Rumen bacteria isolated from Indian goat transform vegetable oil into conjugated linoleic acids

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Abstract: *Conjugated linoleic acids (CLA) comprise a group of positional and geometric isomers of octadecadienoic acids, naturally occurring polyunsaturated fatty acids which are synthesized in the rumen of cattle, deer, sheep and goat by microbial biotransformation of forage derived unsaturated fatty acids such as oleic acid (OA) and linoleic acid (LA). The ability of many bacteria to produce CLA has been probed during the last years. India is abundant with vegetable oils like coconut oil, sunflower oil, cotton seed oil and groundnut oil which are rich in OA and LA, the precursors of CLA. If such cheap vegetable oils could be enriched by microbial intervention by adding CLA, that would increase the nutritional value of these oils and boost oil production as well as oil based industries. Owing to the functional food status and proven health benefits, CLA have become a multi-dollar business in the developed countries. Considering these facts, we have investigated the ability of nineteen bacteria isolated from goat rumen for CLA production in mineral salt medium supplemented with vegetable oils as sole carbon source. Four of them were able to produce CLA as the biohydrogenation intermediate, which was confirmed by GC equipped with Stabil wax capillary column and flame ionization detector. CLA fractions were extracted from the growth medium and CLA peaks were identified by comparison with the retention times of the reference high-purity CLA standard.*

Key words: rumen bacteria, conjugated linoleic acids, CLA.

INTRODUCTION

Conjugated linoleic acids (CLA) comprise a group of positional and geometric isomers of octadecadienoic acids, naturally occurring polyunsaturated fatty acids with conjugated double bonds. These are synthesised in the rumen of cattle, deer, sheep and goat by biotransformation of forage derived unsaturated fatty acids such as oleic acid (OA) and linoleic acid (LA) (Benjamin *et al*., 2005). They are mostly ω-6 fatty acids with conjugated double bonds (last double bond is on the 6^{th} carbon from CH_3 end of the fatty acid chain, hence the name ω-6 fatty acids (Benjamin and Spener, 2009). The discovery of a "functional food" role for CLA occurred over two decades ago when Pariza and coworkers found that ground beef contained an anticarcinogenic factor that consisted of a series of conjugated dienoic isomers of linoleic acid (Pariza *et al*., 2001). As the biomedical studies with CLA expanded, it became apparent that CLA had a range of positive health effects in experimental animal models. These included beneficial effects on reducing body fat accretion, delaying the onset of type II diabetes, retarding the development of atherosclerosis, improving the mineralization of bone and modulating the immune system (McGuire and McGuire, 2000; Whigham *et al*., 2000; Pariza *et al*., 2001). Like neutraceuticals,, being minor lipids with supposed functional food status, CLA are getting momentum in alleviating major killer diseases such as cancer, atherosclerosis and diabetes in humans (Benjamin and Spener, 2009). Bhattacharya *et al*. (2006) reviewed the inhibitory effects of CLA on chemically induced skin, stomach, mammary or colon tumours in mice and rats. *In vitro* studies in murine myeloid leukaemia and human colorectal (HT-29, MIP-101) and prostate (PC-3) colorectal cells as well as *in vivo* human studies on breast and prostate cancers showed CLA's best anti proliferative effects.

Several strains of *Propionibacterium, Bifidobacterium, Enterococcus,*most of the lactic acid bacteria namely *Lactobacillus acidophilus, L. delbrueckii* ssp. *bulgaricus, L. delbrueckii* ssp. *lactis, Lactococcus lactis* ssp. *cremoris, L. lactis* ssp. *lactis* and *Streptococcus thermophilus* and rumen bacteria *B. wbrisolvens* have the ability to convert linoleic acid into conjugated linoleic acid (Kim *et al*., 2002). Two strains of *Propionibacterium freudenreichii* ssp. *freudenreichii* and one strain of *P. freudenreichii* ssp. *sheramnii* were found to be capable of converting free linoleic acid to extracellular CLA (Jiang *et al*., 1998). Cheap vegetable oils available in India are rich in oleic acid (OA) and linoleic acid (LA), the precursors of CLA. Hence this study mainly involves the isolation of CLA microorganisms from the rumen of Malabari goat.

