Rapid and simple DNA extraction protocol from goat rumen digesta for metagenomic analysis

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Abstract: In contrast to the traditional culturing techniques and microscopy that have led to the identification and characterization of only about 15-20 % of the rumen microbes till date, nucleic acid-based molecular approaches are rapid, reproducible, and allow both the qualitative and quantitative assessment of microbial diversity. The aim of this study was to develop a simple, rapid and effective extraction protocol for the recovery of high-molecular-weight and cloneable metagenomic DNA (mDNA) from goat rumen contents. An efficient method was devised to isolate high-molecular-weight mDNA (>23kb) that was pure and cloneable after isolation in a relatively short period (3.5 h). This is the first report wherein purification of isolated mDNA could be passed. The purity and cloneability of mDNA was found to be possible with the successful restriction digestion, 16S rDNA PCR amplification of the isolated mDNA and mDNA library construction.The screening of 1600 clones from the metagenomic library revealed one clone with adistinct hydrolytic activity on carboxymethyl cellulose (CMC) agar suggesting its endoglucanase activity. Agarose gel electrophoresis showed aDNA insert of ~1.5kb size on digestion with *Bam*H1. The metagenomic clones offer a prodigious non-conventional means to explore the genetically untapped resources from nature.

Keywords: Metagenomics, Goat rumen, Metagenomic DNA, Cloneable, Polymerase chain reaction, Metagenomic DNA library.

INTRODUCTION

Microbial populations have conventionally been described either by the culture-based techniques like the selective plate counts, or by specific direct counts like immunofluorescence. The drawbacks of these techniques are that the plate counts monitor only culturable organisms, whereas immunofluorescence enumerates dead cells and is limited by the lack of specificity (Smalla *et al*., 1993). The fact that more than 99% of prokaryotes in any given environment cannot be cultured in the laboratory drew attention to the unculturable microbial world, and thus came the concept of metagenomics, the culture-independent cloning and analysis of microbial DNA (metagenome) isolated directly from an environmental sample (Handelsman *et al*., 1998). Metagenome analysis is an increasingly popular approach and has become a powerful tool for studying phylogeny and functional characterization of the microbes present in the environment (Stefanis *et al*., 2013). The diverse ecosystems that have been explored for metagenomic analyses include soil (Jiang *et al*., 2011), marine sediments (Gray and Herwing 1996), compost (Howeler *et al*., 2002), rumen (Rosero *et al*., 2012) etc.

Ruminants like goats feed on the tips of woody shrubs, lignocellulosic agricultural by-products such as cereal straws and stovers. Symbiotic microbes in the gut of these ruminants play pivotal roles in providing the hosts with various nutrients (Hungate 1996). Enzymes secreted by the ruminal microbes are needed for the conversion of complex cellulose and hemi-cellulose into simple sugars. These sugars serve as an energy source for these animals (Miyagi *et al*., 1995). There are also fermentative bacterial populations that transform simple sugars into low molecular weight fatty acids, which are also used as energy source by the ruminants. The rumen being a very complex microbial eco-system, the enumeration of a specific bacterial species and the quantification of its role in the rumen fermentation is difficult with the traditional techniques. It is often limited by large number of biochemical tests to be performed and imprecision of techniques even for the most predominant ruminal microbe (Kamra 2005). Furthermore, the rumen is one of the best microbial habitats to explore for sources of industrially important enzymes like cellulases that may find its use in the biofuel industry (Hess *et al*., 2001). The pre-requisite for these studies is the isolation of pure and high-molecular-weight DNA with high recovery efficiency so that the final genomic DNA represents total genomic DNA within this ecosystem. Also the DNA extraction and purification protocol should be simple so that the whole DNA recovery process is rapid and cost effective (Hurt *et al*., 1996). The objective of this study

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was to develop a simple, effective and rapid extraction protocol for the recovery of high-molecular-weight and cloneable mDNA from goat rumen digesta. A comparative study of five different DNA extraction methods revealed a rapid method providing with higher quality and yield of mDNA from goat rumen digesta for the restriction digestion, 16S rDNA based polymerase chain reaction and mDNA library construction.

MATERIALS AND METHODS

Sampling

The goat rumen digesta was sampled from a slaughter house located at Napaam, Tezpur, Assam, India. The sample was collected and stored at −80ºC until theDNA extraction was performed.

