ISSN 1819-1878

Asian Journal of Animal Sciences

http://knowledgiascientific.com

Knowledgia SCIENTIFIC
A Place to Publish Outstanding Research

Asian Journal of Animal Sciences 5 (3): 166-174, 2011
ISSN 1819-1878 / DOI: 10.3923/ajas.2011.166.174

Improved Extraction of Quality DNA from Methanogenic Archaea Present in Rumen Liquor for PCR Application

P. Prashant Chaudhary, S. Kumar Sirohi and S. Kumar
Nutrition Biotechnology Lab, Dairy Cattle Nutrition Division, Animal Biotechnology Centre, National Dairy Research Institute, Karnal-132001, India

Corresponding Author: Dr. Sunil Kumar Sirohi, Nutrition Biotechnology Lab, Dairy Cattle Nutrition Division, Animal Biotechnology Centre, National Dairy Research Institute, Karnal-132001, India

ABSTRACT

The present study has been planned to standardize a simple and effective method for the isolation of good quality as well as quantity of methanogenic DNA in total genomic DNA from rumen liquor of *Bubalus bubalis*. Methanogens are a diverse group of organisms found in anaerobic environments such as anaerobic sludge digester, wet wood of trees, sewage, rumen, black mud, black sea sediments , etc which utilize carbon dioxide and hydrogen and produce methane. Methanogens exhibit great diversity in cell envelopes, ranging from simple, non rigid surface layers consisting of protein or glycoprotein subunits to a rigid "pseudomurein" sacculus, analogous to eubacterial murein. Methanogens having different chemical composition of cell wall and known for tough cell wall which is difficult break to isolate good quality and quantity of genomic DNA. Various DNA extraction methodologies have been used but problems are most often encountered in terms of low DNA yields and quality of DNA for further application. Method of DNA isolation based on guanidine thiocynate lysis buffer was compared with commonly used phenol chloroform method and commercial kit based methods, results showed that modified protocol generated high molecular weight genomic DNA while the other two methods resulted in considerable DNA degradation. Further, the isolated genomic DNA was tested for downstream applications such as PCR and Real-Time PCR using methanogens specific primers. In both the cases, the genomic DNA isolated by our protocol was comparatively better than rest of two protocols tested.

Key words: Genomic, methanogen, PCR, real-Time PCR, downstream applications

INTRODUCTION

One of the major sources of methane release in the environment is from livestock, mainly ruminants including cows and buffaloes. Enteric methane is produced by methanogenic archaea as a by-product of fermentation of feed in the gut. One way of reducing global methane emission is to check the release of methane produced by livestock (Wright *et al*., 2007). It is possible only when, we control the growth of methanogens present in the rumen. Recent biotechnological approaches like PCR, Real time analysis enable us to study these methanogens in more detail, so that effective strategies could be find out in order to reduce their population in the ruminants (Tajima *et al*., 2001; Tokura *et al*., 1999; Whitford *et al*., 1998; Yanagita *et al*., 2000). The above said molecular approaches are very sensitive and require high quality as well as purity of DNA for the analysis. Methanogens present in rumen and involved in methane production utilizing carbon dioxide and hydrogen. Methanogens exhibit great diversity in cell envelopes, ranging from simple,

non rigid surface layers consisting of protein or glycoprotein subunits to a rigid "pseudomurein" sacculus, analogous to eubacterial murein. Muramic acid or D-amino acids have not been detected till date (Benner and Kaiser, 2003). According to their major cell wall constituent, cell envelopes of Methanogens may be categorized into three characteristic classes: (i) pseudomurein layer (ii) protein or glycoprotein layer and (iii) heteropolysaccharides layer (Kandler and Konig, 1985). Methanogens are strict anaerobes and therefore it is very difficult to culture them in lab and if cultured it is very costly. So for the experimentation, the total genomic DNA from rumen digesta or liquor is isolated and then methanogenic DNA is amplified by using gene specific primers. But due to tough cell wall composition of methanogens, it is not very easy to isolate good quality DNA by the conventional methods (e.g., Phenol-Chloroform based method). Here, in present study we describe an improved methodology for the isolation of good quality methanogenic genomic DNA from rumen liquor and results are compared other method like Phenol-Chloroform method as well as with a good quality commercial kit (Genomic DNA Purification kit, Fermentas.,USA) based isolation method. Further, DNA isolated by all the three methods for compared downstream applications like PCR and Real Time PCR.