MATERIALS AND METHODS

1. Preparation of inoculums

Goat rumen content was aseptically collected in screw capped tubes from slaughter houses (Prive *et al*., 2010). Rumen liquor was anaerobically transferred to centrifuge tubes and centrifuged at 150g for 5 minutes at 4°C. The supernatant was collected in screw capped tubes with O_2 free head space. The samples were then subjected to logarithmic dilution using anaerobic dilution solution. 1ml of the sample was transferred to 9ml of anaerobic dilution medium, flushed with CO_2 and closed tightly. The tube was marked as 10^{-1} dilution. 0.1 ml of the sample from the 10^{-8} dilution was used as inoculum.

2. Isolation of microbes by spread plate method

15ml of the de Man Rugosa Sharpe (MRS) agar medium was dispensed into sterile petriplates and allowed to solidify. 0.1ml of the inoculums was spread uniformly on the medium and incubated at 37°C using anaerobic jar for 4-5 days. Isolated colonies were picked by sterile loop, streaked on mineral salt agar medium (MSM) containing (%)K_2HPO_4 0.4, NaCl 0.4, NH_4NO_3 0.3, NH_4SO_4 0.3, $MgSO_4$ 0.01 and cysteine-HCl 0.05, agar 2, pre-coated with groundnut oil as sole source of carbon. The plates were incubated at 37°C anaerobically for 5-7 days.

3. Submerged fermentation

Isolated colonies were inoculated to Mineral Salt Medium (MSM) enriched with 0.01% of groundnut oil and incubated anaerobically at 37°C at 150rpm. The culture was inoculated into fresh MSM containing higher concentration of oil. The subculturing procedure was repeated until the microbes had adapted to high concentration of oil (0.1%).

4. Extraction and methylation of fatty acids

The culture broth (20 mL) was centrifuged at 3,000×g for 10 min. at room temperature. The supernatant (15mL) was then vigorously shaken with organic solvents (30 mL n-hexane and 22 mL isopropanol) in a separating funnel, the upper layer collected, and then concentrated by evaporation to 3mL. The concentrated lipids were hydrolyzed with 2.0 mL of 1.0 M sodium hydroxide in methanol for 10 min in a water bath at 70°C. The free fatty acids in the mixture were methylated by the addition of 2mL of 14% Boron trifluoride in methanol in a water bath at 60°C for 10min. (Bligh and Dyer, 1959).

5. Gas chromatography

Methyl esters of CLA were analyzed as described by Alonso *et al*. (2000). The specific equipment and materials included a gas chromatograph (Shimadzu GC 2010 plus series, Japan) equipped with a flame ionization detector. Analyses were performed with a Stabilwax (30m × 0.25mm, Restek, USA). The conditions used were 200-250˚C Oven temperature and 200˚C Column temperature. The split ratio was 1:50, and the carrier gas was Nitrogen. The injection volume was 1µl, and the CLA peaks were identified by comparison with the retention times of the reference standards.