Isolation of high-molecular-weight metagenomic DNA

The metagenomic DNA (mDNA) from the goat rumen digesta was extracted by using five different methods. P1 method was performed according to protocol of Sharma *et al*. (2003). It is a CTAB extraction method using liquid nitrogen to grind the samples and buffers AP1, AP2, AP3, AW and AE to purify and precipitate the DNA. P2 method was performed according to Yu and Morison (2004). It uses zirconia beads to lyse the sample and a conventional ammonium acetate/isopropanol DNA precipitation method. P3 method was performed according to Krause *et al*. (2001). It utilizes zirconium beads, methanol, phenol-chloroform, Polyethylene glycol (PEG) and isopropanol to isolate DNA from rumen digesta. P4 method was performed according to Popova *et al*. (2010). This method employs bead beating, phenol–chloroform extraction and saline–alcohol precipitation of DNA. Method P5 is a modified CTAB extraction method developed in our laboratory.

P5 method: Weigh 0.5 g rumen digesta and place the sample in a 15-mL Falcon tube. Add 5 mL CTAB extraction buffer [1% (w/v) CTAB; 100 mM Tris-HCl, pH 8.0; 50 mM EDTA; 1.5 M NaCl; and 100 µg/mL proteinase K]. Incubate at 65°C for 2 h with occasional mixing (Note that proteinase K is added to the buffer after pre-warming it to 65°C]. Centrifuge the lysate at 10000× g for 5 min and retain the supernatant. Add 500 µL of 25:24:1 phenol: chloroform: isoamyl alcohol and 100 µL of 35% polyethylene glycol (PEG, M_r = 4000) and centrifuge at 15 000× g for 10 min at 4°C. Precipitate the DNA from the recovered aqueous phase with 0.6 volumes of isopropyl alcohol. Recover the DNA by centrifugation at 10000× g for 15 min at 4°C. Measure the DNA concentration spectrophotometrically or by gel electrophoresis.

Yield, purity and integrity of mDNA

DNA was quantified spectrophotometrically (A_{260} nm, Cecil 7400, Cambridge) and the purity of DNA was assessed from A_{260} nm/A_{230} nm and A_{260} nm/A_{280} nm ratios spectrophotometrically to check possible contamination of DNA with tannins and proteins, respectively. Integrity was determined by agarose (0.8%, w/v) gel electrophoresis using GeneRuler 1 kb DNA ladder (Thermo Scientific, USA) as molecular weight markerand illumination under UV light.

Assessing suitability of mDNA for PCR-based applications

The purity of mDNA for downstream applications was checked by partial amplification of 16S rDNA by using the universal primers {(fD1, AGA GTT TGA TCC TGG CTC AG and rP1, ACG GTT ACC TTG TTA CGA CTT); (UD Scientific) (Weisburg *et al*., 1991)}. PCR amplification was doneusing a 20 µl reaction mixture containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Fermentas), 10 pmole of each forward and reverse primer, 10 ng mDNA template and 1U of Taq polymerase (Bangalore Genei, India) with reaction buffer supplied by the manufacturer. Amplification was performed with a thermal cycler (Applied Biosystems) by using the following program: 95 ºC for 3 min; 30 cycles consisting of 95ºC for 30 sec; 55 ºC for 30 sec; 72 ºC for 1 min; and a final extension step consisting of 72°C for 5 min. The amplification was determined by electrophoresis of reaction product in 1% agarose gel.

Restriction digestion susceptibility testing

The susceptibility of mDNA for restriction digestion was tested by incubating 0.25 µg of each DNA sample separately with 2.5 U of *Eco*RI and *Bam*HI (MBI Fermentas, Germany) restriction enzymes in a 25 µl reaction mixture. The mixtures were incubated at 37ºC for 4 hours followed by inactivation of the restriction enzymes by heating at 70ºC for 10 min. The digested products were resolved on 0.8% agarose gel.

mDNA library construction

The cloneability of the isolated mDNA was confirmed by construction of mDNA library in pUC19 cloning vector. mDNA partially digested with *Bam*HI (MBI Fermentas, Germany) was resolved on 0.8% agarose gel and DNA fragments ranging about 0.5–4.0 kb were fractionated by using Qiaquick gel extraction kit (Qiagen, Germany). The purified mDNA fragments were incubated with *Bam*HI digested and CIP dephosphorylated pUC19 cloning vector at 16 °C overnight for ligation using T4 DNA ligase (MBI Fermentas, Germany). The ligated mixture was transformed into *E. coli*DH5α by electroporation (200 Ω, 25 µF and 2.5 kV) using gene pulser (Biorad, USA). Transformed cells were cultured on Luria Bertani (LB) agar plates supplemented with ampicillin (100 µg/ml), and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside) (20 µg/ml). The recombinants were scored by blue–white screening after overnight incubation at 37°C. The resulting library was stored in 15% glycerol at −80°C.

