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ORIGINAL ARTICLE

Isolation and characterization of reductive acetogens from rumen fluid samples of Murrah buffaloes

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Abstract

In the present study, attempts have been made to isolate reductive acetogens from the rumen fluid samples of Murrah buffaloes (*Bubalus bubalis*). Out of 32 rumen samples 51 isolates were isolated, and based on autotrophic growth for production of acetate and presence of formyltetrahydrofolate synthetase gene (FTHFS) 12 isolates were confirmed as reductive acetogens. Microscopic observations showed that ten isolates as Gram-positive rods (ACB28, ACB29, ACB66, ACB73, ACB81, ACB91, ACB133, ACB229, ACB52, ACB95) and two isolates as Gram-positive cocci (ACB19, ACB89). All isolates tested negative for catalase, oxidase, and gelatin liquefaction, whereas the production of H_2S was detected for two (ACB52 and ACB95) of the above isolates. All these isolates showed autotrophic growth from H_2 and CO_2, and heterotrophic growth with different fermentable sugars, viz., D-glucose, D-fructose, and D-trehalose but failed to grow on salicin, raffinose, and L-rhamnose. Out of the isolates, two showed amylase activity (ACB28 and ACB95), five showed CMCase activity (ACB19, ACB28, ACB29, ACB73 and ACB91), three showed pectinase activity (ACB29, ACB52 and ACB89), whereas none of the isolates was found positive for avicellase and xylanase activity. Based on 16S rDNA gene sequence analysis, the isolates showed their phylogenetic relationship with maximum similarity up to 99% to different strains of earlier reported known acetogens of clostridia group including *Clostridium* sp. (6), *Eubacterium limosum* (1), *Ruminococcus* sp. (1) and *Acetobacterium woodii* (1) except one, i.e., *Vagococcus fluvialis*. The results indicate that reductive acetogens isolated from the rumen fluid samples of Murrah buffalos are both autotrophic and heterotrophic in nature and further investigations are required to exploit and explore their potential as an alternate hydrogen sink.

Keywords Alternate hydrogen sinks · Formyltetrahydrofolate synthetase gene · Methane mitigation · Reductive acetogenesis · Rumen anaerobe

Introduction

The enteric fermentation of ruminants results in methane (CH_4) emissions that account for a significant loss of gross energy intake, ranging from 2 to 12% (Ungerfeld 2018; Beauchemin et al. 2020; Choudhury et al. 2022). This has prompted considerable interest in reducing CH_4 production in the rumen. Over the last few decades, numerous strategies and factors that affect CH_4 abatement have been studied, including the use of reducing agents and inhibitors (Kumar et al. 2014; Malik et al. 2015; Ungerfeld 2018; Garsa et al. 2019; Kim et al. 2020; Pereira et al. 2022; Króliczewska et al. 2023). However, the use of toxic chemicals and antibiotics as inhibitors is increasingly discouraged due to their adverse effects on the environment and residues in animal-derived products such as meat and milk (Pereira et al. 2022). As a result, successful CH_4 abatement strategies must focus on natural processes, such as non-methanogenic H_2 sinks in the rumen (Choudhury et al. 2022; Króliczewska et al. 2023). By identifying and promoting these natural processes, we may be able to reduce CH_4 emissions without the need for harmful inhibitors. For example, recent research has focused on identifying reductive acetogens as a potential alternative to hydrogenotrophic methanogens for H_2 utilization in the rumen (Kim et al. 2020; Li et al. 2020; Tseten et al. 2022).

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These natural strategies not only reduce CH_4 emissions but also have the potential to improve animal productivity and health. Thus, it is critical to continue researching and developing natural CH4 abatement strategies to mitigate the negative impacts of enteric fermentation on the environment and improve the sustainability of livestock production.

Non-methanogenic hydrogenotrophic bacteria residing in the rumen include acetogens that reduce CO_2 to form acetate by the Wood–Ljungdahl pathway during reductive acetogenesis an energetically profitable alternative to methane production (Malik et al. 2015; Choudhury et al. 2022; Pereira et al. 2022). H_2 and CO_2 utilizing acetogens have been isolated from the rumen environment, and these bacteria may be the natural microflora of the rumen, able to occupy the niche of methanogens as terminal sinks for ruminal H_2 (Mackie et al. 1994; Joblin 1999; Kim et al. 2020). Several studies have indicated that reducing methane emissions through the promotion of acetogenesis in the rumen while inhibiting methanogens could be a viable approach (Yang et al. 2015; Tseten et al. 2022). Research on the fore-stomach of a low-methane-emitting kangaroo species (*Macropus giganteus*) has suggested that acetogens play a significant role in H_2 removal (Godwin et al. 2014). Although methanogens are favored thermodynamically for H_2 capture compared to acetogens (Fonty et al. 2007), they contribute significantly to H_2 sequestration when methanogens are reduced or removed. Gagen et al. (2012) observed that in methanogen-free rumen, reductive acetogenesis accounted for up to 26% of H_2 removal. Similarly, in methanogen-free meroxenic lambs, reductive acetogenesis contributed to 21–25% of rumen fermentation, accounting for about 66% of total H_2 recovery (Fonty et al. 2007). In a previous experiment, newborn lambs reared in isolation from their dams produced 30–40% less CH_4 compared to conventional animals, as H_2 gas was utilized for acetate production via reductive acetogenesis (Faichney et al. 1999). These findings suggest that there may be potential for utilizing isolates within the rumen to enhance reductive acetogenesis and either inhibit or bypass ruminal methanogenesis. However, despite these promising results, there is currently a lack of information on reductive acetogens isolated from domesticated ruminants in India. Therefore, this study aimed to isolate and characterize reductive acetogens from rumen fluid samples of Murrah buffaloes. By identifying and studying these microorganisms in greater detail, we may gain a better understanding of their potential as a solution for reducing methane emissions in livestock production. In addition, this information could be utilized to develop new strategies for improving the sustainability of agricultural practices in India and beyond.