MATERIALS AND METHODS

DNA extraction: Rumen liquor samples were obtained after manual mixing of rumen contents from a rumen fistulated buffalo (*Bubalus bubalis*) maintained on standard diet (60 parts roughage:40 parts concentrate) from the cattle yard of National Dairy Research Institute, Karnal, Haryana, India. The experiment was conducted from March to June, 2010 in Nutrition Biotechnology laboratory, Dairy Cattle Nutrition Division, NDRI, Karnal Roughage part consists of wheat straw and concentrates as normal farm concentrate mixture. Rumen liquor was collected from all the sides of rumen, squeezed and filtered through four layers of muslin cloth and bring to for further processing. Then 1.5 mL of rumen liquor was centrifuged at 12000 rpm for 5 min at room temperature (22-24°C). Pre-weighing of the empty centrifuge tubes was done, to ensure the equal weight (150 mg) of all the pellets, before the start of the experiment. After getting the bacterial pellet the pellet was re-suspended in 100 μL of 1X TE buffer (pH 8.0). Fifty microliter of 2% TritonX-100 and 50 μL of lysozyme (50 mg mL^{-1}) were added and incubated for at least 30 min at 37°C (with gentle vortexing for 10 sec after every 5 min). After this 50 μL proteinase K (50 mg mL^{-1}) and 250 μL of lysis buffer (100 Mm Tris-HCl-pH-8.0; 100 mM EDTA; 5 M GuSCN and 0.5% NP-40) were added. Mixed by vortexing and incubated at 56°C for 30 min and then for 15 min at 95°C (vortex gently after every 5 min for 10 sec) and centrifuge for 30 sec at 5000 rpm. The aqueous phase part was then taken out without disturbing the cell pellet in a 2 mL fresh tube and added 150 μL of binding buffer (50 Mm Tris-Cl-pH-8.0, 3 M GuSCN, 20 mM EDTA-pH-8.0) and 50 μL of isopropanol. Mixed by pipetting up and down before loading on silica based column (supplied with Fermentas Genomic DNA purification Kit, USA) and centrifuged at 10000 rpm for 1 min at room temperature. Material collected from column was discarded and column was placed into the same collection tube. Add 500 μL of 20 mM Tris-HCl (pH 8.0), 3M GuSCN and 10 mM EDTA and centrifuge at 10000 rpm for 1 min at room temperature. The material comes out after passing through column was discarded and column was placed again in the same collection tube again. Again add 700 μL of 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM EDTA and 80% ethanol and centrifuged at 13000 rpm for 1 min at room temperature. Discard the flow through and placed the column in the same collection tube. Centrifuge for additional 2 min at 12000 rpm at room temperature to remove the ethanol traces completely. Placed the column in fresh 1.5 mL centrifuge tube and elute the DNA with pre-warmed (55°C) nuclease free water.

Bacterial DNA isolation procedure popularly known as phenol-chloroform method applied as per Sambrook *et al.* (1989) and Kaufman *et al.* (1995) and kit method applied as per details given by manufacturer for isolation of genomic DNA from rumen liquor in this study.

DNA analysis: The quality and quantity of the DNA extracted by the above mentioned three methods were assessed by spectrophotometry (Bowtell, 1987; Kaufman *et al.*, 1995). In this, the Optical Density (OD) of the DNA samples was measured at 260 and 280 nm on Nanodrop (Thermo-scientific). Since the light absorption of nucleic acid peaks at 260 nm and that of the protein at 280 nm, the ratio of the two indicate the purity.

PCR and qPCR conditions: primers used, medium composition, run setup: The quality of the DNA yield was further assessed by agarose gel electrophoresis, PCR amplification and Real-Time analysis. The genomic DNA's were amplified with *mcr-A* gene (a functional gene restricted to methanogenic archaea) specific primers (*mcrA* F 5'-GGTGGTGTMGGATTCACA CARTAYGCWACAGC-3 and mcrA R5'-TTCATTGCR TAGTTWGGRTAGTT-3') by using PCR. The PCR reaction consisted of an initial denaturation step of 95°C for 5 min followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 90 sec followed by a final extension step of 10 min. The starting volume of genomic DNA taken was 1 μL in all the reactions and the amplified product was run on 1.5% agarose gel to check the quality of DNA isolated.

