Indian Journal of Animal Sciences **92** (1): 96–100, January 2022/Article
https://doi.org/10.56093/ijans.v92i1.120932

Isolation and characterization of sulphate reducing bacteria from goat rumen and its inclusion to improve *in vitro* feed fermentation

SANDEEP UNIYAL[1], LAL CHAND CHAUDHARY[2]✉, ANJU KALA[2] and NEETA AGARWAL[2]

ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

Received: 20 February 2021; Accepted: 25 October 2021

ABSTRACT

In the present study sulphate-reducing bacteria (SRB) were isolated from the rumen of goats fed a diet of wheat straw and concentrate in 50:50 ratio using specific medium followed by phenotypic and phylogenetic characterization. Based on the biochemical characteristics, four best SRB isolates were evaluated for their efficacy to reduce *in vitro* CH_4 production and stimulate fibre digestion. It was found that true dry matter digestibility (IVTD) and production of metabolites were higher but methane emission was low by inclusion of live culture of SRB4 isolate as compared to control. Sequencing of 16S rDNA revealed 99% homology of SRB4 with *Streptococcus caviae* strain NR156902. The isolate also exhibited expression of dissimilatory sulphite reductase gene (*dsR*) gene substantiating sulphate reducing ability of the isolate. The results indicate the ability of SRB4 to reduce *in vitro* CH_4 emissions and improve fibre digestibility, hence can be explored as an effective candidate for microbial feed additive to modify rumen fermentation, so that enteric methane production can be controlled.

Keywords: Dissimilatory sulphite reductase gene, Goat rumen, *In vitro* feed fermentation, Methane, Sulphate reducing bacteria

Methane (CH_4) is a potent greenhouse gas (GHG) having 25 times higher global warming potential than that of carbon dioxide and globally contributing about 40% of the emissions produced by human-related activities (Kumari *et al.* 2016). During fermentation process in the rumen, the majority of methanogens use H_2 as electron donor and CO_2 as an electron acceptor to from CH_4 (Janssen and Kirs 2008). Carbon dioxide constitutes up to 65% of total gas in the rumen (Ellis *et al*. 1991) and it is not a limiting substrate of rumen methanogenesis. Therefore, H_2 is a key compound for controlling CH_4 production so, diversion of H_2 produced during feed fermentation towards the metabolic pathways other than methanogenesis seems to be a sustainable strategy to reduce methane production without affecting the animal performance (Yatoo *et al*. 2018, Lakhani *et al*. 2019, Uniyal *et al.* 2020). Competitive and co-operative relationships between methanogens and sulphate-reducing bacteria (SRB) have been described in anaerobic environments including in the rumen (Uniyal *et al.* 2020). The first sulphate reducing bacteria was isolated by Colemn in 1960 and was supposed to belongs the genus *Desulfovibrio,* later bacteria belonging to genera *Desulfotomaculum* and *Fusobacterium* was also isolated from the rumen fluid (Howard and Hungate 1976, Paul *et al.* 2011). The SRBs reduce sulphur containing compounds into hydrogen sulphide by utilizing ruminal hydrogen is either eructed from gas space or utilizes as a source of sulphide for the rumen microorganisms which are unable to directly utilize sulphate. It also stimulates cellulose degrading bacteria and fungi (McSweeny and Denman 2007) as minimum level of sulphide is required for maximal microbial growth. Sulphur in the form of is also required for the synthesis of sulphur containing amino acids which further enhance growth of rumen microbes hence, the performance of the animal (Lakhani *et al*. 2019). According to Droge *et al*. (2005), some SRBs can produce hydrogen sulphide, whereas, some can oxidize sulphide into sulphate and prevent accumulation of hydrogen sulphide in the rumen. But due to very thin population of SRBs in the rumen, it is not serving as hydrogen sink to an extent which could help in methane mitigation. So, the present study was conducted to identify elite SRB from the goat rumen which could be further explored as a microbial feed supplement as methane mitigation agent.