Materials and methods

Sampling of rumen liquor

Rumen liquor samples were collected from three adult fistulated male Murrah buffaloes (*Bubalus bubalis*) maintained at Livestock Research Centre, ICAR-National Dairy Research Institute, Karnal-132001, Haryana, India on a standard diet with roughage to concentrate ratio of 60:40. Samples were immediately brought to the laboratory in pre-warmed sterile CO_2 flushed reagent bottles and processed immediately following standard anaerobic cultivation techniques. The samples were flushed with O_2 free CO_2 passed through a Gassing manifold system (Biosystems, Pune, India) for 10 min and blended to separate microbes from the surface of feed particles followed by isolation. Serial dilution procedure was carried out in serum bottles containing anaerobic diluents solution [Mineral solution I-150 mL (K_2HPO_4-3 g/L), Mineral solution II-150 mL (KH_2PO_4-3 g, $(NH_4)_2SO_4$-6 g, NaCl-6 g, $MgSO_4 \bullet 7H_2O$-0.6 g and $CaCl_2 \bullet 2H_2O$-0.6 g dissolved in 1 L distilled water), Resazurin (0.1%)-1 mL, Na_2CO_3-5 g and L-cysteine-HCl-0.5 g in a final volume to 1 L adjusted with distilled water].

Isolation of reductive acetogens

Roll tubes were prepared by injecting 0.5 mL of inoculum from 10^{-3} to 10^{-5} dilution of each rumen liquor sample into 30 mL serum bottles containing 10 mL acetogen agar medium (Boccazzi and Patterson 2013). Care was taken to add the filter sterilized vitamin solution (10 mL/L) maintained at 4 ℃ containing Pyridoxine.HCl (0.02 g), Ascorbic acid (0.0112 g), Choline chloride (0.01 g), Thiamine–HCl (0.01 g), D, L-6,8-thioctic acid (0.01 g), Riboflavin (0.01 g), D-Calcium pantothenic acid (0.01 g), *p*-Amino benzoic acid (0.01 g), Niacinamide (0.01 g), Nicotinic acid (0.01 g), Pyridoxal–HCl (0.01 g), Pyridoxamine (0.01 g), Myo-Inositol (0.01 g), Biotin (0.004 g), Folic acid (0.004 g), and Cyanocobalamin (0.0002 g) in 200 mL solution and inocula only after cooling of medium to ≈45–50 °C. Inoculated serum bottles were incubated at 39 °C for 3–7 days for the development of colonies. The colonies were transferred to acetogen broth (Boccazzi and Patterson 2013) using sterile loops in an anaerobic glove box (IMSET, Mumbai, India). The individual cultures were subjected to Gram staining and examined under the microscope (Meiji, Saitama, Japan) to observe the morphology and purity. Shape and arrangement of the cells of the isolates were recorded. Catalase and oxidase activity of the isolates were performed. The isolates were designated

as ACB followed by their isolation number. Cultures were maintained by routine sub-culturing and for preservation; all the cultures were preserved in serum bottles (5 mL) containing 2 mL of anaerobically prepared 20% glycerol stock at -80 °C.