RESULTS AND DISCUSSION

India is abundant with vegetable oils like coconut oil, sunflower oil, cotton seed oil and groundnut oil which are rich in OA and LA, the precursors of CLA. If such cheap vegetable oils could be enriched by microbial intervention by adding CLA, that would increase the nutritional value of these oils and boost oil production as well as oil based industries. Owing to the functional food status and proven health benefits, CLA have become a multi-dollar business in the developed countries. Considering these facts, we have investigated the ability of nineteen bacteria isolated from goat rumen for CLA production in mineral salt medium supplemented with vegetable oils as sole carbon source. The anaerobic condition was maintained with the help of anaerobic work station and anaerobic jars (Fig 1). Fifteen isolates based on colonial morphology of the isolated cultures were identified as cream colour, circular shape and approximately 0.5-mm diameter (Fig 2). All the isolates were found to be Gram positive rods of varying shape. Culture 1 was small bacilli whereas culture 2 was micromorphologically similar to yeast with oval shape (Fig 3). The GC analysis of the standards of LA and CLA showed characteristic peaks at retention times of 6.18min. and 6.92min. respectively (Fig 4). CLA production potential of the two isolates in vitro was observed in MSM supplemented with 0.1% linoleic acid as sole source of carbon. At regular intervals of 3 hours the samples were analysed for the production of CLA. At 12th hour the culture 2 showed maximum conversion of LA to CLA with reduced LA and increased CLA concentrations (Fig 5).

Conclusion

From the present work, it could be concluded that the rumen constitutes a rich source of CLA producing potential microorganisms. A potential CLA producer with an ability to produce significant level of CLA at lower concentration of groundnut oil has been isolated. As there is a high demand and scope for CLA enriched health-promoting foods because of various functional properties associated with CLA, the isolate extends a possibility to be used as CLA source. Hence, *in vivo* feeding trials are needed to validate the results obtained under laboratory conditions.

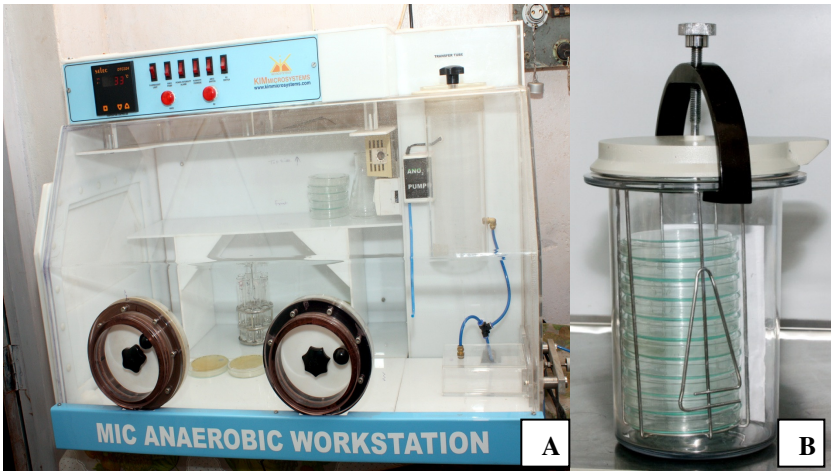

Fig. 1. Equipments for the maintenance of anaerobic condition. A. Anaerobic workstation; B. Anaerobic jar

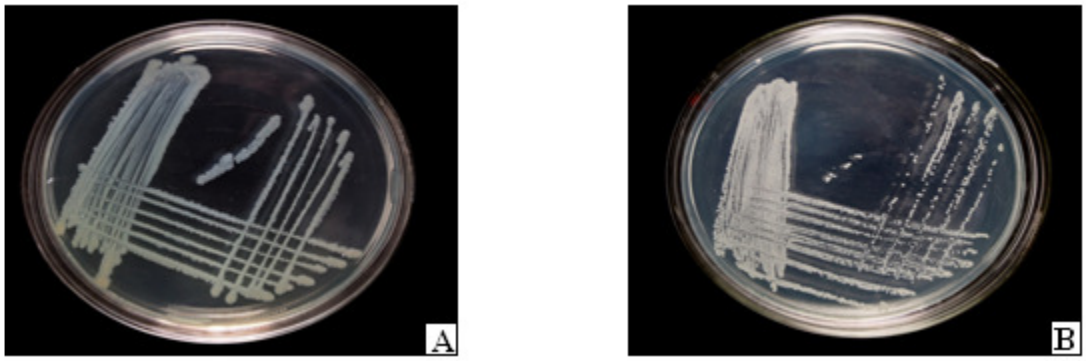

Fig. 2. Isolates on MRS agar plates after incubation at 37^0C for 4-5 days. A. Isolate 1; B. isolate 2

