Appl Biochem Biotechnol (2012) 168:1265–1276
DOI 10.1007/s12010-012-9855-9

Appraisal of Conjugated Linoleic Acid Production by Probiotic Potential of *Pediococcus* spp. GS4

Vinay Dubey · Asit Ranjan Ghosh · Badal Kumar Mandal

Received: 9 May 2012 / Accepted: 21 August 2012 /
Published online: 13 September 2012

Abstract Probiotics with ability to produce conjugated linoleic acid (CLA) is considered as an additive health benefit property for its known role in colon cancer mitigation. The conversion involves the biohydrogenation of the unsaturated fatty acid into conjugated form. Probiotic strain *Pediococcus* spp. GS4 was efficiently able to biohydrogenate linoleic acid (LA) into its conjugated form within 48 h of incubation. Quantum of CLA produced with a concentration of 121 μg/ml and sustained cell viability of 8.94 log cfu/ml maximally. Moreover, antibacterial effect of LA on the strain ability for biohydrogenation was examined at different concentrations and concluded to have a direct relationship between LA and amount of CLA produced. The efficiency of the strain for CLA production at different pH was also estimated and found maximum at pH6.0 with 149 μg/ml while this ability was reduced at pH9.0 to 63 μg/ml. Sesame oil, which is rich in the triacylglycerol form of LA, was also found to act as a substrate for CLA production by *Pediococcus* spp. GS4 with the aid of lipase-catalyzed triacylglycerol hydrolysis and amount of CLA produced was 31 μg/ml at 0.2 % while 150 μg/ml at 1.0 % of lipolysed oil in skim milk medium. Conjugated form was analyzed using UV scanning, RP-HPLC, and GC-MS. This study also focused on the alternative use of lipolysed sesame oil instead of costly LA for biohydrogenation and could be a potential source for the industrial production of CLA.

Keywords Probiotic · Linoleic acid · CLA · Biohydrogenation · Lipolysed oil

Electronic supplementary material The online version of this article (doi:10.1007/s12010-012-9855-9) contains supplementary material, which is available to authorized users.

V. Dubey · A. R. Ghosh (✉)
Center for Infectious Diseases and Control, School of Bio Sciences and Technology (SBST), VIT University, Vellore 632014, India
e-mail: asitranjanghosh@vit.ac.in

V. Dubey
e-mail: vinaydubey@vit.ac.in

B. K. Mandal
School of Advanced Sciences (SAS), VIT University, Vellore 632014, India
e-mail: badalmandal@vit.ac.in

Introduction

Probiotics is considered as an important component of functional food which has several health benefit properties including anti-inflammatory as well as anti-cancer activity [3, 10]. Studies on probiotics provide evidence that probiotics are able to hydrogenate long-chain unsaturated fatty acids [10]. During hydrogenation process, the free fatty acid is converted into conjugated form [8]. There are several isomers of conjugated linoleic acid (CLA; C18:2, *cis*-9, *cis*-12) consisting of positional (9:11, 10:12) and geometric isomers (*cis* or *trans*). These isomers were found to have several health benefit properties such as anti-inflammatory, anti-diabetic, anti-cancer activity, immuno-modulatory, anti-atherosclerotic, and anti-obesity activities [8, 9, 17, 21, 23, 25]. Due to their potential health benefit properties, CLA has drawn much more attention in research. Several strains of lactic acid bacteria (LAB) have been isolated with ability to produce CLA from human as well as non-human sources [2, 11, 25]. Several factors were found influencing biohydrogenation ability of probiotic strains [8] including incubation time, initial pH of the medium, and initial concentration of the substrate (free fatty acid). There were several reports suggesting that LA have antibacterial effects on gram-positive bacteria and involved in interference with cytoplasmic permeability, leading to cell death [22]. Separate studies also conducted on probiotic CLA-forming strains like *Lactobacillus freudenreichii* and *Lactobacillus plantarum*, and found that free fatty acid had inhibitory effects on their growth [18]. Akalin et al. [1] demonstrated that *Lactobacillus acidophilus* significantly increased the proportion of CLA (9:11, C18:2) in yogurt and suggested that strain might be able to utilize unsaturated fatty acid from natural food sources. Yadav et al. [27] found that Dahi produced by probiotic strains *L. acidophilus* and *Lactobacillus casei* had higher amount of CLA in compared to normal Dahi and concluded that *Lactobacillus* spp. involved in the lipolysis of milk fat to free fatty acid which in turn was further converted to CLA. LA is naturally present in dairy and non-dairy sources like milk, poppy seed, soyabean oil, sunflower oil, sesame oil, safflower oil, coconut oil, etc. In a separate study, *Lactobacillus lactis* on milk supplemented with sunflower oil produced CLA which was found influenced by oil concentration [15]. Indeed, bacterial strains belonging to a few genera, such as *Lactobacillus*, *Propionibacterium*, and *Bifidobacterium* have been reported to produce CLA in either synthetic media or in milk [25, 26]; however, the ability to produce CLA can vary from strain to strain.

Sesame seed oil (SSO) has been used as healing oil and nutraceuticals for thousands of years [20]. SSO is composed of several fatty acids including linoleic acid (C18:2) with maximum of 50.0 %. Thus, SSO could be a good source for the bioproduction of CLA and, this could be used as an alternative economical source of linoleic acid after lipolysation. Therefore screening for the ability to form CLA from free fatty acids could be an additive property in probiotics.

