8], Biofilms [9], volcanic environments [10] and art objects. Thus, soil DNA has been used to detect DNA adducts and to determine mutagenic compounds present in soil without the need for analytical chemistry [11], and also to obtain genetic profiles from past environments [3]. But the most application of environmental DNA remains the study of natural microbial communities without the need for cultivation [12, 13]. Current estimates indicate that more than 99% of the microorganisms present in many natural environments are not readily culturable [14, 15, 16], and the development of methods to isolate nucleic acids from environments has opened a window to a previously unknown diversity of microorganisms.

There are many published methods for extracting DNA from soil and sediment samples [17,18,19]. Commercial Kits are also available for extraction and purification of soil DNA. However, none of the methods is universally applicable and every type of environmental samples, because of its own nature, requires optimization of DNA extraction methods [4,10,20]. All methods developed for extracting environmental DNA are based on two basic approaches: cell extraction methods and *in situ* lysis extraction methods. Comparative studies showed that the second one provides, from small sample size, less biased microbial DNA with higher yields compared to protocols based on indirect cell lysis [21, 7, 22, 23, 24]. The *in situ* lysis of cells involves chemical and/or enzymatic and/or mechanical treatments, such as bead-beating, sonication, lysozyme, proteinase K, SDS … [25,26, 27, 28]. Advantages and disadvantages of using various combinations of these treatments have also been studied [29]. Generally, combination of treatments should be required, according to samples properties, to obtain the DNA in high yield.

DNA extraction from environmental samples results in the co-extraction of contaminants mainly humic acids and polysaccharides that exhibit similar solubility properties to DNA [30,31,32]. Some of these contaminants affect almost all molecular biological methods and could inhibit polymerase chain reaction (PCR) amplification by chelating the mg^{2+} or by binding to target DNA [33, 34]. Many methods have been used to remove these inhibitors from environmental samples and to obtain purified DNA. Some of the post-extraction purification methods include electroelution [35], Sephadex™ spin column [36] size exclusion chromatography [37] and silica-based DNA binding [38]. Simultaneous purification and extraction procedures are also used such as the addition of polyvinylpolypyrrolidone (PVPP),

hexadecyltrimethylammonium bromide (CTAB) and bovine serum albumin (BSA) to the lysis buffer [39,4, 40].

Extraction of DNA from phosphate deposit samples will permit, through molecular microbiology methods, to understand the diversity and characteristics of microbial life that they harbour and to identify potential functional genes. In fact, Moroccan phosphate deposits are worldwide known. Morocco has ¾ of the world phosphate reserves; it is the first exporter in the world and the second producer after the USA [41]. Unfortunately, very few microbiological studies on phosphate deposit have been carried out. Iddar et al. [42] have studied the elevated of inorganic phosphate concentrations on primary metabolism of a *bacillus cereus* strain isolated from a phosphatic layer containing more than 65% w/w belonging to basin phosphate of Khouribga (Morocco). They revealed a phosphate-stimulated $NAD(P)^+$-dependent GAPDH in *B. cereus*, which indicates that this bacterium can modulate its primary carbon metabolism according to phosphate availability. Moroccan phosphate mines are also sources of Actinobacteria showing abilities to solubilize insoluble natural phosphate rock [43]. These strains are related to *streptomyces griseus* and *Micromonospora antiaca* and were isolated from three different phosphate mines centres in Morocco: Benguerir, Khouribga and Youssoufia. They have also shown multiple plant growth properties under laboratory conditions [44].

The aim of the present study was to optimize a DNA extraction method adapted to the nature of Moroccan phosphate deposit. We also investigated both efficiency and purity of DNA isolated by our improved method and also its detection by electrophoresis in comparison with the most cited protocol of DNA extraction from soils and sediments [4] (Zhou et al., 1996).

II. MATERIALS AND METHODS

A. Phosphate deposit sampling

El HALASSA phosphate deposit site (32°40'60N, 6°49'60W) is within the phosphate basin of the province of Khouribga about 12 Km south-west of it. The sampling site consists of a phosphate reserve deposit made up of 3 major phosphatic layers.