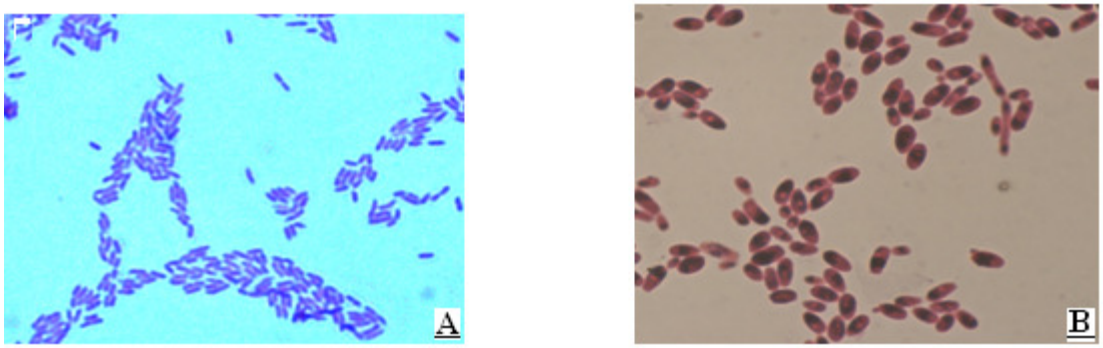

Fig. 3. Gram staining of the isolates. A. Isolate 1- Gram positive bacilli; B. Isolate 2- oval cells similar to yeast

