Journal of Microbiological Methods 148 (2018) 155–160

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Optimisation of protocol for effective detachment and selective recovery of the representative bacteria for extraction of metagenomic DNA from *Eucalyptus* spp. woodchips

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ARTICLE INFO

Keywords:
Bacteria
Detachment
DNA
Metagenomic
Woodchips

ABSTRACT

For some environments such as planktonic/aqueous environments, the separation of bacteria cells from eukaryotic cells prior to DNA extraction using filtration is relatively straightforward. However, for woodchips, the bacteria are attached/embedded within the wood matrix, which prevents easy removal of bacterial cells. In this study, a method for the selective extraction of DNA from bacteria inhabiting *Eucalyptus* spp. woodchips has been developed. The objective was to compare milled and unmilled woodchips processed via three detachment methods, viz., sonication, vortexing and shaking followed by filtration using Teflon filters according to three relevant criteria: DNA yield, DNA purity and quality of DNA. Highest DNA yield was obtained by milling and vortexing for 10 min (77.50 ± 5.17 ng/μl), followed by milling and vortexing for 2 min (61.00 ± 6.56 ng/μl), unmilled and vortexing for 10 min (38.67 ± 5.17 ng/μl) and milled and shaking for 2 h (31.62 ± 5.17 ng/μl). The lowest DNA yield was obtained by using unmilled woodchips and 5 min of sonication treatment (7.00 ± 1.22 ng/μl). There was no significant difference in DNA purity for milled or unmilled woodchips processed via the three detachment methods. Duration of cell detachment treatment did not significantly influence DNA yield and purity. Following optimisation experiments, it was possible to extract bacterial DNA using milled woodchips and 10 minute vortexing devoid of DNA from the host background and other associated eukaryotes and of sufficient quality and quantity for metagenomic analysis.

1. Introduction

Wood is often inhabited by a diverse and complex community of bacteria that can produce biologically active molecules (Clausen, 1996; Kallioinen et al., 2003; Folman et al., 2008; Gelbrich, 2009). Most studies exploring the biocatalytic capabilities of bacterial communities have been carried out using cultured isolates (Rogers and Baecker, 1991; Warnick et al., 2002; Roux et al., 2004; Kluczek-Turpeinen et al., 2007; Bandounas et al., 2011; Huang et al., 2013; Liang et al., 2014). However, the majority of bacteria existing in the environment are not culturable (Stewart, 2012; Fakruddin et al., 2013; Neelakanta and Sultana, 2013; Pienaar et al., 2016). Therefore, comprehensive studies of bacterial communities are mostly based on the analysis of total environmental DNA, which is required in high yield and quality (high molecular weight) and ideally should be devoid of eukaryotic or host DNA (Burke et al., 2009).

For some environments including planktonic/water environments, using filtration for the separation of bacterial cells from eukaryotic cells and environmental debris prior to DNA extraction is relatively straightforward (LaMontagne and Holden, 2003; Rusch et al., 2007; Ghiglione et al., 2009; Kellogg and Deming, 2009; Smith et al., 2013; D'Ambrosio et al., 2014; Ganesh et al., 2014; Mohit et al., 2014; Orsi et al., 2015; Padilla et al., 2015). This undertaking usually involves collection of suspended biomass by passing the water through a filter (Padilla et al., 2015). To collect planktonic bacteria in the filtrate, it is common to use any filter during the collection step, often with a prefilter of larger pore size (Padilla et al., 2015).

However, the cells of the bacterial inhabitants of woodchips are attached/embedded within the wood matrix, which prevents their easy removal (Lindahl and Bakken, 1995; Liu et al., 2010). These cells have to be detached from the woodchip matrix prior to filtration and thereafter DNA extraction. Moreover, to collect bacteria in the filtrate, the heterogeneity of the microbial and/or particle suspension and hydrophobicity and pore size of the filter material influence the amount of bacteria collected in the filtrate (Zierdt, 1979; Nnadozie et al., 2015). In wood chip suspensions and suspensions of other environmental samples where the particulate component is predominantly debris hydrophobic filters are suggested for filtration, in order to collect bacteria in the

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https://doi.org/10.1016/j.mimet.2018.04.009
Received 5 March 2018; Received in revised form 13 April 2018; Accepted 13 April 2018
Available online 17 April 2018
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filtrate (Sharpe et al., 1979; Millipore, 2003; Nnadozie et al., 2015). Taking advantage of the weaker resistance of hydrophobic filters to adhesion (Jarusutthirak et al., 2002), wood particles tend to outcompete bacteria for binding capacity because of the effect of electrostatic forces of attraction, and thus adsorb more strongly onto the filter membrane, while allowing bacteria to pass through. It has been reported that hydrophobic Teflon membrane filters adsorbed bacterial cells the least, with highest recovery of cells within the filtrate (Zierdt, 1979). Up to 100% recovery of bacterial cells (*E. coli*) within the filtrate was achieved by Teflon membrane with pore size of 10 μm.

The aim of this study was to develop a protocol that would allow for effective detachment and selective recovery of the representative bacteria from *Eucalyptus* spp. woodchips, for extraction of pure and sufficient quantities of genomic DNA amenable for next generation sequencing. Apart from two studies by Cabrol et al. (2010) and Ramnath et al. (2014), who explored methodological aspects of direct nucleic acid recovery from microbial communities involved in a gas biofilter filled with pine bark woodchips and *Eucalyptus* spp. woodchips, respectively to the best of our knowledge, there is no documented and existing standard protocol for pre-processing woodchips for selective isolation of bacteria for DNA extraction. In addition, the difference in the preprocessing protocol (milling and unmilled) of the woodchip material between the Cabrol study and the current study could lead to differing results. In the Cabrol study, the effect of detachment methods was not assessed in combination with milling. A full systematic evaluation of the effects of sonication, vortexing, and shaking on the indirect recovery of nucleic acids from microbial communities in woodchips has never been reported.