A total of eleven composite Samples (from P1 to P11) were collected randomly in aseptic conditions using sterile bags of the raw phosphatic layers from the EL HALASSA deposit then taken back to our laboratory at the temperate of 4°C where sieved under sterile conditions (2mm nominal pore size) and kept at -22°C

B. . Soil sampling

A composite soil samples were used to evaluate the efficiency of DNA extraction procedures according to the samples properties. Three samples S1; S2 and S3 were collected under the ground at the depth of 0 – 20 cm, 20 – 40 cm and 60 – 80 cm respectively from an agriculture area near the University of Settat. These samples were stored at -22°C during the experiment period.

C. DNA extraction procedures

The Zhou protocol:

Soil and phosphate samples of 5 g were mixed with 13.5 ml of DNA extraction buffer (100 mM Tris-HCL [pH 8.0], 1.5 M NaCL,) and 100 µl of proteinase K (10 mg/ml) in Oakridge tubes by horizontal shaking at 225rpm for 30 min at 37°C. After the shaking treatment, 1.5 ml of 20% SDS was added, and the samples were incubated in a 65°C for 2 h with gentle end-over-end inversion every 15 to 20 min. The supernatant were collected after centrifugation at 6,000 x g for 10 min at room temperature and transferred into 50-ml centrifuge tubes. The soil pellets were extracted two more times by adding 4.5 ml of the extraction buffer and 0.5 ml of 20% SDS, vortexing for 10 s, incubating at 60°C for 10 min. and centrifuging as before. Supernatants from three cycles of extractions were combined and mixed with an equal volume of chloroform-isoamylalcohol (24:1, vol/vol). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1h. The pellet of crude nucleic acids was obtained by centrifugation at 16,000xg for 20 min at room temperature, washed with cold 70% ethanol, and resuspended in sterile deionized water, to give a final volume of 500 ml.

The improved protocol:

In order to obtain the sample mixture, 5g of samples was mixed with 13,5 mL of DNA extraction buffer (100mM tris pH 8,0 ; 100 mM Sodium EDTA pH 8,0 ; 1,5 M NaCl ; 1% CTAB. A first dispersion was carried out using an ultrasonic bath FRITSCH during 30min. we added 100µL of proteinase K (10mg/mL) and we incubated the mixture at 37°C for 30min. a second mechanical treatment consisted of ultasonication lysis in a glace bath using SONOPLUS (Bundelin) HD2070 max power during 3 min. we added immediately 1,5mL of Sodium Dodecyl Sulfate SDS 20% and we incubated at 65°C for 2hours with gentle inversion each 20 minutes. Samples mixtures were then extracted by centrifugation at 6000g for 10 minutes and the supernatants were collected. They were mixed with an equal volume of phenol– chloroform–isoamyl alcohol (25:24:1, vol/vol). The aqueous phase was recovered by centrifugation and precipitated with 0,6 volume of isopropanol at -20°C for 2 hours. The pellet of crude nucleic acids was obtained by centrifugation at 16000g for 20 minutes at room temperature washed with cold 70% ethanol 2 times and re-suspended in sterile deionized water, to bring a final volume of 3ml.

D. Evaluation and quantitative analysis of total DNA

The DNA purity was estimated spectrophotometrically using the NANODROP 8000 (Thermo Scientific), by calculating A_{260}/A_{280} and A_{260}/A_{230} ratios for respectively protein evaluation and humic compounds impurities [45]. Generally, the A_{260}/A_{280} and A_{260}/A_{230} of pure DNA are about 1,7 and more than 2 respectively.

DNA concentrations and yields were also determined by the Nanodrop spectrophotometer, about one unit of 260nm equals to 50µg (double chains DNA)/mL.

The detection of DNA isolated from all samples was run on agarose gel electrophoresis (0,8%). The gel was stained with 0,1µg of ethidium bromide and revealed under UV light Transilluminator [45].

E. Statistical analysis

Statistical analyses were performed using SPSS 13 (for windows) and Microsoft office excel 2003 software. Monofactorial analysis of variance (ANOVA) was used to detect statistical differences in the yields and purity of DNA.

III. RESULTS AND DISCUSSIONS

A. DNA extraction by Zhou et al. method

The above mentioned Zhou technique has been applied twice to extract the DNA respectively from the phosphate and the agricultural soil samples collected DNA yield Results are reported in the table 1.

TABLE 1: COMPARATIVE DATA OF THE DNA CRUDE YIELDS ISOLATED BY THE ZHOU PROTOCOL FROM AGRICULTURAL SOIL SAMPLES AND PHOSPHATE SAMPLES USING SPECTROFLUOROMETRIC DETERMINATIONS.