Selection of cellulolytic clones from metagenomic library

The plasmid transformants were grown for 24-48 h on 0.5% CMC agar plates at 37˚C. The colonies with yellow halos (zone of clearing) around them were selected as cellulase-positive clones. The plasmids of the clones were prepared and analysed for the insert DNA.

RESULTS

The P5 extraction method was found to be effective for the extraction of high-molecular-weight mDNA from goat rumen digesta. The mDNA from 0.5g of the rumen digesta was isolated with a final yield of approximately 240 µg of high quality DNA per gram of the sample. An important criterion for environmental microbiology studies is high-molecular-weight mDNA as sheared DNA is not suitable for cloning and can cause PCR amplification artifacts (Hurt *et al*., 1996).P5 method was consistently able to recover high-molecular-weight mDNA (>23kb) with high integrity represented as non-sheared DNA on agarose gel electrophoresis (fig. 1). The highest DNA yield was obtained in the case of P5, followed by P4, P2, and P3 and the lowest in the case of the P1 method (table 1). A_{260}/A_{230} has been used to evaluate the purity of mDNA and rule out the presence of tannins and other phenolics that may inhibit its downstream applications. The protocols P1, P2, and P3 did not prove successful in obtaining pure mDNA as it was not susceptible to restriction digestion and no PCR product was observed (table 1). The protocol of Popova *et al*. (2010) did yield pure DNA but the processing time was too long (>8h). P5 protocol took only 3.5 h and resulted in pure DNA that was susceptible to restriction digestion, PCR amplification, and cloning.

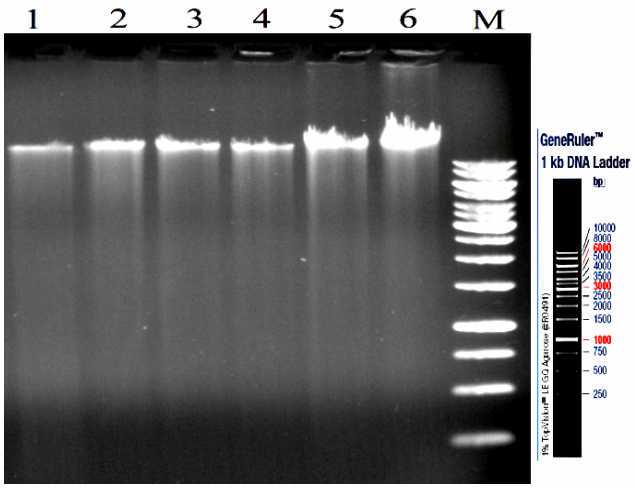

Fig. 1: mDNA extracted from goat rumen digesta using different methods: lane 1: genomic DNA extracted from *E. coli* (MTCC 40) using method P5; lanes 2 to 6: mDNA extracted using methods P1–P5 respectively; lane M: 1 kb DNA ladder (Thermo Scientific, USA).

P5 method was also used for extracting genomic DNA from *E. coli* (MTCC 40) to validate its utility for cultivable bacteria. It was observed that the method also gave high yield of DNA in case of *E. coli* (MTCC 40), thus expanding the scope of usage for P5 extraction method to cultivable bacteria as well (fig. 1).

The isolated mDNA was subjected to 16S rRNA gene amplification and it showed an amplicon of ~1.5kb size on 1% agarose gel. The isolated mDNA was successfully digested with *Eco*RI and *Bam*HI restriction enzymes and a metagenomic library was constructed by using pUC19 cloning vector. The transformation efficiency was calculated to be ~1x10^{6}transformants/µg DNA. Further, the recombinants were selected on the basis of blue–white screening after overnight incubation at 37 °C temperature. On screening 1600clones, one clone showed cellulolytic activity. Restriction analysis of the plasmids of the clone showed that they had ~1.5 kb insert DNA (fig. 2) that conferred cellulolytic activity on the clone. The ability to degrade CMC suggested that the clone has endoglucanase activity.