Present address: [1]Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. [2] ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh. ✉Corresponding author email: lcchaudhary1@rediffmail.com

MATERIALS AND METHODS

Isolation of sulphate reducing bacteria: Sulphate reducing bacteria were isolated from the rumen of goats fed a diet of wheat straw and concentrate in 50:50 ratio using specific medium modified from Howard and Hungate (1976). The specific medium comprised of solution A, 170 mL/L; solution B, 170 mL/L; resazurin (1.0 g/L), 1 mL;

yeast extract, 1.0 g/L; sodium bicarbonate, 5.0 g/L; DL sodium lactate, 0.8 ml/L; $FeSO_4 \cdot 7H_2O$ (10%), 430 µl/L; cysteine hydrochloride 0.05 g/L and ascorbic acid, 100 mg/L. Solution A was composed of Na_2SO_4, 5.0 g/L; NaCl, 2.0 g/L; $(NH_4)_2SO_4$, 3.0 g/L; KH_2PO_4, 3.0 g/L; $CaCl_2 \cdot 2H_2O$, 0.6 g/L; $MgSO_4 \cdot 7H_2O$ 0.6 g/L. Solution B contained K_2HPO_4, 3.0 g/L. The medium components excluding reducing agent (($FeSO_4 \cdot 7H_2O$ added at the time of inoculation after sterilization using 0.25 µ syringe filter and made anaerobic by bubbling CO_2 in it for 10 min) were prepared in a conical flask, degassed by boiling, cooled under oxygen free gas and dispensed anaerobically in Hungate anaerobic culture tubes under a steam of carbon dioxide. The tubes were stoppered with butyl rubber stopper and then autoclaved for 20 min at 15 psi pressure. The rumen liquor collected from sulphur adapted goats by stomach tube was used for the isolation of sulphate reducing bacteria. Hungate role tubes containing medium were inoculated with 1 ml of fresh rumen liquor and incubated at 39°C for 5–7 days till the inoculated medium turned black. Then, 1 ml of 5–7 days old enriched culture was diluted in anaerobic dilution medium up to 10^{-6} dilution and 0.3 ml of finally diluted culture was spread uniformly on the surface of agar by gently rotating the vials. The vials were incubated at 39°C for 7–10 days keeping upside down. The black colonies appeared on the agar medium were picked up using sterile inoculating needle under the stream of CO_2. The colonies were immediately transferred into fresh medium and incubated at 39°C for 7–10 days. Anaerobic plating, colony picking and trans-inoculations were repeated until monocultures were obtained.

Morphological and biochemical characterization of SRB isolates: The morphological characterization of isolates were done by performing Gram staining and examined under the microscope for Gram's reaction, shape and size. The individual cultures were also tested for growth pattern. The Biochemical characterization of the isolates were done for, sugar utilization ability, hydrogen sulphide production and catalase activity by inoculating in specific media. Based on the biochemical characterization including intensity of H_2S production and colour of colonies, four best isolates were selected for sugar utilization test.

Sugar utilization test: Thirteen sugars were tested for their utilization by the isolates as energy source. Each culture was inoculated in Hungate tubes containing specific sugar. Inoculated tubes containing medium without sugar served as control. The tubes were incubated for 24 h at 39ºC and observed visually for the growth. Based on morphological and biochemical characteristic four isolates (SRB1, SRB2, SRB3 and SRB4) were selected for their effect on *in vitro* feed fermentation.

In vitro *gas production test: In vitro* gas production test was conducted as per the procedure of Menke and Steingass (1988). The substrate (200±2 mg per syringe) used was wheat straw and concentrate mixture in 1:1 ratio and pooled rumen liquor collected from two adult male fistulated buffaloes (body wt 500±50 kg, maintained at Animal Nutrition sheds, ICAR-IVRI, Izatnagar) fed on the same diet which was used as inoculum. Incubation medium (30 ml) was dispensed anaerobically in each syringe. One set of syringes was comprised of three syringes each with 1.5 ml un-inoculated culture medium, with 1.5 ml un-inoculated culture medium + 0.09 mg sulphur, with 1.5 ml of live culture of SRBs (SRB1, 2, 3 or 4) + 0.09 mg sulphur and blanks, respectively, and three such sets were run so n=3. The syringes were incubated for 24 h at 39°C, thereafter, syringes were withdrawn from the incubator and all the analyses were done.