Samples	**OD**				**DNA yield µg/g**
	260 nm	***280 nm***	***A260/A280***	***A260/A230***	
Soil samples:					
S1	0,64	0,42	1,64 (±0,4)	0,73 (±0,1)	46,88 (±20,6)
S2	1,87	1,37	1,4 (±0,07)	1,21 (±0,02)	78,81 (±40,6)
S3	0,51	0,32	1,5 (±0,09)	1,41 (±0,19)	36,18 (±14,2)
Phosphate samples:					
P1	0,128	0,173	0,74 (±0,16)	1,4 (±1,06)	0,62 (±0,007)
P2	0,167	0,169	1,03 (±0,007)	4,38 (±5,21)	0,87 (±0,05)
P3	1,016	0,756	1,34 (±0,004)	1,28 (±0,05)	5,08 (±0,007)
P4	0,162	0,139	1,175 (±0,03)	1,41 (±1,17)	0,81 (±0,16)

Values in the table are mean of two replicate samples with the standard deviations in parentheses.

A highly statistically significant difference was evidenced while analyzing the DNA concentrations obtained using the zhou protocol according to the sample nature ($p<0,01$). Indeed, DNA crude yields are ranged from 36.18 ± 14,2 to 78.81 ± 40,6 $\mu g.g^{-1}$ in agricultural soil samples and from 0,62 ± 0,007 to 5,08 ± 0.007 $\mu g.g^{-1}$ in phosphate deposit samples. However, gel electrophoresis did not reveal the extracted DNA in phosphate samples.

Consequently, the DNA extraction method strongly depends on the sample nature and its properties. The Zhou protocol, based only on an enzymatic lysis in a high salt extraction buffer and extended heating seems to be not adapted to the nature of phosphate samples. Several factors can limit the efficiency of direct DNA extraction such as incomplete cell lysis, physicochemical characteristics of the samples and adhesion and adsorption of autochthonous bacteria to the deposit particles [46]. Thus, for these samples, more vigorous extraction protocol may overcome these difficulties.

B. DNA extraction by our improved protocol

The combination of mechanic lysis (e.g. cell dispersion and ultrasonication) significantly increased DNA yields from phosphate deposit samples. As represented in figure 1 our improved protocol displayed DNA extraction yields ranging from 19,36±3 to 57,19±26,87$\mu g.g^{-1}$ in the samples P8 and P10 respectively.

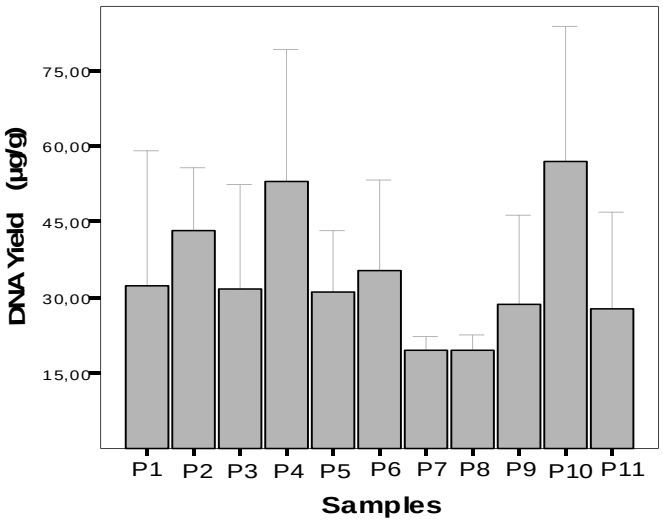

FIGURE 1: THE DNA CRUDE YIELDS OBTAINED WITH THE IMPROVED PROTOCOL USING SPECTROFLUOROMETRIC DETERMINATIONS.
P1 – P11: PHOSPHATE DEPOSIT SAMPLES

For all samples, the extracted DNA was detected by agarose gel electrophoresis as shown in Figure 2.