2. Materials and methods

2.1. Sample collection and preparation

Woodchips were randomly sampled from a pile from South African Pulp and Paper Industries (SAPPI) Dissolving Cellulose, Umkomaas, KwaZulu-Natal in plastic bags. The chip pile had been generated with several *Eucalyptus* species, namely: *Eucalyptus dunnii*, *Eucalyptus nitens* and *Eucalyptus grandis* and transported back to the laboratory and temporarily stored at 4 °C until the samples could be analysed and stored at −20 °C. One part was milled into sawdust sized particles (0.1 to 0.3 cm) in a Wiley-type mill, whereas, the other part was left unmilled. The Wiley-type mill was decontaminated by spraying with 70% ethanol to avoid cross-contamination of the sawdust. Approximately 10 g each of the unmilled or 5 g each of milled wood chip were suspended in 25 ml phosphate buffered saline (PBS, pH 8). A different ratio of unmilled and milled wood chips was used due to the concern about the obvious difference in physical characteristics of the milled (sawdust) and unmilled (woodchip) (Briggs, 1994). The milled chips absorbed the liquid (PBS) and so in order to obtain a sort of slurry a bigger liquid to chip ratio was required. In addition, the milled woodchips (sawdust) weighs lesser than (about half the weight of) the unmilled. Moisture is lost in the milling process, causing the sawdust to weigh lesser. Wood is hygroscopic: it will lose moisture until its moisture content is in equilibrium with the temperature of the air in the mill (Ahmed et al., 2016).

2.2. Detachment methods

Three detachment methods were investigated, including vortexing, shaking, and sonication. Vortexing was performed using a vortex mixer-ZX3 (Velp scientific, Milano, Italy) at 40 Hz. Sonication was performed using an Ultrasonic homogenizer (Sonic Ruptor 400, OMNI International the Homogenizer Company, GA, USA) at a power setting of 200 W (with 5 s intermediate pulse), while shaking was performed at 250 rpm on an orbital shaker (Innova® 44/44R, New Brunswick™).

Three durations were tested for sonication (5; 15 and 30 min). One duration was tested for shaking (60 min), whereas two durations were tested for vortexing (2 and 10 min). Each detachment method and treatment condition was repeated on triplicate samples.

2.3. Selective separation of cells from sample matrix by filtration and DNA extraction

After detachment, bacterial cells were selectively separated from wood chip particles by vacuum filtration with Teflon membrane filters (10 μm Millipore MITEX, Ireland) (Nnadozie et al., 2015). Filter paper together with residual woodchips was further rinsed with 15 ml of the PBS solution (pH 8) to recover any bacterial cells within the sample matrix. The filtrate was collected and aliquoted into 2 ml centrifuge tubes. Cells were harvested by centrifugation (Eppendorf Microfuge 5418, Germany) at 7000 rpm for 30 min. The supernatants were discarded by decanting and the cell pellets were pooled and re-suspended in up to 200 μl of the PBS solution to be used for DNA extraction. Total bacterial genomic DNA was extracted using the MoBio soil DNA isolation kit (Inqaba BiotecTM, South Africa) as per manufacturer's instructions.

2.4. Evaluation of DNA yield and purity

DNA concentration was determined by measuring absorbance at 260 nm using a NanoDrop 1000 Spectrophotometer (ThermoScientific, USA). DNA purity was determined by the ratio of absorbance at 260 nm and 280 nm, considering that the absorbance at 280 nm was mainly due to protein contamination. It is commonly assumed that DNA is devoid of protein contamination if this ratio is higher than 1.7 (Stach et al., 2001). Extracted DNA (5 μl) was visualized by gel electrophoresis on 1% agarose gel.

For subsequent experiments (16S and 18S PCR), the optimized protocol for wood chip processing and cell detachment was as follows: milled woodchips and vortexing for 10 min.

2.5. 16S and 18S rRNA polymerase chain reactions

Amplification was performed on 5 μl DNA template in a final volume of 25 μl containing 2 μl $MgCl_2$, 2.5 μl reaction buffer (1 ×), 1 μl of dNTP mix, 2.5 μl of each primer and 0.25 μl Supertherm DNA polymerase (LPI, UK). PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems, United States). The genomic DNA was PCR amplified using 16S primers 63F and 1387R (Marchesi et al., 1998). Conditions for 16S rRNA PCR were: denaturing temperature: 94 °C for 1 min; annealing at 55 °C for 1 min; and extension temperature 72 °C for 2 min.

18S PCR was carried out to ascertain that there was no contamination arising from eukaryotic DNA using IT5S and IT4S forward and reverse primers, respectively (White et al., 1990). Conditions for 18S PCR were: denaturation temperature at 96 °C for 2 min followed by 35 cycles consisting of denaturation at 96 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. A final extension at 72 °C for 10 min was done at the end of the amplification (Ristaino et al., 1998).

PCR products were visualized after electrophoresis on 1% agarose gels stained in 0.5 μg/ml ethidium bromide in a Chemi-Genius 2 BioImaging System (Syngene, United States).

2.6. Statistical analysis

DNA yields and DNA purity, means and standard deviations were evaluated in triplicate samples. Differences between milled and unmilled woodchips processed via three detachment methods in DNA yield and DNA purity were determined using ANOVA with Bonferroni post hoc test (Microsoft Excel). Results were considered statistically significant for *p*-values ≤ .05.

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