Confirmation of the isolates as reductive acetogens

Autotrophic acetate production

The isolates were checked for autotrophic acetate production incubated anaerobically containing a gas proportion of H_2: CO_2 (80:20) prepared by passing through a Gassing manifold gas mixture (Biosystems, Pune, India) by adjusting the flow rate of H_2 and CO_2. The anaerobic serum bottles were inoculated from the glycerol stocks and incubated for a period of 6 days at 39 ℃ in acetogen broth in a rotatory shaker (Shel Lab, Cornelius, USA) at 120 rpm with a control without any bacterial inoculation. After incubation, the supernatant of each culture was analyzed by gas chromatograph (Nucon 5765, Nucon Engineers, New Delhi, India) to determine Volatile Fatty Acids (VFAs) composition. VFAs in samples were estimated according to method given by Erwin et al. (1961). Supernatant (4.0 mL) collected from the centrifuged incubated material was transferred to a tube containing 1 mL of 25% meta-phosphoric acid and was kept overnight and centrifuged at 3000 rpm for 15 min at room temperature. The supernatant was transferred to another tube and stored in a freezer (− 20 °C) till estimation of VFA. VFA in the rumen fluid samples were determined using Gas chromatograph equipped with Flame Ionization Detector (FID) and glass column packed with chromosorb101 (length 4′; o.d. ¼′′; i.d. 3 mm; mesh range 80–100). Temperature of injection port, column and detector was set at 200, 180 and 210 °C, respectively. The flow rate of carrier gas (N_2) through the column was 40 mL/min; and the flow rate of H_2 and air through FID was 30 mL/min and 300 mL/min, respectively. Sample (5.0 µL) was injected through the injection port using a 10.0 µl Hamilton syringe (Hamilton Company, Nevada, USA). Different VFAs were identified on the basis of their retention time area covered on monitor and their concentration (mM/L) was calculated by comparing the retention time as well as the peak area of standards after deducting the corresponding blank values. Standard VFAs solution was prepared with various concentrations of VFAs (mM/100 mL) with acetic acid, 60.00; propionic acid, 20.00; butyric acid, 8.00; isobutyric acid, 4.00; valerate, 4.00 and iso-valerate, 4.00. The analysis and calculations were performed using Aimil Chromatography Data System (WINACDS).

Formyltetrahydrofolate synthetase amplification

The isolates were further analyzed by functional gene, formyltetrahydrofolate synthetase (FTHFS) PCR targeted methods for the confirmation of the isolates as reductive acetogens. For this, genomic DNA was extracted from all the isolates (Jena et al. 2017) and the concentration was checked using Nano-drop spectrophotometer (ND-1000; V3.5.2, Cambridge, UK). PCR-based detection for partial FTHFS gene was carried out as per the methods of Leaphart and Lovell (2001) with little modifications. Briefly; PCR reaction mixtures (50 µL) contained dNTPs each 2.5 mM; 20 pmol each primer; 5 µL 10 × PCR buffer (Genetix, India); 1U *Taq* DNA polymerase (Genetix, India) and template DNA approximately 50–100 ng. PCR reaction was carried out using forward primer 5′-TTY ACW GGH GAY TTC CAT GC-3′ and reverse primer 5′-GTA TTG DGT YTT RGC CAT ACA-3′ in a thermal cycler (Veriti® Applied Biosystems, California, USA) with initial denaturation for 5 min at 94 °C followed by 9 cycles of denaturation at 94 °C for 45 s, annealing at 63 °C for 45 s (decreased by 1 ℃ per cycle to 55 ℃), extension at 72 °C for 1 min. After the touchdown PCR reaction of the protocol was finished, 25 additional cycles with annealing temperature of 55 °C was used, and this was followed by a final elongation step consisting of 72 °C for 7 min. Amplified DNA fragments were examined by horizontal electrophoresis in 1% agarose gel (Sigma, USA) with 5 µL aliquots of PCR products. The gel images were digitized through the gel documentation system (Syngene G-Box, Cambridge, UK).

Biochemical characterization

Sugar fermentation

The isolates were biochemically characterized for sugar fermentation, gelatin liquefaction and H_2S production. For sugar fermentation test, ten different sugars, viz., D-glucose, sucrose, D-fructose, D-maltose, salicin, D-galactose, arabinose, L-rhamnose, D-trehalose and raffinose were individually added at a rate of 0.5% to anaerobic basal medium (Rieu-Lesme et al. 1996a) containing CO_2 as gas phase without H_2. The basal medium contains [Mineral solution I-150.0 mL, Mineral solution II-150.0 mL, Resazurin solution (0.1%, w/v)-1.0 mL, Tryptone-15.0 g, Yeast extract-1.0 g, L-Cysteine hydrochloride-0.5 g, Na_2CO_3-4.0 g, Clarified Rumen Fluid 100.0 mL and rest with distilled water maintained to Final pH-6.7 ± 0.1 per L]. Control tubes were prepared by the same method but without sugar in the medium to check the growth of bacteria influenced by the sugars present in clarified rumen liquor included in the medium. About 0.2 mL of 48–72-h-old culture was inoculated to each tube containing respective sugar along with the

control tube and incubated at 39 °C for 7–8 days. Development of turbidity in the medium was indicated positive for the utilization of the sugar supplemented.

H_2S production and gelatin liquefaction test

H_2S production test was performed to characterize for the production of FeS by blackening of the medium. For this, inoculum from 48 to 72 h old culture was inoculated to each serum bottle and incubated at 39 °C for 3–7 days in basal medium supplemented with glucose (0.2 g/L), cellobiose (0.2 g/L), ferric ammonium citrate (0.05 g/L) and sodium thiosulphate (0.008 g/L). Change in color of the medium to jet-black, indicates positive for H_2S production. For gelatin liquefaction test, 0.2 mL of inoculum was inoculated to each serum bottle containing basal medium supplemented with glucose (0.2 g/L), cellobiose (0.2 g/L gelatin (15 g/L) and kept at 39 °C for 2 weeks along with the control (without inoculum). After 2 weeks of incubation, all the serum bottles were stored at 4 °C and observed for liquefaction of gelatin by inverting the bottles.