Further the genomic DNA isolated by all the three protocols was subjected to Real-Time analysis. The reaction mixture (25 μL) consisted of 12.5 μL SyBR green PCR Master Mix (Fermentas, USA) 20 pmol. Each of *mcr-A* forward (F 5'-TTCGGTGGATCDCARAGR GC-3') and reverse (5'-GBARGTCGWAWCCGTAGAATCC-3') primers and 1 μL of genomic DNA isolated by all the three protocols. The PCR reaction consisted of an initial denaturation step of 95°C for 5 min followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 90 sec. Data was collected using the ABI Prism 7700 sequence detection system (Applied Biosystems, USA). The Ct value, the cycle at which the fluorescence increases beyond a threshold level, correlates with the input target gene level (Winer *et al.*, 1999).

RESULTS

Comparison of total genomic DNA for purity and concentration: The DNA obtained using the above methods were analyzed on the basis of yield and purity. Isolated DNA by all the three protocols was run on 0.8% agarose gel to check the quality of genomic DNA. Results indicated that our improved method given an intact genomic DNA band similar in case of kit based method while, phenol chloroform method resulted in the shearing of genomic DNA. The isolated DNA from all methods were checked for purity by taking absorbance at 260/280 nm. The DNA isolated by improved extraction method gives the O.D equivalent to 1.8 which shows high purity DNA, whereas, the O.D of the other two samples falls within the range of 1.6-1.8 which shows the presence of some impurities in the isolated samples. The concentrations of the DNA samples were also checked by Nanodrop (Thermo-scientific) measurement. A result of concentration of DNA sample isolated by the improved protocol was 233 ng μL^{-1} while the concentration of DNA sample isolated by kit based method was 212 and 144 ng μL^{-1} by phenol-chloroform method, respectively.

Detection of methanogenic DNA in total genomic DNA by conventional PCR: The genomic DNA isolated by all the three protocols was further subjected to conventional PCR assay by *mcr-A* gene specific primers to determine the quantity and quality of methanogenic DNA present in the isolated DNA samples. The intensity of the amplified product was checked by Spot Denso (Alpha Image tech) and the good amplified band was obtained with improved extraction method protocol in comparison to other methods which gives faint bands as depicted in Fig. 1.

Quantitative RT- PCR analysis: All the three isolation methods which yielded genomic DNA were also evaluated by qPCR for their ability to recover methanogenic DNA from total genomic DNA samples. Figure 2 and Table 1 showed the limit of detection of real-time PCR using DNA extracted from rumen liquor of *Bubalus bubalis* with improved protocol, kit based method and Phenol Chloroform based method. Of the three extraction methods evaluated, improved protocol yielded methanogenic DNA detected at the lowest concentration by real-time PCR.

Comparative evaluation of all methods for cost, processing time and other parameters: Details of different parameters of evaluation presented in Table 2 which showed comparisons among extractions methods for cost, processing time, and sample extract volume. Improved DNA extraction protocol was the least expensive ($1.12 per extraction) than that of the three methods evaluated in present experiment. The cost per extraction for the Phenol Chloroform methods was $1.78 per extraction. However, the kit based method was considerably more expensive than the two other extraction methods, costing $9.40 per extraction. Although our proposed protocol was the least expensive on account of a cost per extraction basis but it requires slightly more processing time (2 h, 30 min) than the kit based method but less than the Phenol Chloroform method. The processing time for the kit based and Phenol Chloroform methods were 1 h, 30 min and 3 h, 10 min, respectively. The recovery volumes for sample extracts prepared using the three isolation methods were ranged from 50 to 100 μL, with improved protocol and the kit based method providing the maximum volume then the Phenol-Chloroform method.