Estimation of gas and methane production: Gas production was estimated after 24 h of incubation by piston displacement. Net gas produced by feed fermentation was calculated by subtracting from total gas produced in blank. For methane estimation, 100 µl of gas sampled from headspace of the syringe was injected into Nucon- 5765 gas chromatograph equipped with Porapak Q column and flame ionization detector (Agarwal *et al*. 2008). A mixture of 50% carbon dioxide and 50% methane was used as standard.

Estimation of metabolites: For VFA estimation, 0.5 ml fermented medium was mixed with 0.1 ml of 25% metaphosphoric acid and allowed to stand at room temperature for 1 h. The mixture was centrifuged at 10,000 rpm for 10 min and 1 µl clear supernatant was injected on Nucon-5765 gas chromatograph equipped with chromosorb 101 column and FID as per the procedure described by Cottyn and Boucque (1968) with some modifications (Agarwal *et al*. 2008). Fermented medium was analyzed for NH_3-N (Weatherburn 1967).

In vitro *true digestibility (IVTD) of substrate:* The IVTD of feed was determined after termination of incubation. The contents of syringes were transferred quantitatively, into spoutless beakers by repeated washings with 100 ml neutral detergent solution. The flask contents were refluxed for 1 h and filtered through pre-weighed Gooch crucibles (Grade G1). The dry matter content of the residue was weighed and in vitro true digestibility of feed was calculated as follows (Van Soet *et al*. 1988).

$$\text{True digestibility (TD \%)} = \frac{\text{(Initial DM of feed taken for incubation} - \text{NDF residue)}}{\text{(Initial DM of feed taken for incubation)}} \times 100$$

Phylogenic characterization of SRB isolates by 16S ribosomal DNA: For DNA extraction, a pure culture (2 ml) of the isolate was taken in 2 ml micro centrifuge tubes with baked zirconium beads and centrifuged at 14,000 ×g for 15 min at 4°C. The pallet was washed with TE-buffer and processed for isolation of genomic DNA (Yu and Morrison 2004) using QiAmp DNA stool mini kit (Cat. No.51504). The 16S rDNA sequence was amplified by PCR using the bacterial universal primers 350f 5'-GTGCCAGCM-GCCGCGG-3′ and 1492r 5′-TACGYTACCTTGTTA-CGACT-3 (An *et al*. 2005). The sequencing of the purified PCR product was got done from Eurofins Genomics India Pvt. Ltd, Bengaluru. The sequences obtained were edited

and checked for chimera by using CHECK_CHIMERA program. The reference sequences were retrieved from the GenBank of National Centre of Biotechnology Information (NCBI) and similarity of isolate sequence with reference sequences were searched by using NCBI Basic Local Alignment Search Tool (BLAST). The alignment of the known and reference sequences were performed using CLUSTAL W and corrections were made manually at places of ambiguous alignment. The phylogenetic tree was constructed by neighbour-joining method with complete deletion option to eliminate all gaps and missing data from datasheet and 1,000 bootstrap using MEGA 5.0 software.

Amplification of DSR gene: To verify the presence of dissimilatory sulphite reductase (*dsR*) gene, genomic DNA of the isolate was amplified for a 221 bp fragment of the *dsR* gene using a specific primer pair, DSR-F5′ACSC-ACTGGAAGCACGGCGG3′ and DSR-R 5′ GTGGMRC-CGTGCAKRTTGG 3′ (Kondo *et al.* 2004).

Statistical analysis: The data were statistically analyzed using IBM SPSS version 16 computer package. For comparison of groups, generalized linear model ANOVA procedure and Duncan's multiple range test were used (Snedecor and Cochern, 1994). Significant difference among the treatments was established at $P<0.05$.