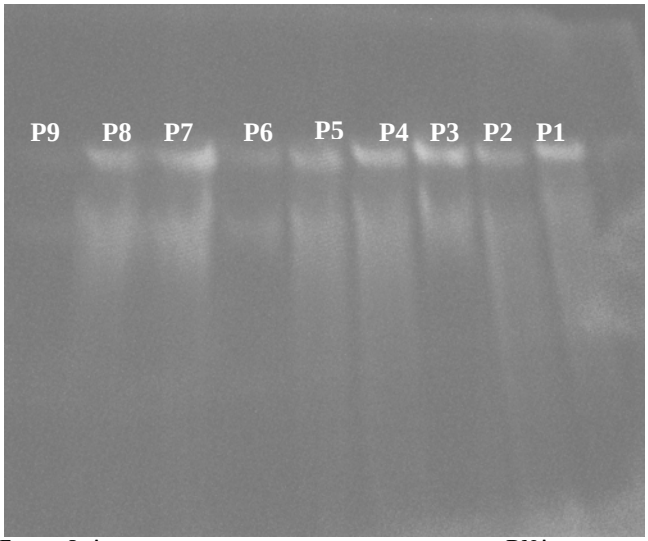

FIGURE 2: AGAROSE GEL ELECTROPHORESIS OF THE TOTAL DNA ISOLATED PHOSPHATE SAMPLES BY THE IMPROVED METHOD
(P1 – P9: PHOSPHATE SAMPLES)

Geologically, the phosphate deposit starts by Maastrichtian phosphatic marls overlain by uncemented phosphatic layers and limestones containing many bony debris [47]. Palaeocene is mainly composed of uncemented phosphates; its submittal

part corresponds to a level of limestones formed of coprolites and silex nodules [48]. Eocene corresponds to an alternation of uncemented phosphates levels, phosphatic marly limestones, discontinuous horizons of silex and silto-pelitic levels. The whole sequence is characterized by characterized by a hard rock structure. The presence of mineral matrix in phosphate deposit containing organic matter, silex and clay particles [49] can lead in certain cases to complete microbial cell sequestration in this hard mineral matrix. Consequently first difficulty in DNA extraction is actually to access the cells and DNA which is trapped in the matrix [50]. The following table 2 summarizes some properties of our phosphate deposit samples:

TABLE 2 : PHYSICAL PROPERTIES AND BACTERIAL COUNT OF THE STUDIED PHOSPHATE DEPOSIT SAMPLES .

Samples	Amt (%) of [a]		Moisture [b] content %	pH[c]	%OM [d]	Bacterial count [e] (CFU x 10^5)
	Sand	*Silt + Clay*				
P1	90,25(±4,5)	9,95(±4,6)	12,78(±0,3)	7,14(±0,07)	2,83(±0,2)	1,77(±2,2)
P2	92,43(±6,4)	4,53(±1,4)	15,31(±3,5)	7,47(±0,5)	2,70(±0,2)	1,72(±0,3)
P3	92,96(±2,8)	7,26(±2,3)	4,14(±3,3)	7,87(±0,1)	2,35(±0,3)	2,44(±1,4)
P4	93,84(±2,09)	6,48(±1,9)	6,68(±1,4)	7,73(±0,2)	2,61(±0,6)	2,42(±0,04)
P5	93,92(±4,3)	6,68(±4,1)	6,54(±0,2)	7,19(±0,2)	2,93(±0,3)	1,48(±0,6)
P6	95,19(±2,2)	4,98(±2,1)	7,07(±2,2)	7,03(±0,4)	2,12(±0,2)	3,74(±0,6)
P7	92,2(±4,2)	5,61(±3,8)	7,35(±3,3)	7,37(±0,17)	2,37(±0,05)	2,21(±1,07)
P8	91,83(±3,06)	4,65(±0,9)	7,34(±2,6)	7,73(±0,01)	2,93(±0,1)	1,37(±0,1)
P9	95,05(±0,8)	4,96(±1,4)	8(±2,1)	7,66(±0,4)	2,09(±0,09)	2,58(±0,3)
P10	92,45(±4,2)	5,45(±1,9)	6,57(±2,8)	7,56(±0,2)	2,66(±0,22)	2,08
P11	96,91	3,08	14,01	7,47	3,09	2,53(±1,09)

Values in the table are mean of three replicate samples with the standard deviations in parentheses

a) Assessment of soil particle size by sieving to separate sand fractions and fine-grained fractions (silt + clay)

b) Moisture contents were determined by drying at 110°C for 48h

c) pH was determined in a slurry (5 parts distilled water, 1 part sample)

d) Organic mater contents were determined by Loss-on-ignition at 430 °C as recommended by Davies [51]

e) colony forming units counts for total bacteria on the Yeast Starch Agar medium