Table 1: Quantitative analysis (Real Time PCR) of the amount of methanogenic DNA isolated by different methods using real-time PCR. Wells A3 and B3 contain DNA samples isolated by kit, C3 and D3 contain DNA sample isolated by our protocol and wells E3 and F3 contain DNA samples isolated by phenol-chloroform method

Well / Set dye	Content	Efficiency (%)	C(t)
A3 SBG1	Sample	82.01	22.07
B3 SBG1	Sample	87.77	21.06
C3 SBG1	Sample	84.44	17.65
D3 SBG1	Sample	84.70	15.79
E3 SBG1	Sample	70.09	24.22
F3 SBG1	Sample	72.17	24.09

Table 2: Comparative evaluation of various critical features of different methods used for DNA isolation.

	Methods		
Parameters	Improved	Phenol chloroform	Kit based
Cost	Very less	High	Very high
Quality	Very Good	Good	Very Good
Efficiency *	High	Low	High

*Efficiency is calculated on the basis of amplification of gene by Real-Time PCR

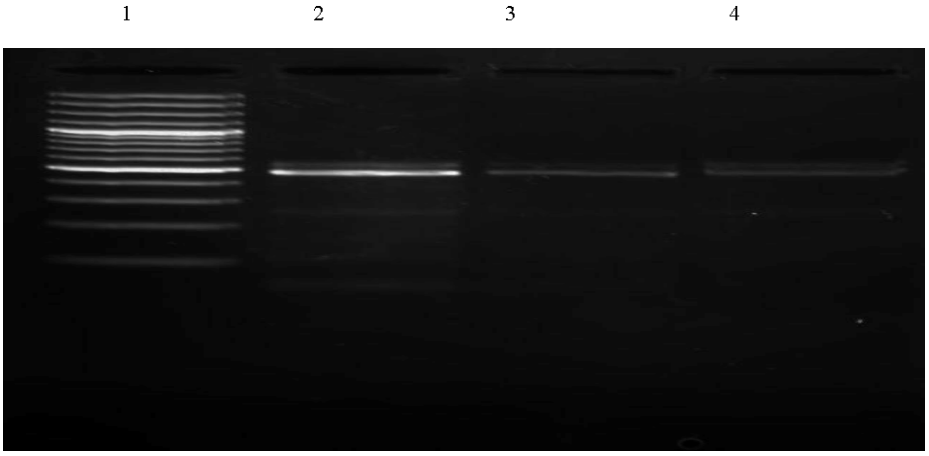

Fig. 1: Amplified PCR products by using all the three protocols. Lane 2: MCR gene product amplified from the genomic DNA isolated by our protocol. Lane 3 and 4: MCR gene product amplified from the genomic DNA isolated by kit based and Phenol chloroform protocol respectively

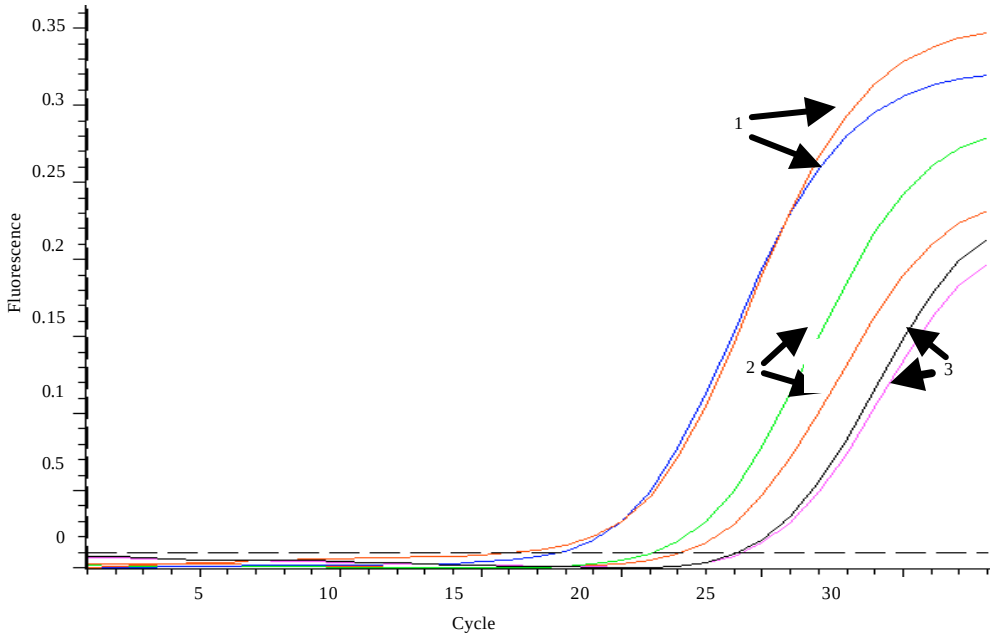

Fig. 2: Quantification of methanogenic DNA isolated by different methods using real-time PCR. (1) DNA sample isolated by our protocol, (2) DNA samples isolated by kit and (3) DNA samples isolated by phenol-chloroform method