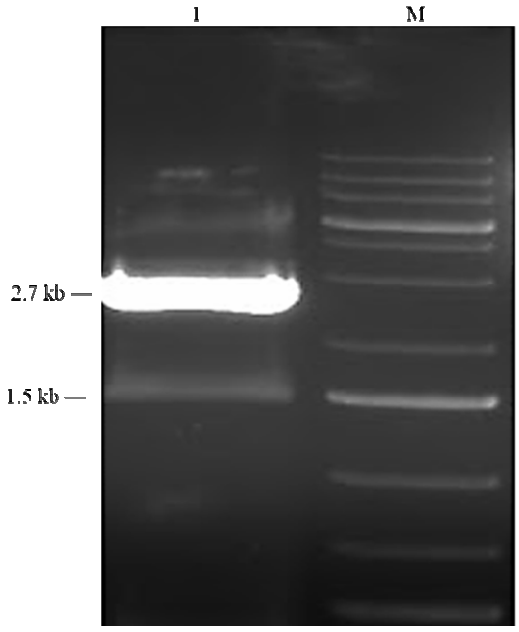

Fig. 2: Agarose gel electrophoresis showing restriction digestion of pUC19. 1: pUC19 digested with *Bam*H1; M: 1 kb Plus DNA ladder (Thermo Scientific, USA).

DISCUSSION

The sample in the P5 protocol was not subject to overly harsh mechanical lysis owing to the trade-off between obtaining sufficient DNA representing total rumen microbial community and shearing the DNA during the extraction process. Moreover, the DNA extraction methods that minimise shearing are best for genome sequencing (Henderson *et al*., 2013). The key step, in our protocol, is the removal of tannins that could inhibit PCR

Table 1: Yield, purity (absorbance ratios) and other useful parameters of crude mDNA isolated by five different methods

Method	Crude DNA yield (µg/g rumen digesta)	Absorbance ratios		Processing time (h)	16S rDNA amplification (PCR)	Endonuclease activity		References
		A_{260}/A_{280}	$A_{260/230}$			*Eco* RI	*Bam* HI	
P1	50±2.4	1.6	0.5	5	−	−	−	Sharma *et al*., 2003
P2	70.2±3.7	1.2	0.8	3	−	−	−	Yu and Morrison, 2004
P3	56.8±3.9	1.3	0.6	2.5	−	−	−	Krause *et al*., 2001
P4	180±9.7	1.65	0.7	>8	+	+	+	Popova *et al*., 2010
P5	240.6±8.1	1.75	0.9	3.5	+	+	+	This study

Values are mean ± standard error of three replicates.+: methods are showing suitability for PCR and endonuclease activity.−: methods are not showing suitability for PCR and endonuclease activity.

(Stefanis *et al*., 2013). This was achieved by using soluble polyethylene glycol (PEG). PEG forms an aggregate with tannins and other phenolics, which bind to the protein and cell debris upon lysis, forming a complex. This lysate, when centrifuged in the presence of phenol, results in the accumulation of protein-tannin complexes at the interface between the organic and aqueous phases. The supernatant, thus obtained, is largely free of tannins (Krause *et al*., 2001).

To our knowledge, this is the first report wherein the mDNA from goat rumen digesta was found to be contaminant free and cloneable with no further need for purification. The available protocols so far may yield high-molecular-weight mDNA but subsequent purification results in loss of DNA thereby negating the prospect of cloning and sequencing. Moreover our protocol recovered approximately 240 µg of community DNA per gram of rumen digesta, which represented 4.8, 3.4, 4.2, and 1.3 fold increase in mDNA yield as compared to P1, P2, P3 and P4, respectively.

mDNA library was constructed successfully using DNA extracted by method P5. This proves its suitability for sequence based and function based metagenomic approaches confirming its cloneability and retrieval of gene(s) of specific biocatalysts.

The high content of lignocellulosic materials and the diversity of indigenous microorganisms make rumen digesta an excellent source of cellulolytic enzymes that convert lignocellulosic materials into fermentable sugars. The screening of metagenomic library revealed one cellulase-positive clone after subjecting 1600 clones to functional metagenomic analysis. Functional screens enable us to select enzymes based on their activity. As the frequency of discovering novel active metabolic pathways from metagenomic DNA libraries is often low, high-throughput functional screening of library clones is the most efficient approach for function-based activity determination (Kakirde *et al*., 2010).

The rumen microbiome is still largely unknown and represents untapped genetic wealth (Cunha *et al*., 2011). Therefore, this study may open up avenues to explore the untapped goat rumen microbiota for biotechnological applications such as the deconstruction of lignocellulosic biomass for ethanol production.