Extracellular enzyme production

The isolates were characterized for the degradation of the complex polysaccharides and/or presence of extracellular enzymes, viz., CMCase, xylanase, pectinase, avicellase and amylase. Basal medium containing the individual polysaccharides at a rate of 5 g/L (carboxymethyl cellulose sodium salt, xylan, pectin, avicel and starch with 2% agar powder) were prepared to study the activity of CMCase, xylanase, pectinase, avicellase and amylase, respectively. Wells of equal diameter were made over the agar surface by keeping the plates in an anaerobic chamber. 100 µL of the respective culture was added in the wells and incubated at 39 °C for 48–72 h. After incubation, the plates first were treated with 1% Congo red (Teather et al. 1982) for 15 min, and then with 1 N NaCl for 15 min to see the zone of hydrolysis around the well for all the tests except for amylase assay for which iodine vapor was treated. To enhance the zone clarity, 1 N HCL was also used in pectinase assay. A clear zone surrounding the agar well indicates a positive reaction.

16S rDNA sequencing and identification

The DNA samples of the positive isolates confirmed for FTHFS gene were further amplified with universal 16S rDNA primers. The universal primers (Weisberg et al. 1961) targeting partial 16S rDNA region were used in PCR to amplify the partial FTHFS gene sequence. The PCR components (10 × PCR buffer; forward primer (10 µM); 20 pmol each primer; dNTPs mix (10 mM each); *Taq* DNA polymerase 1 U; template DNA 50 ng) were mixed properly and reaction was performed in 50 µL of PCR reaction mixture under a normal PCR cycling conditions for 35 cycles. The amplified products were electrophoresed in 1.5% agarose gel (Sigma, USA) with 5 µL aliquots of PCR products. The samples were gel purified with QIAquick gel extraction kit (Cat#28,704) according to the manufacturer's instructions (QIAGEN, Germany) and outsourced for sequencing in both strands (Merck, Millipore, Bangalore). The sequences received from the chromatogram were retrieved and both strands were aligned with CLUSTAL W (Thompson et al. 1994). Common aligned sequences were deleted and sequence chromatogram was corrected for no base calling (N). Then, the sequences were blasted (http://blast.ncbi.nlm.nih.gov/) to identify the isolate and different parameters including similarity percentage, nearest valid taxon and query coverage. All the sequences were submitted to NCBI database (GenBank) using BankIt submission tool (BankIt1879847) and the accession numbers received for the isolates were recorded from KU316938 to KU316947.

Results and discussion

The isolation of reductive acetogens involved processing 32 samples, and detectable counts of reductive acetogens were observed at 10^{-5} dilutions. In a study by Doré et al. (1995), the number of acetogenic bacteria utilizing H_2/CO_2 was reported to range from 10^2 to above 10^8 per gram of wet weight gut content. Nonetheless, the precise population density of reductive acetogens in Murrah buffaloes has yet to be quantified. The AC-11 medium was utilized for the isolation of reductive acetogens (Rieu-Lesme et al. 1998; Fonty et al. 2007). The inclusion of rumen fluid can enhance the growth of acetogens present in the rumen. Moreover, rumen fluid contains various nutrients and minerals that are necessary for the growth of reductive acetogens. Rieu-Lesme et al. (1996a; 1998) employed roll-tube techniques under H_2:CO_2 conditions to isolate acetogens and determined the optimal temperature and pH to be 37–40 °C and 6.3–6.8, respectively. H_2–CO_2 is a commonly used selective medium for the isolation of various acetogens (Leigh et al. 1981), including *Acetobacterium woodii* and *Sporomusa termitida* (Breznak et al. 1998). Chaucheyras et al. (1995) isolated the homoacetogenic bacterium Ser 8 in a gas mixture of H_2 and CO_2 (80%/20%; v/v) at 39 °C, using the medium reported by Balch et al. (1979). The growth of *Ruminococcus productus*, an acetogen, can also be selectively facilitated using CO–CO_2 medium (Lorowitz and Bryant 1984). Following incubation in an acetogen medium for 3–7 days, colonies appeared on the surface of serum bottles and the prolonged incubation period for isolation may be attributed due to the presence of inhibitory factors that exert selective pressure on growth. A total of 51 isolates were obtained from rumen