DISCUSSION

In the present study three protocols were evaluated for the isolation of good quality and quantity of methanogenic DNA in total genomic DNA isolated from rumen liquor. Isolated DNA from different methods was examined for its utility in carrying out downstream applications like

PCR and qPCR. Cellular disruption is the first step in DNA isolation and one of the most critical and important step, affecting yield and quality of the isolated DNA. Typically, cell disruption needs to be fast and thorough. Slow disruption, for example placing cells or tissue in lysis solution without any additional physical shearing, may cause DNA degradation. Incomplete disruption may also result in lower yield because some of the DNA in the sample remained trapped in intact cells and, therefore, is unavailable for subsequent purification. For the most samples, thorough disruption can be monitored by close inspection of the lysate after disruption. There should be no visible particulates, except when disrupting materials containing hard components, such as cell wall of methanogens. Finding the most appropriate method of cell or tissue disruption for specific starting material is important for maximizing the yield and quality of your DNA preparation.

Many laboratories carry out the kit based method or traditional Phenol-Chloroform method for the isolation of total genomic DNA from rumen liquor and further uses this genomic DNA to amplify the methanogenic DNA from it. In this study, the improved methodology as we described, for the isolation of good quality and quantity of methanogenic DNA in total genomic DNA was better than the other two methods in comparison to purity, yield, cost, recovery and time. The results of this study indicated that proposed protocol is much better than the two other extraction methods in most of the parameters stated above.

The large differences in the amounts of methanogenic DNA recovered with the different DNA extraction methods may be due to the use of Guanidine thiocyanate for cell lysis. It has been shown that the best way to lyse bacterial cells is guanidine thiocyanate. In this protocol we have used the above chemical for the lysis of the bacterial cell membrane. After Pre-digestion of the cell wall cells should be lysed efficiently. Only limited information was available for the use of guanidine thiocyanate for lysing the bacterial cell membrane.

Selection of method for DNA isolation to be used in routine works the protocol should be cost effective.The main motive behind designing the present study is to provide an inexpensive method for successful isolation of total DNA as well as methanogen specific DNA.This DNA would be further used explore unique microbial community with high diversity and abundance (Lysnes *et al*., 2004; Santelle *et al*., 2008). In terms of cost also our extraction protocol is the least expensive of the three methods evaluated. Cost wise per extraction for improved method for DNA extraction was $1.12 per extraction in comparison to phenol chloroform method being $1.78 per extraction. The kit based method however, was considerably expensive than the two other extraction methods, costing $9.40 per extraction. Although our proposed improved method was the least expensive on the basis of cost per extraction, it required slightly more processing time (2 h, 30 min) than the kit based method but less than the phenol chloroform method. The processing time for the kit based and phenol chloroform methods were 1 h, 30 min and 3 h, 10 min, respectively. The recovery volumes for sample extracts prepared using the three isolation methods were ranged from 50 to 100 μL, with improved protocol and the kit based method also providing the good amount volume but phenol-chloroform method providing the minimum volume. However, some of the researchers have observed and suggested that the addition of skimmed milk to goethite enhances archeal DNA yield by 6.5% (Ogram *et al*., 1994; Rochelle *et al*., 1994; Zhou *et al*., 1996; Frostegard *et al*., 1999; Purdy *et al*., 1996; He *et al*., 2005; Hoshino and Matsumoto, 2005).