Contrary to the Zhou protocol, the first physical disruption using ultrasonic bath induces the rupture of the most strongly linked bacterial cells to the deposit particle and micro-aggregate [52]. This mechanical dissolution in a high salt extraction buffer containing EDTA leads also to the liberation of microbial cells from their mineral encasing. In fact, Wade and Garcia-Pichel [50] clearly showed that the chelator-mediated dissolution of carbonate matrix is the appropriate method that yields sufficient amounts of high quality DNA. Our second mechanical treatment using the sonics' Vibra-Cell with its variable intensity capability allowed the breaking of cell membranes and releasing the contents. In a recent study, Guobin et al. [8] showed that the highest yields of DNA extracted from activated sludge were obtained using sonication and SDS. Nevertheless, it caused severe DNA shearing. In fact, Picard et al. [27] suggested that a high level of sonication intensity during a repeated cycle of 150s caused DNA fragmentation. Thus we applied 10 cycles of 20s with 50Hz sonication intensity to avoid the DNA shearing.

The combination of these treatments significantly increased the yields of DNA extracted from phosphate deposit samples. The Figure 3 clearly showed that the highest yields of DNA were achieved by our improved protocol in comparison to the Zhou protocol ($p<0,001$). In fact, the DNA yields obtained by the Zhou protocol were practically zero except for the sample P3 (5,08±0,007 µg.g^{-1}). This result can be explained by the properties of this sample. As showed in table.2, this sample is characterized by the lowest moisture content (4,14%) and a high fine-grained fractions which avoid formation of aggregates, and that was clearly visible to the naked eye contrary to the other samples.

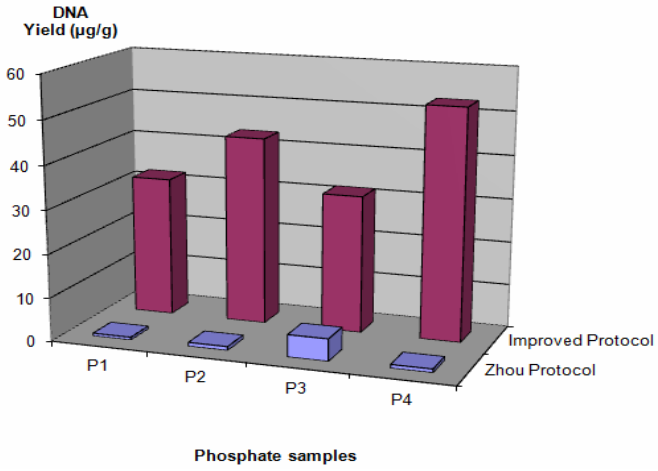

FIGURE 3: COMPARISON OF DNA YIELDS EXTRACTED FROM FOUR PHOSPHATE DEPOSIT SAMPLES WITH THE ZHOU PROTOCOL AND OUR IMPROVED PROTOCOL

C. Purity of DNA extracted by the improved protocol from phosphate samples

Results in Table 3 showed that the purity of DNA extracts by the improved protocol were low. A_{260} /A_{280} and A_{260} /A_{230} ratios were ranged between 1,23 – 1,52 and 0,43 – 0,93 respectively. In particular, the level of humic acid-like impurities, represented by A_{260} /A_{230} ratios was very low. Thus, the ability of CTAB to precipitate and remove humic contaminant was poor. Similar low ratios at absorbance A_{260}/A_{230} have been reported in other studies [53], whereas these absorbance ratios are not sufficient as a guide of purity [54] since their extracted DNA was accessible to Taq DNA polymerase enzyme for a successful PCR reaction without any

purification step. Furthermore, contrary to the soil samples we noted the absence of the brown color of the crude DNA solutions in the case of the phosphate samples.

TABLE 3 : THE OPTICAL DENSITY OF THE DNA EXTRACTED BY THE IMPROVED PROTOCOL FROM PHOSPHATE DEPOSIT SAMPLES.