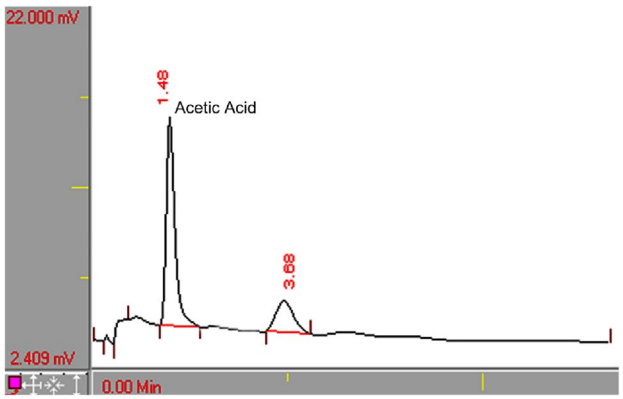

Fig. 1 Photograph showing gas chromatogram peak for acetate production from a reductive acetogen isolate cultured broth

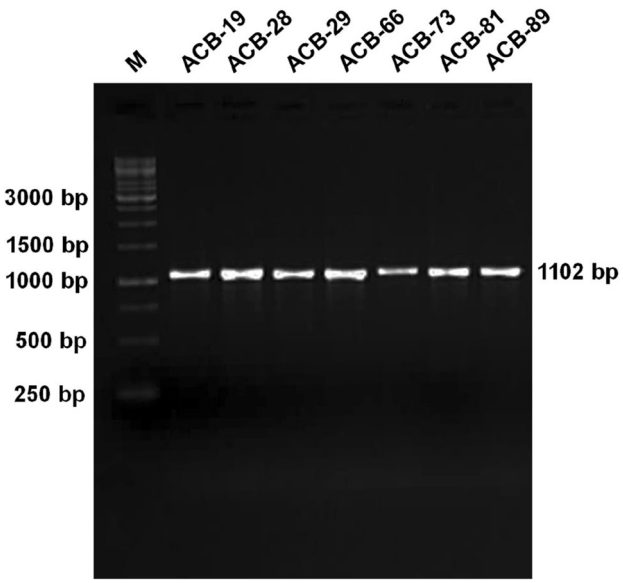

Fig. 2 Gel photograph showing amplified PCR products of the formyltetrahydrofolate synthetase gene (FTHFS gene) [Lane 1–7 FTHFS gene amplified products of marked respective isolates, M-1 kb DNA size ladder]

fluid samples and further subjected to molecular analysis for confirmation.

The production of acetate from CO_2 and H_2 via autotrophic acetate production was quantified by gas chromatography, and the resulting peak in acetate production is shown in Fig. 1. To confirm the isolates as reductive acetogens, the PCR-based method targeted the FTHFS functional gene. Among all the isolates, only 12 (19.84%) were found to exhibit a band size of approximately 1102 bp (Fig. 2). This particular gene serves as a functional marker for reductive acetogenesis and has been utilized for detection and quantification in various rumen studies (Matsui et al. 2008; Henderson et al. 2010; Singh et al. 2020). The low percentage of isolates confirmed at the molecular level may be attributed to the composition of the selective medium. One potential reason is that the rumen fluid present in the medium may provide sufficient carbon sources to support the growth of other microbes. To overcome this, Boccazzi and Patterson (2013) developed MAC-19, a rumen fluid-free medium incorporating casein hydrolysate and betaine, which was not utilized in this study. Among all the isolates, ACB19, ACB28, ACB29, ACB52, ACB66, ACB73, ACB81, ACB89, ACB91, ACB95, ACB133, and ACB229 were selected for further biochemical characterization and identification.

The isolates obtained in this study exhibited diversity in terms of colonial sizes, shapes, and colors, indicating variations among different isolates. Microscopic examination also revealed variations in cell sizes, shapes, and arrangements. Specifically, the cells of the acetogen isolates were characterized as Gram-positive cocci or rods that occurred singly or in chains. Drake et al. (2008) reported that reductive acetogens represent a diverse group comprising 22 genera and over 100 species that inhabit various ecological niches. *Acetobacterium* and *Clostridium* are the most commonly known genera among the 22 reported for acetogens. These bacteria exhibit great variation in their morphological, nutritional, and physiological characteristics as a group (Drake et al. 2008). The majority of the known isolates are mesophilic or thermophilic, appearing as rods or cocci (Drake et al. 2013). However, *Treponema primitia* sp. ZAS-2, a spirochete isolated from termite hindgut, is an exception. Although the rumen's temperature is in the mesophilic range, so our isolates were cultured at typical bovine rumen temperature, and their optimal growth temperatures and tolerance limits were not investigated. Previous studies on acetogenic bacteria isolated from the rumen have shown that they typically grow at mesophilic temperatures (Rieu-Lesme 1996a, 1996b; Genthner et al. 1981; Krumholz et al. 1985, 1986; Greening and Leedle 1989). To the best of our knowledge, none of the isolates reported in this study was from the buffalo rumen.