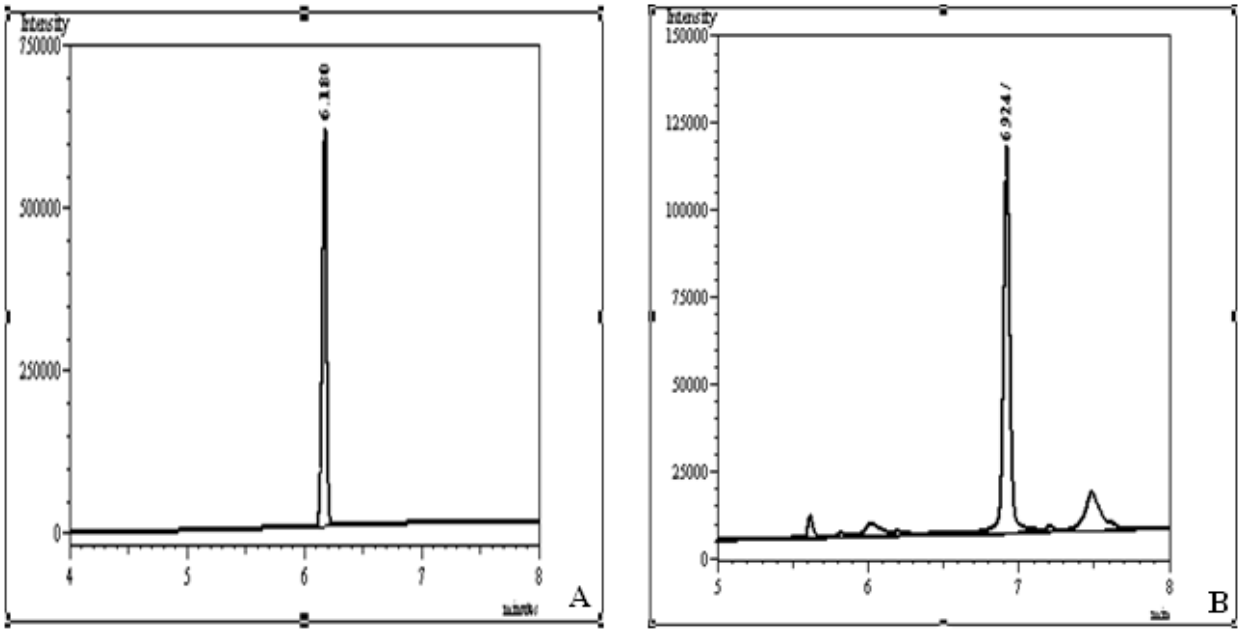

Fig. 4. GC profile of standard fatty acids. A. Linoleic acid showing characteristic peak at a retention time of 6.18min.; B. Conjugated linoleic acid showing characteristic peak at a retention time of 6.92min.

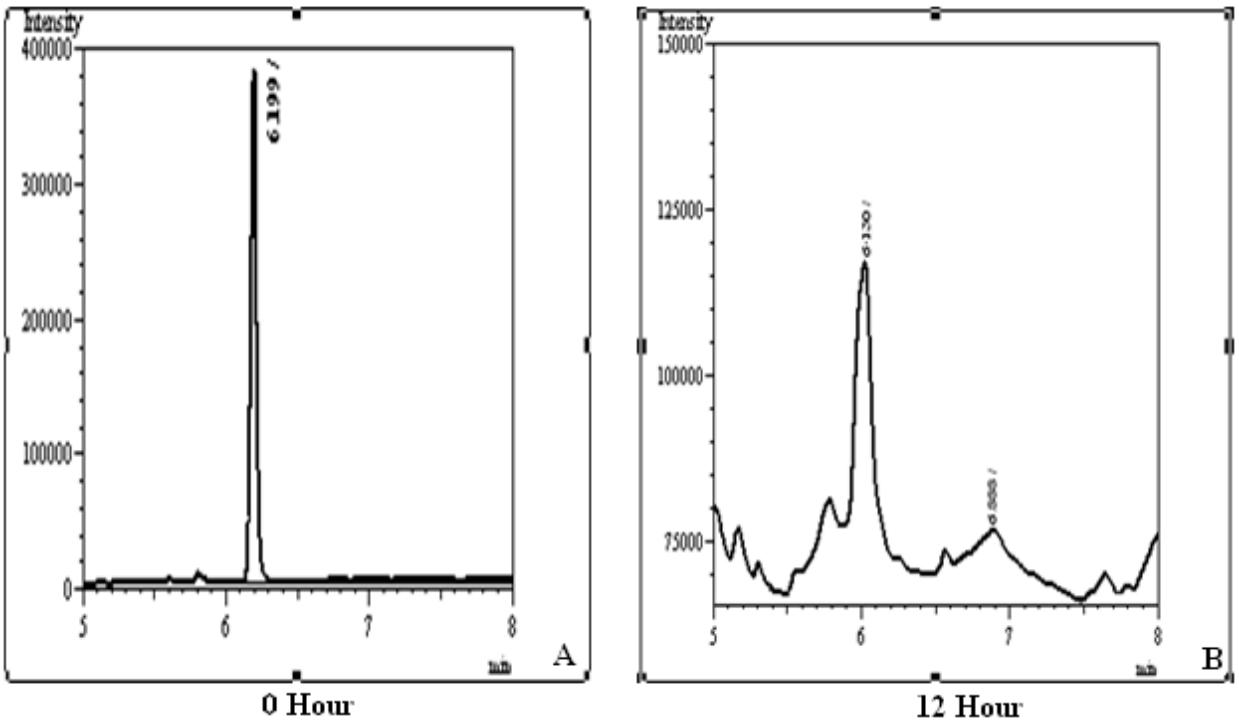

Figure 5. GC profile of sample A: GC profile of the extacted fatty acids from MSM with groundnut oil as sole source of carbon before inoculation. B. GC profile of the culture after 12 hours of incubation at 37^{o}C and 150rpm showing decease in the height of LA peak with the appearance of CLA peak.

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