Samples	OD 260 nm	OD 280 nm	Ratios	
			A260/A280	***A260/A230***
P1	1,071(±0,9)	0,766(±0,7)	1,52(±0,2)	0,86(±0,07)
P2	1,439(±0,4)	1,04(±0,3)	1,39(±0,08)	0,84(±0,02)
P3	1,053(±0,6)	0,995(±0,6)	1,23(±0,5)	0,43(±0,2)
P4	1,767(±0,8)	1,691(±1,07)	1,19(±0,12)	0,65(±0,17)
P5	1,037(±0,4)	0,82(±0,2)	1,25(±0,08)	0,69(±0,09)
P6	1,183(±0,6)	0,973(±0,5)	1,24(±0,08)	0,65(±0,04)
P7	0,614(±0,07)	1,436(±0,05)	1,44(±0,04)	0,9(±0,06)
P8	0,645(±0,1)	0,487(±0,06)	1,32(±0,04)	0,83(±0,1)
P9	0,953(±0,5)	0,701(±0,4)	1,39(±0,08)	0,81(±0,02)
P10	1,906(±0,8)	1,526(±0,7)	1,25(±0)	0,77(±0,03)
P11	0,962(±0,6)	0,668(±0,5)	1,32(±0,1)	0,93(±0,2)

OD: Optical density

Values in the table are mean of three replicate samples with the standard deviations in parentheses

IV. CONCLUSION:

The main approach of the present work was to elaborate an adequate Environmental DNA extraction procedure particular to the Moroccan phosphate deposit samples. A successful DNA extraction protocol is perfect springboard for further microbiological study of such environment. The delicate balance of extracting DNA from Complex environment with achievement of high DNA yield and purity poses real challenge during the manipulation.

In our study, the elaborated combined approach of DNA extraction based, on a physical disruption, ultrasonication lysis and enzymatic treatment, has proved itself capable of efficiently extract DNA easily detectable by the agarose gel eletophoresis along with high yields in all phosphate deposit samples . Furthermore, it has been demonstrated that the DNA extraction method strongly depends on the sample nature and its properties.

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CONFLICT OF INTEREST: NONE

REFERENCES

[1] Kotik M, Stepanek V, Maresova H, Kyslik P, Archelas A (2009). Environmental DNA as a source of a novel epoxide hydrolase reacting with aliphatic terminal epoxides. J. Molecular. Catalysis. B: Enzymatic, 56, pp-288–293.

[2] Botero LM, D'Imperio S, Burr M, McDermott TR, Young M, Hassett DJ (2005). Poly(A) polymerase modification and reverse transcriptase PCR amplification of environmental RNA. Appl. *Environ*. *Microbiol*. 71, pp:1267– 1275.

[3] Haile J, Holdaway R, Oliver K, Bunce M, Thomas M, Gilbert P, Nielsen R, Munch K, Simon Y, Ho W, Shapiro B, Willerslev E (2007). Ancient DNA Chronology within Sediment Deposits: Are Paleobiological Reconstructions Possible and Is DNA Leaching a Factor Mol. Biol. Evol. 24(4) pp:982–989.

[4] Zhou J, Bruns MA, Tiedje JM (1996). DNA recovery from soils of diverse composition, Appl. Environ. Microbiol. 62 pp:316–322.

[5] Fabiani A, Gamalero E, Castaldini M, Cossa GP, Musso C, Pagliali M, Berta G (2009). Microbiological polyphasic approach for soil health evaluation in an Italian polluted site. Science of the Total Environment. 407 pp:4954–4964.

[6] Mitchell KR, Takacs-Vesbach CD (2008). A comparison of methods for total community DNA preservation and extraction from various thermal environments. J. Ind. Microbiol. Biotechnol. 35 pp:1139–1147.

[7] Luna GM, Dell'Anno A, Danovaro R (2006). DNA extraction procedure: a critical issue for bacterial diversity assessment in marine sediments. Environ. Microbio. 8(2) pp:308–320.