The 12 selected isolates in this study were evaluated for their oxidase and catalase production capabilities (Table 1). The results indicated that all isolates were catalase-negative as they did not produce any air bubbles when treated with H_2O_2. In additon, all isolates were negative for oxidase production. These findings are consistent with previous reports on catalase negativity of *Acetobacterium* genus (Balch et al. 1998) and of rumen acetogenic bacteria by Greening and Leedle (1989). Rieu-Lesme et al. (1996a) also reported the catalase and oxidase negativity of two strains of acetogenic bacteria (B and Bie 41). However, it was documented that acetogens can tolerate small amounts of O_2 (Karnholz et al. 2002; Takors et al. 2018) and can grow in both semi-solid and liquid media. They also found that low concentrations

Table 1 Microscopic and biochemical test characteristics of the isolated reductive acetogens

Name of the isolates	Gram staining	Catalase	Oxidase	H_2S production	Gelatin liquefaction
ACB19; ACB89	+ ve Cocci	− ve	− ve	− ve	− ve
ACB28; ACB29; ACB66; ACB73; ACB81; ACB91; ACB133; ACB229	+ ve Rods	− ve	− ve	− ve	− ve
ACB52; ACB95	+ ve Rods	− ve	− ve	+ ve	− ve

of O_2 can cause a lag phase in growth but do not affect the ability of acetogens to produce acetate via the acetyl CoA pathway. The same authors detected peroxidase and NADH oxidase activities were in cell-free extracts of *Moorella thermoacetica*, *Sporomusa silvacetica*, and *Clostridium magnum*, whereas catalase and superoxide dismutase activities were not detected. Other studies have shown that acetogens present in aerated soils and the rhizosphere of seagrass can tolerate and consume O_2 (Küsel et al. 2001; Karnholz et al. 2002). For example, *Clostridium glycolicum* RD-1, an aerotolerant acetogen isolated from seagrass roots, can tolerate up to 4% O_2 and possesses peroxidase and NADH oxidase activity (Küsel et al. 2001). However, none of the isolates from rumen origin has been reported to tolerate molecular O_2 or possess oxidative machinery, which supports the characteristics of our isolates being negative for catalase and oxidase activity.

Sulfur reduction activity is a characteristic feature of sulfur-reducing bacteria, but most of the isolates in the present study did not produce H2S except for two isolates (ACB52 and ACB95) that showed blackening of the medium supplemented with sodium sulfate. Rieu-Lesme et al. (1999b) reported 13 isolates of reductive acetogens, but did not observe any H_2S production under cultivable conditions, possibly due to the lack of additional sulfur source in the culture medium. Although metagenomic libraries of reductive acetogens have shown the presence of sulfur-reducing bacteria in many studies (Henderson et al. 2010), there are few reports characterizing the H_2S production activity of reductive acetogen isolates from rumen fluid samples. Gelatin liquefaction was not observed in any of the rumen reductive acetogen isolates, which is consistent with previous studies (Rieu-Lesme et al. 1996a, b) that reported negative results for gelatin liquefaction in reductive acetogens.

The sugar utilization patterns of various reductive acetogen isolates were determined using a medium without a carbon source but containing 0.1% yeast extract as a control. After 8 days of incubation with the substrate, growth was observed and confirmed by turbidity in the medium. All isolates were able to utilize D-glucose, D-fructose, and D-trehalose, but none was able to ferment salicin, arabinose, L-rhamnose, and raffinose. Isolates ACB19, ACB28, ACB52, and ACB133 were able to metabolize sucrose, while isolates ACB19, ACB73, and ACB95 metabolized D-maltose. These results were consistent with previous studies (Rieu-Lesme et al. 1996a, b; Breznak et al. 1998; Katsyv A and Müller 2020). Acetogens are known to be heterotrophically grown with other carbon sources in most studies. Greening and Leedle (1989) studied the sugar utilization pattern of five different strains and observed positive results for glucose and cellobiose utilization, but variation in fructose, esculin, and ferulic acid utilization. Drake et al. (2013) reported that acetogens can oxidize a wide range of substrates, including CO, H_2, carbohydrates, alcohols, carboxylic acids, dicarboxylic acids, aldehydes, substituent groups of aromatic compounds, and numerous other organic and halogenated substrates, delivering reductant to the acetyl-CoA pathway and facilitating the reductive synthesis of acetate. Due to their ability to use a wide range of substrates, acetogens have numerous trophic links to other organisms under in situ conditions. Isolates, namely ACB19, ACB28, ACB29, ACB73 and ACB91 showed positive reaction for CMCase production (Fig. 3A). Three of the isolates showed the utilization of pectin [ACB29, ACB52 and ACB89; (Fig. 3B)] and two isolates (ACB28 and ACB95) exhibited starch hydrolysis. Acetogens were also reported to degrade complex polymers, such as lignin or cellulose (Drake et al. 2008). An acetogen *Bryantella formatexigens* isolated from human feces utilized amorphous cellulose and carboxymethyl cellulose when isolated but lost this ability upon storage under frozen conditions (Wolin et al., 1994). Similarly, in another study, Karita et al. (1989) reported the ability of *M. thermoacetica*; strain F21 for CMCase and xylanase activities. The cellulolytic capabilities of these two organisms suggest that acetogens may be able to degrade certain complex polymers. An organism referred to as *Moorella thermoautotrophica* was also reported to degrade inulin (Budavari et al. 1989). However, Rieu-Lesme et al. (1996a) observed absence of utilization of cellulose by *Ruminococcus schinkii.* The findings of the present study also suggested the variability of lignocellulosic material degrading enzyme production. Although the biochemical results did not allow for clear classification of the different strains based on their properties, it was observed that most of the isolates shared common traits such as their ability to tolerate strict anaerobic conditions, grow under CO_2 and H_2, and utilize glucose. As a result, molecular