RESULTS AND DISCUSSION

Isolation of sulphate reducing bacteria (SRB): The sulphate-reducing bacteria (SRB) were isolated from the rumen of goats adapted for sulphur containing (0.25% of DMI) maintenance ration (ICAR 2013) using specific medium. Growth of SRB isolates was evidenced by the development of black colour in the medium by rapid precipitation of ferrous sulphide and generation of H_2S within 5–7 days of inoculation which is specific for SRB. A black precipitate was formed due to the formation of ferrous sulphide as a result of reduction of ferrous sulphate to H_2S which is a strong indicator of the presence of sulphate reducing bacteria in the medium. The inoculation of this active culture on agar plates resulted in appearance of round shaped, smooth colonies on the surface of agar plates after 7–10 days of incubation (Fig. 1). After repeated plating on agar medium and picking the black colonies in broth, 30 isolates were obtained which were pure when examined under the microscope. Till date, very few studies have been

Fig. 1. Black colonies of sulphate reducing bacteria on agar plate.

carried out on the isolation of sulphate reducing bacteria from the rumen. The first SRB from sheep rumen was isolated by Coleman in 1960 and later by Howard and Hungate (1976) using various culture-based techniques. Paul *et al*. (2011) isolated an elite sulphate reducing bacteria from rumen liquor of buffalo fed on wheat straw, green forage and concentrate and identified the isolate as *Fusobacterium* spp. However, sulphate reducing bacteria from other anaerobic environments like soil, sewage, marine inhabitant are more popular (Kondo *et al*. 2012, Babu *et al*. 2014, Tkachuk *et al*. 2020).

Morphological and biochemical characterization of SRB isolates: The purity of the isolates was confirmed by microscopic examination after Gram staining and it was observed that the shape of all the isolates was coccoid. Most of the isolates were single. All the isolates were Gram positive and non-motile which gives an initial indication that the bacteria belong to genus *Streptococcus*. All the isolates produced black precipitate in the culture medium which is an indicator of generation of hydrogen sulphide and confirm the presence of sulphate reducing bacteria in the medium. All the isolates were negative for catalase activity which means they were anaerobic in nature and of rumen origin. Paul *et al*. (2011) isolated an SRB from the

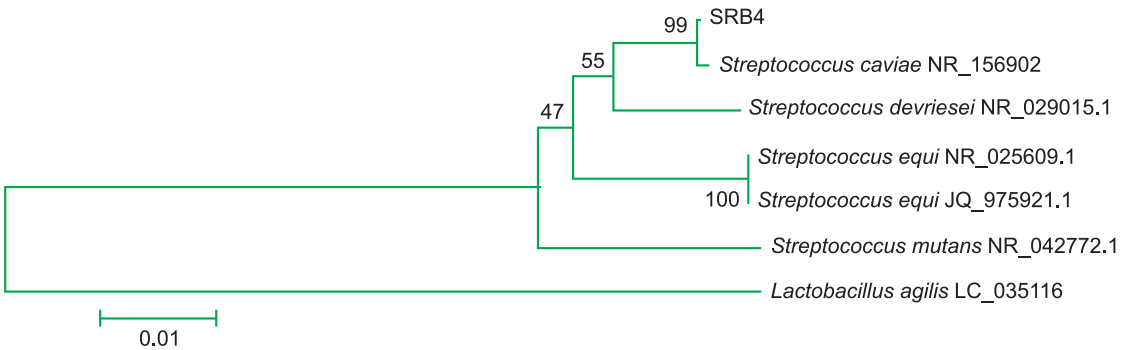

Fig. 2. Phylogenic analyis of 16S rDNA sequence of SRB4.