[8] Guobin S, Wenbiao J, Lam EKH, Xinhui X (2008). Purification of total DNA extracted from activated sludge. Journal of Environmental Sciences. 20 pp:80–87

[9] Ahn Y, Park EJ, Oh YK, Park S, Webster G, Weightman AJ (2005). Biofilm microbial community of a thermophilic trickling biofilter used for continuous biohydrogen production. FEMS. Microbiol. Lett. 249(1) pp: 31–38

[10] Herrera A, Cockell CS (2007). Exploring microbial diversity in volcanic environments: A review of methods in DNA extraction. Journal of Microbiological Methods. 70 pp:1–12

[11] Hua G, Lyons B, Killham K, Singleton I (2009). Potential use of DNA adducts to detect mutagenic compounds in soil. Environmental Pollution. 157, pp:916–921

[12] Amman RI, Ludwig W, Schleifer KH (1995). Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. Microbiol. Rev. 59(1):143–169

[13] Riesenfeld CS, Goodman RM, Handelsman J (2004). Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ. Microbiol. 6(9), pp: 981–989

[14] Pace NR (1997). A molecular view of microbial diversity and the biosphere. Scien. 276, pp: 734-740.

[15] Torsvik V, Ovreas L (2002). Microbial diversity and function in soil: from genes to ecosystems. Curr. Opin. Microbiol. 5, pp: 240–245

[16] Schleper C, Jurgens G, Jonuscheit M (2005). Genomic studies of uncultivated archaea. Nature. Rev. Microbiol. 3, pp: 479–488

[17] Rajendhran J, Gunasekaran P (2008). Strategies for accessing soil metagenome for desired applications. Biotechnol. Advances. 26, pp:576–590

[18] Robe P, Nalin R, Capellano C, Vogel TM, Simonet P (2003). Extraction of DNA from soil. Europ. J. of Soil Biology. 39, pp :183–190

[19] Roose-Amsaleg CLE, Garnier-Sillam EM, Harry M (2001). Extraction and purification of microbial DNA from soil and sediment samples. Appl. Soil Ecology 18, pp: 47–60

[20] Lakay FM, Botha A, Prior BA (2006). Comparative analysis of environmental DNA extraction and purification methods from different humic acid-rich soils. J. Appl. Microbiol.. 102, pp:265–273

[21] Roh C, Villatte F, Kim BG, Schmid RD (2006). Comparative Study of Methods for Extraction and Purification of Environmental DNA From Soil and Sludge Samples. Appl. Biochem. and Biotech. 134, pp:97–112

[22] Gabor EM, De Vries EJ, Janssen DB (2003). Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. FEMS Microbiol Ecol. 44, pp:153–163

[23] Lamontagne MG, Michel FC, Holden PA, Reddy CA (2002). Evaluation of extraction and purification methods for obtaining PCRamplifiable DNA from compost for microbial community analysis. J. Microbiol. Methods. 49, pp:255–264

[24] Tien CC, Chao CC, Chao WL (1999). Methods for DNA extraction from various soils: a comparison, J. Appl. Microbiol. 86, pp:937–943

[25] Marilley L, Vogt G, Blanc M, Aragno M (1998). Bacterial diversity in the bulk soil and rhizosphere fractions of *Lolium perenne* and *Trifolium repens* as revealed by PCR restriction analysis of 16S rDNA. Plant and Soil 198, pp: 219–224

[26] Miller DN (2001). Evaluation of gel filtration resins for the removal of PCR-inhibitory substances from soils and sediments, J. Microbiol. Methods. 44, pp: 49–58

[27] Picard C, Ponsonnet C, Paget E, Nesme X, Simonet P (1992). Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction, Appl. Environ. Microbiol. 58, pp:2717–2722

[28] Burgmann H, Pesaro M, Widmer F, Zeyer J (2001). A strategy for optimizing quality and quantity of DNA extracted from soil. J Microbiol Methods. 45, pp:7-20

[29] Carrigg C, Rice O, Kavanagh S, Collins G, O'Flatherty V (2007). DNA extraction method affects microbial community profiles from soils and sediment. Appl Microbiol Biotechnol. 77, pp:955–964

[30] Donn S, Griffiths BS, Neilson R, Daniell T (2007). DNA extraction from soil fauna. J Nematol. 39, pp:81–81

[31] Zhang L, Xu Z, Patel BKC (2009). An improved method for purifying genomic DNA from forest leaf litters and soil suitable for PCR. J Soils Sediments. 9, pp:261–266

[32] Saano A, 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

[33] Wilson IG (1997). Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63, pp:3741–3751

[34] McGregor DP, Forster S, Steven J, Adair J, Leary SEC, Leslie DL (1996). Simultaneous detection of microorganisms in soil suspension based on PCR amplification of bacterial 16S rRNA fragments. BioTechniques. 21, pp:463–471