CONCLUSION

Although many DNA extraction methods have been published the effective recovery of mDNA from environmental samples has still remained to be a challenge. The purity, molecular weight and yield of the isolated mDNA from the goat rumen digesta with the method presented here (P5) is substantially superior to other protocols reported in the literature to date. The protocol is able to isolate pure mDNA free from any contaminants viz. tannins etc. that might inhibit downstream applications. Further studies are needed to reveal the exact nature of the enzyme screened from the goat rumen metagenomic library.

ACKNOWLEDGEMENTS

YB is grateful to University Grants commission, India for providing financial assistance in the form of Basic Science Research (UGC-BSR) fellowship. A very special thanks to Tezpur University, Assam, India for providing the necessary R&D infrastructures.

REFERENCES

Smalla K, Cresswell N, Mendonca-Hagle LC, Wolters A and Van Elsas JD (1993). Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. *J. Appl. Bacteriol.,* **74**: 78-85.

Handelsman J, Rondon MR, Brady SF, Clardy J and Goodman RM (1998). Molecular biological access to the chemistry of unknown soil microbes: A new frontier for natural products. *Chemistry & Biology,* **5**: 245-249.

Stefanis C, Alexopoulos A, Voidarou C, Vavias S and Bezirtzoglou E (2013). Principal methods for isolation and identification of soil microbial communities. *Folia Microbiol.,* **58**: 61-68.

Jiang CJ, Chen G, Huang J, Huang Q, Jin K, Shen PH, Li JF and Wu B (2004). A novel β-glucosidase with lipolytic activity from a soil Metagenome. *Folia Microbiol.,* **56**: 563-570.

Gray JP and Herwig RP (1996). Phylogenetic analysis of the bacterial communities in marine sediments. *Appl Environ Microbio.*. **62**: 4049-4059.

Howeler M, Ghiorse WC and Walker LP (2002). A quantitative analysis of DNA extraction and purification from compost. *J. Microbiol. Methods.,* **54**:37-45.

Rosero JA, Štrosová L, Mrázek J, Fliegerová K and Kopečný J (2012). PCR detection of uncultured rumen bacteria. *Folia Microbiol.,* **57**:325-330.

Hungate RE (1966). The rumen and its microbes, Academic Press, New York.

Miyagi T, Kaneichi K, Aminov RKY, Sakka K, Hoshino S and Ohmiya K (1995). Enumeration of transconjugated Ruminococcus albus and its survival in the goat rumen microcosm. *Appl. Environ. Microbiol.,* **61**:2030-2032.

Kamra DN (2005). Rumen microbial ecosystem. *Current Science*, **89**: 124-134.

Hess M, Sczyrba A, Egan R, Kim TW, Chokhawala H, Schroth G, Luo S, Clark DS, Chen F, Zhang T, Mackie RI, Pennacchio LA, Tringe SG, Visel A, Woyke T, Wang Z and Rubin EM (2011). Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. *Science*, **331**: 463-467.

Hurt RA, Qiu X, Wu L, Roh Y, Palumbo AV, Tiedje JM and ZhouJ (1996). Simultaneous recovery of RNA and DNA from soils and sediments. *Appl. Environ .Microbiol.,* **67**: 4495-4503.

Sharma R, John SJ, Damgaard M and McAllister TA (2003). Extraction of PCR-quality plant and microbial DNA from total rumen contents. *Biotechniques,* **34**: 92-97.

Yu Z and Morrison M (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques.,* 36: 808-812.

Krause DO, Smith WJ and McSweeney CS (2001). Extraction of microbial DNA from rumen contents containing plant tannins. *Biotechniques.*, **31**:294-298.

Popova M, Martin C and Morgavi DP (2010). Improved protocol for high-quality co-extraction of DNA and RNA from rumen digesta. *Folia Microbiologica., **55***: 368-372.

Weisburg WG, Barns SM, Pelletier DA and Lane DJ (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol.,* **173**: 697-703.

Henderson G, Cox F, Kittelmann S, Miri VH, Zethof M, Noel SJ, Waghorn GC and Janssen PH (2013). Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. *PLoS One.,* **8**: e74787.

Kontanis EJ and Reed FA (2006). Evaluation of real-time PCR amplification efficiencies to detect PCR inhibitors. *J. Forensic Sci.,* **51**: 795-804