Table 1. Effect of inclusion of sulphate reducing bacteria isolates on *in vitro* feed fermentation

Treatment	Gas (ml/g DM)	Methane (ml/g DM)	IVTD (%)	TVFA (mM/dl)	NH_3-N (mg/dl)
Control	135.3^{abc}	30.06	61.35^{a}	4.10	6.88^{a}
C+M	130.6^{ab}	28.89	62.37^{ab}	4.78	7.21^{ab}
C+M+S	125.3^{a}	28.02	61.41^{a}	4.51	7.84^{ab}
SRB1	140.8^{abcd}	28.76	62.68^{ab}	4.74	7.89^{abc}
SRB1+S	144.1^{abcde}	28.58	62.79^{ab}	5.21	7.95^{abc}
SRB2	157.7^{cde}	29.52	63.20^{ab}	4.97	8.05^{abc}
SRB2+S	143.5^{abcde}	28.39	62.31^{ab}	5.17	8.14^{abc}
SRB3	155.0^{bcd}	29.41	63.13^{ab}	4.77	8.06^{abc}
SRB3+S	144.4^{abcde}	26.92	63.37^{ab}	4.55	8.66^{ab}
SRB 4	165.0^{de}	27.94	64.23^{b}	4.84	9.45^{bc}
SRB 4+S	167.8^{e}	26.54	64.18^{b}	5.06	10.73^{c}
SEM	2.23	0.36	0.19	0.15	0.16
P Value	<0.001	0.084	0.03	0.953	<0.001

^{abc}values bearing different superscript in a column differ significantly. S, sulphur @ 0.3% of substrate; M, 1.5 ml broth; SRB, live culture 1.5 ml.

rumen of buffalo which was Gram negative, non-motile rods and produce H_2S as indicated by formation of black precipitate in the isolation medium.

Sugar utilization test: Based on intensity of hydrogen sulphide production and colour of colonies, four isolates (SRB1, SRB2, SRB3 and SRB4) were selected and subjected for sugar utilization test. Thirteen sugars were tested for their utilization by the isolates as energy source. All the four isolates utilized cellobiose, lactose, glucose, xylose, raffinose, arabinose, trehalose, galactose, salicin maltose, sucrose and starch but unable (-) to utilize inulin as source of energy. The sugar utilization test indicated that the isolates can sustain on variety of sugars. No such study has been performed on SRBs of rumen origin, only Paul *et al*. (2011) conducted this test with few carbon sources and observed that isolates were not able to utilize acetate, butyrate or maltose as a source of energy. But the present isolates had good ability to utilize maltose as energy source reflecting the difference between two isolation studies.

In vitro *feed fermentation:* The results of total gas production (ml/g DM), methane production (ml/g DM), *in vitro* true digestibility (IVTD), ammonia nitrogen (NH_3-N) and TVFA after 24 h of incubation are presented in Table 1. The total gas production in 24 h ranged from 125.3 to 167.8 ml/g DM and was significantly (P<0.001) higher with the isolate SRB4 along with sulphur (167.8 ml/g DM) as compared to control. The mean values of methane ranged from 26.54 to 30.06 ml/g DM and it was 11.7% lower (P<0.08) in the treatment with SRB4 along with sulphur as compared to control. *In vitro* true digestibility (IVTD) and ammonia nitrogen (NH_3-N) were significantly higher, whereas, TVFA production was numerically higher (P=0.09) with SRB4 either alone or with sulphur as compared to control. Paul *et al*. (2011) also demonstrated reduction (P<0.05) in *in vitro* methane production by inoculating sulphur reducing bacteria isolated from buffalo rumen. These finding suggests that SRBs use H_2 for the reduction of sulphate to sulphide and divert H_2 away from the methanogens and competition between these two groups reduce ruminal CH_4 synthesis. Jeyanathan *et al*. (2014) were also of the view that when sulphate is not limiting in the environment, SRBs can compete methanogens for the common substrates like formate acetate, etc. and also advocated use of SRBs as microbial feed additive to check methanogenesis in the rumen. Also thermodynamically sulphate reduction is slightly more favourable than methanogenesis (Gibson *et al*. 1993). The threshold values of H_2 (mM/L) for SRBs is lower than inhibition of methanogenesis by SRBs has been demonstrated in a variety of environments including landfills (Beeman and Suflita 1987) and the gut of termites (Dröge *et al*. 2005).