[35] Kallmeyer J, Smith DC (2009). An improved electroelution method for separation of DNA from humic substances in marine sediment DNA extracts. FEMS. Microbiol. Ecol. 69, pp:125–131

[36] Miller DN, Bryant JE, Madsen EL, Ghiorse WC (1999). Evaluation and Optimization of DNA Extraction and Purification Procedures for Soil and Sediment Samples. Appl. Environ. Microbiol. 65(11), pp:4715-4727

[37] Hurt RA, Qiu X, Wu L, Roh Y, Palumbo AV, Tiedje JM, Zhou J (2001). Simultaneous recovery of RNA and DNA from soils and sediments, Appl. Environ. Microbiol. 67, pp: 4495–4503

[38] Moré MI, Herrick JB, Silva MC, Ghiorse WC, Madsen EL (1994). Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. Appl. Environ. Microbiol. 60, pp:1572–1580

[39] Fortin N, Beaumier D, Lee K, Greer CW (2004). Soil washing improves the recovery of total community DNA from polluted and high organic content sediments. J. Microbiol. Methods. 56, pp:181–191

[40] Poussier S, Cheron JJ, Couteau A, Luisetti J (2002). Evaluation of procedures for reliable PCR detection of Ralstonia solanacearum in common natural substrates. J. Microbiol. Methods. 51:349–359

[41] Jasinski SM (2002). Phosphate rock: U.S. Geological Survey Mineral Commodity Summaries 2002, pp: 122-123

[42] Iddar A, Serrano A, Soukri A (2002). A phosphate-stimulated NAD(P)+-dependent glyceraldehyde-3-phosphate dehydrogenase in Bacillus cereus. FEMS Microbiol. Lett. 211, pp:29-35

[43] Hamdali H, Bouizgarne B, Hafidi M, Lebrihi A, Virolle MJ, Ouhdouch Y (2008a). Screening for rock phosphate solubilizing Actinomycetes from Moroccan phosphate mines. Applied. Soil. Ecolog. 38, pp:12–19

[44] Hamdali H, Hafidi M, Virolle MJ, Ouhdouch Y (2008b). Growth promotion and protection against damping-off of wheat by two rock phosphate solubilizing actinomycetes in a P-deficient soil under greenhouse conditions. Applied. Soil. Ecolog. 40, pp:510–517

[45] Sambrook J, Russell DW (2001). Spectrophotometry of DNA or RNA. In: Molecular Cloning. 3rd ed. Cold Spring Harbor, New York: Cold spring Harbor Laboratory Press. 3: A8.20-A8.21

[46] Henneberger RM, Walter MR, Anitori RP (2006). Extraction of DNA from acidic, hydrothermally modified volcanic soils. Environ. Chem. 3, pp:100–104.

[47] Ghoubert G, Salvan HM (1949). Essai sur la paléogéographie du Sénonien au Maroc, Notes et Mém. Serv. géol. Maroc 2(74) pp:18–50

[48] Salvan HM (1960). Étude préliminaire du gisement de Sidi Daoui, rapport inédit., Serv. Carte géol. Maroc

[49] El Ouardi EM (2008). Étude de la calcination du phosphate clair de youssoufia (Maroc). Afrique Scien. 04(2), pp:199 – 211

[50] Wade BD, Garcia-Pichel F (2003). Evaluation of DNA extraction methods for molecular analyses of microbial communities in Modern calcareous microbialites. Geomicrobiol. J. 20, pp:549–561.

[51] Davies BE (1974). Loss-on-Ignition as an Estimate of Soil Organic Matter. Soil. Sci. Soc. Am. J. 38, pp:150-151.

[52] Bakken LR, Lindahl V (1995). Recovery of bacterial cells from soil, in: J.D. Van Elsas, J.T. Trevors (Eds.), Nucleic Acids in the Environment: Methods and Applications, Springer-Verlag, Heidelberg, Germany. pp. 9–27

[53] Gothwal RK, Nigam VK, Mohan MK, Sasmal D, Ghosh P (2007). Extraction of bulk DNA from Thar Desert soils for optimization of PCR DGGE based microbial community analysis. E. J. Biotechnology. 10(3), pp:400-408

[54] Clegg CD, Ritz K, Griffiths BS (1997). Direct extraction of microbial community DNA from humified upland soils. Letters in Applied Microbiology. 25(1), pp:30–33