The amount of methanogenic DNA as quantified by PCR showed significance difference, with our protocol being the maximum followed by kit and phenol-chloroform method (Fig. 1). The results were further confirmed by amplification of *mcr*-A gene using methanogen specific primers qPCR

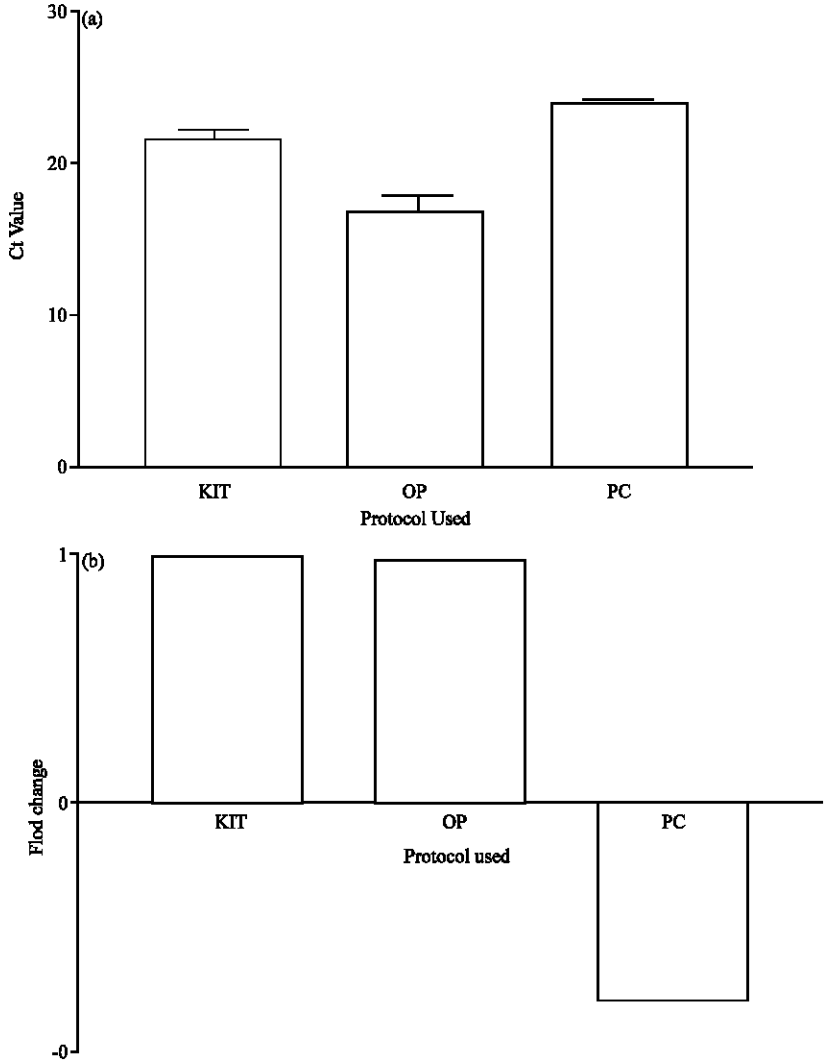

Fig: 3: Graphical representation for the quantitative analysis of the amount of methanogenic DNA isolated by different methods using real-time PCR. (a) Ct values (b) fold change of: (KIT) DNA sample isolated by kit, (OP) DNA samples isolated by our protocol and (PC) DNA samples isolated by phenol-chloroform method

(Table 1, Fig. 2). The large Ct value of PCR product obtained from DNA isolated by using kit and phenol chloroform method further confirmed the efficacy of protocols isolating less amount of methanogenic DNA in the total DNA. The relative amount of PCR product as quantified by fold change, taking the PCR product of DNA isolated by kit as 1 show a significant difference between improved extraction method and phenol-chloroform method (Fig. 3a, b). New improved method was found at par with the kit based method.

As far as the efficiency of the qPCR reaction is concerned, it has been found that the efficiency of reaction carried out by using DNA isolated by new protocol was similar to that of DNA isolated by kit based method and was quite good presented in Table 1. The efficiency of PCR reaction by DNA isolated from phenol chloroform method is however, comparatively less than both the other

methods. It means that DNA isolated by proposed protocol was quite good and is free from contamination for the PCR reaction and suitable for absolute and precise quantification of methanogenic archaea. The DNA isolated from this improved protocol therefore, can be successfully used for many downstream applications.

It was concluded from present study that improved DNA isolation protocol from rumen liquor for specific microbes like methanogenic archaea provides easy, cost effective and efficient for routine use.