Molecular characterization of the isolates: Based on the results of *in vitro* gas production test, isolate number SRB4 was selected for molecular identification. The PCR amplicon produced by using primers targeting 16S rDNA was of desired size, i.e. 1.0 kb. Phylogenetic analysis of the 16S rDNA sequence of SRB4 identified the isolate as the member of *Streptococcus* genus and showed 99% homology with *Streptococcus caviae* strain NR156902 (Fig. 2). Amplification of *dsr* gene substantiates the sulphate reducing ability of the isolate. Confirmation of the presence of the dissimilatory sulphite reductase gene and the production of hydrogen sulphide in the culture medium strongly suggest that the SRB isolates are the members of sulphate reducing bacteria community of rumen microbial ecosystem. Paul *et al*. (2011) also considered hydrogen peroxide production as the one of major criteria for the isolation of SRBs the isolate was identified as the member of *Fusobacteria*. These finding suggest that very little information is available about sulphate reduction in the rumen. Interestingly, nearly all SRB isolates identified from human and animal gut systems belong to the genus *Desulfovibrio* (Dröge *et al*. 2005). Wagner *et al*. (1998) did phylogenic analyses of sulphate educing organisms using *dsr* gene sequences and establish the presence of uncharacterized SRBs in gastrointestinal tract. In conclusion, among the sulphate reducing bacteria isolated from goat rumen, the isolate number SRB4 identified as a member of genus *Streptococcus* and possess the *dsr* gene had ability to improve *in vitro* feed digestibility and decrease methane emission. The culture can be further explored as microbial feed additive for ruminants for reducing methane emission and improving overall performance.

ACKNOWLEDGEMENTS

The first author is grateful to Director, ICAR-Indian Veterinary Research Institute, Izatnagar for providing necessary facilities to carry out the research work.

REFERENCES

Agarwal N, Kamra D N, Chatterjee P N, Kumar R and Chaudhary L C. 2008. *In vitro* methanogenesis, microbial profile and

fermentation of green forages with buffalo rumen liquor as influenced by 2-bromoethane sulphonic acid. *Asian Australasian Journal of Animal Science* **21**: 818–23.

An D, Dong X and Dong Z. 2005. Prokaryote diversity in the rumen of yak (*Bos grunniens*) and Jinnan cattle (*Bos taurus*) estimated by 16S rDNA homology analyses. *Anaerobe*. **11**: 207–15.

Beeman R E and Suflita J M. 1987. Microbial ecology of a shallow unconfined ground water aquifer polluted by municipal landfill leachate. *Microbiology Ecology* **14**: 39–54.

Coleman G S. 1960. A sulphate-reducing bacterium from the sheep rumen. *Journal of General Microbiology* **22**: 423–36.

Cottyn B G and Boucque C V. 1968. Rapid method for the gas chromatographic determination of volatile fatty acids in rumen fluid. *Journal of Agriculture and Food Chemistry* **16**: 105–07.

Dröge S, Limper U, Emtiazi F, Schönig I, Pavlus N, Drzyzga O, Fischer U and König H. 2005. *In vitro* and *in vivo* sulphate reduction in the gut contents of the termite *Mastotermes darwiniensis* and the rose-chafer *Pachnoda marginata*. *Journal of General and Applied Microbiology* **51**: 57–64.

Ellis J E, McIntyre P S, Saleh M, Williams A G and Lloyd D. 1991. Influence of CO_2 and low concentrations of O_2 on fermentative metabolism of the ruminal ciliate *Polyplastron multivesiculatum*. *Applied and Environmental Microbiology* **57**: 1400–07.

Gibson G R, Macfariane G T and Cummings J H. 1993. Sulphate reducing bacteria and hydrogen metabolism in the large intestine. *Gut* **34**: 437–39.