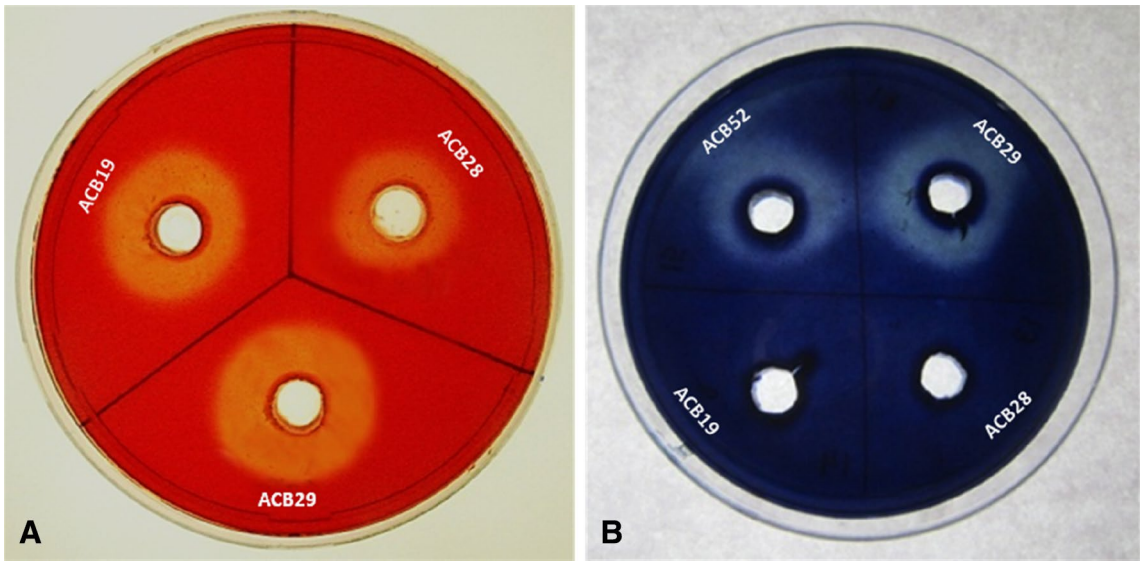

Fig. 3 Extracellular enzyme production assay of the reductive acetogen isolates: **A** cellulose hydrolysis test and **B** pectin hydrolysis assay

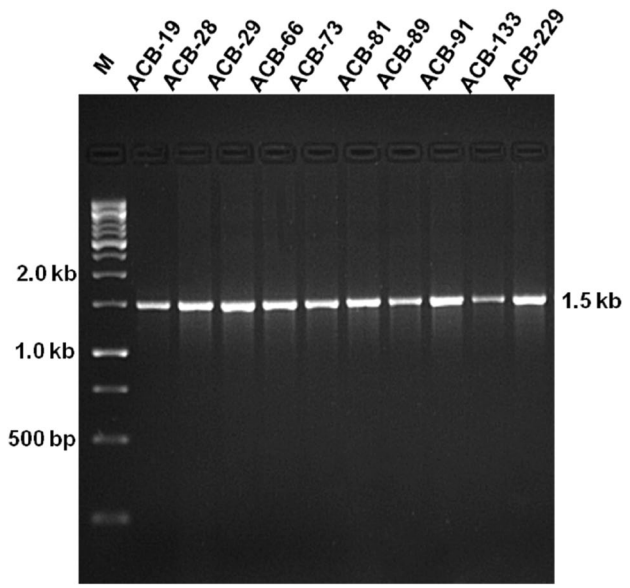

Fig. 4 16S rDNA PCR amplification of the marked respective reductive acetogen isolates (M-1 kb DNA size ladder, Lanes 1–10 amplified PCR product of the respective isolates)

approaches targeting the 16S rDNA were employed for identification purposes.

The 16S rDNA sequencing technique was employed to identify the isolates, and the size of the amplified products was evaluated using 1.5% agarose gel electrophoresis (Fig. 4). After purification, the amplified DNA products were sequenced from both strands and aligned. ACB52 and ACB95, which produced H2S, were excluded from molecular identification. Upon conducting a BLAST search of the ACB19 sequence, it showed up to 99% similarity with *Vagococcus fluvialis* M-19 strain with query coverage up to 99%. The sequencing results indicated that out of the ten isolates, six strains exhibited a maximum similarity of 99% to different strains of *Clostridium* sp., while the remaining strains showed similarity to *Vagococcus fluvialis*, *Eubacterium limosum*, *Ruminococcus sp*., and *Acetobacterium woodii*. Detailed results of the sequencing data and BLAST search are presented in Table 2.