ACKNOWLEDGMENT

Authors are thankful to Director, National Dairy Research Institute, Karnal for providing all facilities to carry out research work and financial assistance provided by National Fund for Basic, Strategic and Frontier Application Research in Agriculture (NFBSFARA), ICAR, New Delhi, India

REFERENCES

Benner, R. and K. Kaiser, 2003. Abundance of amino sugars and peptidoglycan in marine particulate and dissolved organic matter. Limnol. Oceanogr., 48: 118-128.

Bowtell, D.L., 1987. Rapid isolation of eukaryotic DNA. Anal. Biochem., 162: 463-465.

Frostegard, A., S. Courtois, V. Ramisse, S. Clerc and D. Bernillon *et al.* 1999. Quantification of bias related to the extraction of DNA irectly from soils. Applied Environ. Microbiol., 65: 5409-5420.

He, Z., L. Wu, X. Li, M. Fields and J. Zhou, 2005. Empirical establishment of oligonucleotide probe design criteria. Applied Environ. Microbiol., 71: 3753-3760.

Hoshino, Y.T. and N. Matsumoto, 2005. Skim milk drastically improves the efficiency of DNA extraction from andisol, a volcanic soil. Jap. Agric. Res. Q., 39: 247-252.

Kandler, O. and H. Konig, 1985. Cell envelopes of archaebacteria. Bacteria, 8: 413-457.

Kaufman, P.B., W. Wu and K. Donghern, 1995. Handbook of Molecular and Cellular Methods in Biology and Medicine. CRC Press, London, pp: 484.

Lysnes, K., I.H. Thorseth, B.O. Steinsbu, L. Oreas, T. Torsvik and R.B. Pedersen, 2004. Microbial community diversity in seafloor basalt from the Arctic spreading ridges. FEMS Microbiol. Ecol., 50: 213-230.

Ogram, A.V., M.L. Mathot, J.B. Harsh, J. Boyle and C.A. Pettigrew, 1994. Effects of DNA polymer length on its adsorption to soils. Applied Environ. Microbiol., 60: 393-396.

Purdy, K.J., T.M. Embley, S. Takii and D.B. Nedwell, 1996. Rapid extraction of DNA and rRNA from sediments by a novel hydroxyapatite spin-column method. Applied Environ. Microbiol., 62: 3905-3907.

Rochelle, P.A., B.A. Cragg, J.C. Fry, R.J. Parkes and A.J. Weightman, 1994. Effect of sample handling on estimation of bacterial diversity in marine sediments by 16S rDNA gene sequence analysis. FEMS Microbiol. Ecol., 15: 215-226.

Sambrook, J., E.F. Fritsch and T.A. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA., ISBN-13: 9780879695774, pp: 21-51.

Santelle, C.M., B.N. Orcutt, E.W. Banning Bach, C.L. Moyer, M.L. Sogin, H. Staudigel and K.J. Edwards, 2008. Abundance and diversity of microbial life in ocean crust. Nature, 453: 653-656.

Tajima, K., T. Nagamine, M. Nakamura, R.I. Aminov and H. Matsui, 2001. Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. FEMS Microbiol. Lett., 200: 67-72.

Tokura, M., I. Chagan, Y. Kojima and K. Ushida, 1999. Phylogenetic study of methanogens associated with rumen ciliates. Curr. Microbiol., 39: 123-128.

Whitford, M.F., R.J. Forster and C.E. Beard, 1998. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. Anaerobe., 4: 153-163.

Winer, J., C.K.S. Jung, P.M. Williams and I. Shacke, 1999. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes *in vitro*. Anal. Biochem., 270: 41-49.

Wright, A.D.G. and C.H. Auckland and D.H. Lynn, 2007. Molecular diversity of methanogens in feedlot cattle from Ontario and Prince Edward Island Canada. Applied Environ. Microbiol., 13: 4206-4210.

Yanagita, K., Y. Kamagata, T. Suzuki, Y. Nakamura, H. Minato and M. Kawaharasaki, 2000. Phylogenetic analysis of methanogens in sheep rumen ecosystem and detection of Methanomicrobium mobile by fluorescence in situ hybridization. Biosci. Biotechnol. Biochem., 64: 1737-1742.

Zhou, J., M.A. Bruns and J.M. Tiedje, 1996. DNA recovery from soils of diverse composition. Applied Environ. Microbiol., 62: 316-322.