Howard B H and Hungate R E. 1976. *Desulfovibrio* of the sheep rumen. *Applied and Environmental Microbiology* **32**: 598–602.

Huisingh J, McNeill J J and Matrone G. 1974. Sulfate reduction by a *Desulfovibrio* species isolated from sheep rumen. *Applied Microbiology* **28**: 489–97.

ICAR. 2013. *Nutrient Requirements of Sheep and Goats*. 3rd edn. Indian Council of Agricultural Research. New Delhi, India

Janssen P H and Kirs M. 2008. Structure of the archaeal community of the rumen. *Applied and Environmental Microbiology* **74**: 3619–25.

Jeyanathan J, Martin C and Morgavi D P. 2014. The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. *Animal* **8**: 250–61.

Kondo R, Nedwell David B, Purdy Kevin J and Silva S Q. 2004. Detection and enumeration of sulphate-reducing bacteria in estuarine sediments by competitive PCR. *Geomicrobiology Journal* **21**: 145–57.

Kondo R, Mori Y and Sakami T. 2012. Comparison of sulphate-reducing bacterial communities in Japanese fish farm sediments with different levels of organic enrichment. *Microbes and Environments* **27**(2): 193–99.

Kumari S, Dahiya R P, Naik S N, Hiloidhari M, Thakur I S, Sharawat I and Kumari N. 2016. Projection of methane emissions from livestock through enteric fermentation: A case study from India. *Environmental Development* **20**: 31–44.

Lakhani N, Kamra D N, Kala A, Agarwal N, Chaudhary L C and Chaturvedi V B. 2019. Effects of dietary supplementation with rumen modifier and sodium sulphate on methane production and performance of buffalo calves. *Animal Nutrition and Feed Technology* **19**: 169–80.

McSweeney C S and Denman S E. 2007. Effect of sulfur supplements on cellulolytic rumen micro-organisms and microbial protein synthesis in cattle fed a high fibre diet. *Journal of Applied Microbiology* **103**: 1757–65.

Menke K H and Steingass H. 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Animal Research Development* **28**: 7–55.

Paul S S, Deb S M and Singh D. 2011. Isolation and characterization of novel sulphate-reducing *Fusobacterium* sp. and their effects on *in vitro* methane emission and digestion of wheat straw by rumen fluid from Indian riverine buffaloes. *Animal Feed Science Technology* **167**: 132–40.

Prasada Babu G, Subramanyam P, Sreenivasulu B and Paramageetham C H. 2014. Isolation and identification of sulfate reducing bacterial strains indigenous to sulphur rich barite mines. *International Journal of Current Microbiology and Applied Science* **3**: 788–93

Snedecor G W and Cochran W G. 1994. *Statistical Method*, 6th edn. The Iowa State University Press, Lowa, USA.

Tkachuk N, Zelena L, Mazur P and Lukash O. 2020. Genotypic, physiological and biochemical features of Desulfovibrio strains in a sulfidogenic microbial community isolated from the soil of ferrosphere. *Ecological Questions* **31**(2): 79–88.

Uniyal S, Chaudhary L C, Kala A, Agarwal N and Kamra D N. 2020. Effect of sulphur supplementation on methane emission, energy partitioning and nutrient utilization in goats. *Animal Nutrition and Feed Technology* **20**: 111–19.

Van Soest P J, Robertson J B and Lewis B A. 1991. Methods for dietary fiber, nuteral ditergent fiber and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* **74**: 3583–97.

Wagner M A, Roger J, Flax J L, Brusseau G A and Stahl D A. 1998. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. *Journal of Bacteriology* **180**: 2975–82.

Wheatherburn M W. 1967. Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry* **39**: 971–74.

Yatoo M A, Chaudhary L C, Agarwal N, Chaturvedi V B and Kamra D N. 2018. Effect of feeding of blend of essential oils on enteric methanogenesis, growth, nutrient utilization in growing buffaloes. *Asian-Australasian Journal of Animal Sciences* **31**: 672–76.

Yu Z and Morrison M. 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* **36**: 808–12.