The species *E. limosum* has been identified in sheep rumen and digester sludge by Genthner et al. (1981). Cells are Gram-positive, non-spore forming, non-motile straight rods and become more pleomorphic after prolonged incubation. *E. limosum* is metabolically very versatile; its substrate range includes sugars, amino acids, methoxylated aromatic compounds, glycine, betaine, lactate and methanol, H_2–CO_2, and CO (Genthner et al. 1981; Jansen et al. 2001). The isolate ACB66 also have the similar type of morphological features. Isolate ACB73 showed similarity with *Clostridium aceticum* which was also described as reductive acetogens by Wieringa (1936) isolated from soil. Although they have reported it to be Gram-negative, spore-forming motile rods, we observed it as Gram-positive spore-formers. There may be Gram variability within the species as the origin of isolation was different in the present case. Isolate ACB91 was identified as *Clostridium carboxidivorans* which was also reported as acetogen from lagoon sediments and reported to be Gram-positive mesophilic rods. Isolate ACB229 confirmed a similarity matrix of around 99% with *A. woodii* a reductive acetogen isolated from marine sediments. Although other *Acetobacterium* species were also reported from different habitats such as rumen (Greening and Leedle

Table 2 Sequence-based identification and percent similarity values of 16S rDNA gene sequences retrieved from the selected reductive acetogen isolates at NCBI nucleotide BLAST search

Name of the isolates	Sequence length (bp)	Similarity to the nearest relative taxon	Similarity (%)	Query coverage (%)	GenBank accession no
ACB19	1407	*Vagococcus fluvialis* strain M-19	99	99	KU316938
ACB28	1437	*Clostridium* sp. *strain* DR6B	99	100	KU316939
ACB29	1403	*Clostridium* sp. 826	100	99	KU316940
ACB66	1431	*Eubacterium limosum* strain JCM 6421	99	99	KU316941
ACB73	1347	*Clostridium aceticum* DSM 1496	99	100	KU316942
ACB81	1345	*Clostridium* sp. CM-C99	100	98	KU316943
ACB89	1407	*Ruminococcus* sp. DSM 100440	99	100	KU316944
ACB91	1462	*Clostridium carboxidivorans* strain P7	99	100	KU316945
ACB133	1440	Uncultured *Clostridium* sp. clone A23	100	99	KU316946
ACB229	1424	*Acetobacterium woodii* strain DSM 1030	99	100	KU316947

1989), sediment (Sleat et al. 1985) and sewage sludge (Traunecker et al. 1991), the presence of *A. woodii* in the rumen of buffalo may be justified as the genera shown to exist in a diverse ecological niche. ACB89 showed 99% sequence similarity with *Ruminococcus* spp. and this genus was also earlier reported as an acetogen. *Ruminococcous hydrogenotrophicus* isolated from human feces (Bernalier et al. 1996), *Ruminococcous productus* isolated from sewage digester (Lorowitz an Bryant 1984) and *Ruminococcous schinkii* isolated from the rumen of 3-day-old lamb (Rieu-Lesme et al. 1996a) were some earlier reported reductive acetogens of this genus. All were reported to be Gram-positive coccus to coccobacilli with mesophilic growth temperature. Isolates ACB28, ACB29 and ACB81 showed sequence similarity to different cultivable species of *Clostridium* genera. More than 18 different species were reported from this genus (Drake et al. 2013) that includes rumen origin isolate, *Clostridium difficile* AA1 (Rieu-Lesme et al. 1998). Other isolates were isolated from freshwater sediments (Schink 1984), chicken waste (Tanner et al. 1993; Barik et al. 1998), swine manure (Schnurer et al. 1996) and human feces (Bernalier et al. 1996; Leclerc et al. 1997a, b). One isolate ACB131 matched with non-cultivable uncultured clone of *Clostridium* species for which no species-specific name was identified. All the isolated strains were previously reported as known acetogens, but for the first time, we observed the strain ACB19, identified as *V. fluvialis* found to be novel to the literature of acetogen database. Although *Clostridium* spp. were observed in earlier reports from different niche, this is the first report of its isolation from Indian context and from Murrah buffalo rumen. Based on the results of the study, it appears that the reductive acetogens derived from rumen fluid samples taken from Murrah buffaloes exhibit a combination of both autotrophic and heterotrophic characteristics. This finding has significant implications for the reduction of CH_4 emissions, as these microorganisms may prove to be a viable alternative to H_2 gas as a sink for mitigating these emissions. However, further research is required to fully understand the potential of these reductive acetogens and how they can be utilized to effectively reduce CH_4 emissions in livestock experiments. By exploring the properties and capabilities of these microorganisms in greater depth, we may be able to identify new strategies for reducing greenhouse gas emissions and improving the sustainability of the animal agricultural practices.

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Data availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no competing interests among them.

Ethical approval The manuscript contains collection of rumen fluid samples from fistulated Murrah breed buffaloes maintained at Livestock Research Complex as per the animal ethics committee of the Institute.

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