Thus, the aim of the study was to assess the ability of probiotic strain *Pediococcus* spp. GS4 to biohydrogenate free fatty acid into its conjugated form and to find an alternative source of free fatty acid instead of costly linolenic acid for biohydrogenation.

Materials and Method

Chemicals

All the chemicals were purchased from Hi-Media, India, and CLA standard from Sigma-Aldrich, USA.

Bacterial Strain

The Khadi (an Indian fermented food) isolated LAB *Pediococcus* spp. strain GS4 (Gene bank ID: HMO44322) was identified previously by Sukumar and Ghosh. [12]. LAB culture was grown at 37 °C and maintained on De Mann Rogosa Sharpe (MRS) agar at 4 °C for subsequent use.

Study of Biohydrogenation Ability of Strain

Biohydrogenation ability of *Pediococcus* spp. strain GS4 was done in MRS broth supplemented with LA. The stock solution for LA (50 mg/ml) was made in distilled water containing 1 % Tween 80. The stock solution was previously filter-sterilized through 0.45-μm syringe filter. LA was added into MRS broth to achieve 0.2 mg/ml concentration. Media without LA served as control. Strain GS4 in exponential phase (OD_{600}=0.237) was inoculated by 1 % (*v*/*v*) and incubated at 37 °C. Samples were withdrawn at different time intervals for the analysis of CLA production. During this experiment, the effect of LA on the bacterial growth was monitored by counting the viable cells (cfu/ml), OD_{600}, and the media utilization by change in pH of the medium.

Biohydrogenation Ability at Different Concentration of Linoleic Acid

Pediococcus spp. GS4 was grown in MRS broth supplemented with different concentration of free LA (0.5, 1.0, 1.5, 2.0, and 2.5)mg/ml and was incubated at 37 °C and monitored up to 48 h.

Effect of pH During the Biohydrogenation

The biohydrogenation ability of strain GS4 was performed at different pH, two above neutral pH and two below (5, 6, 7, 8, and 9) of the medium with 0.2 mg/ml concentration of LA. Growth of organism (GS4) as well as CLA formation was monitored by similar way as mentioned above.

Assessment of the Biohydrogenation Ability of Strain GS4 in Lipolysed Skim Milk

Lipolysed skim milk was prepared according to the El-Salam et al. [7] with slight modification. Briefly, skim milk was reconstituted (10 % *w*/*v*) and sesame oil was added at 10 % (*w*/*w*) and homogenized at 50 °C for 20 min, for the formation of free fatty acid, lipase enzyme (5 mg/ml) was added and incubated at 40 °C for 8 h. Samples were withdrawn at regular intervals for the determination of acid formation, and the acid value was determined by titration method by using 0.1 N KOH [14]. The acid number was increased from 11.20 to 28.62 after 8 h. The pH of the lipolysed milk was adjusted to 7.0 and sterilized before use. Different amount of lipolysed sesame oil was added in sterilized reconstituted skim milk to make the lipolysed milk concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 %. The starter culture for *Pediococcus* spp. strain GS4 was prepared twice by subculturing in reconstituted skim milk. After second subculture, the medium was inoculated with 1 % (*v*/*v*) culture followed by incubation at 37 °C in still condition. Culture without lipolysed milk served as a control. Growth was monitored by similar way as mentioned above.

Lipid Extraction from the Cell-Free Supernatant

LA hydrogenation was determined by lipid extraction from cell-free supernatant according to the method described by Coakley et al. [6]. In brief, 6 ml cell-free supernatant was mixed with isopropanol/hexane (4:3), followed by centrifugation at 1,910×*g* for 5 min. Organic layer was collected and dried over anhydrous Na_2SO_4. Vacuum-dried sample was mixed with 100 μl of hexane and kept at 4 °C for further analysis.

Analysis of the Biohydrogenation Product

The biohydrogenation ability of *Pediococcus* spp. strain GS4 was primarily determined according to Raes et al. [23], by using UV spectrum analysis method. Briefly, the fat-soluble organic layer was scanned from 200 to 400 nm in 1-cm quartz cuvettes by using UV spectrophotometer (JASCO V-670, Japan) and fatty acids with conjugated double bonds was characterized by an absorption peak at 234 nm. Standard graph for CLA concentration was made by using standard with concentration range 0–0.1 mg/ml and absorbance was recorded at 234 nm and plotted the graph between concentrations of CLA versus absorbance at 234 nm. Standard graph was used to determine the concentration of CLA in samples. UV spectrophotometer absorbs all dienoic bonds at 234 nm without specific differentiation among isomers. To differentiate and to confirm biohydrogenated end products (isomers), reverse-phase high-performance liquid chromatography (RP-HPLC) was performed. A C-18 Water 1525 (5-μm particle size, 4.6×150 mm; Waters 1525 Binary HPLC, Australia), was used with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (70/30/0.12, *v*/*v*/*v*) at flow rate of 1.5 ml/min up to 60 min [5]. Conjugated diene unsaturated fatty acids were detected at 234 nm, the results were matched with the characteristic peak obtained in standard CLA during HPLC analysis with same conditions.

Gas Chromatography and Mass Spectroscopy Analysis of the Lipid Sample

The extracted lipid sample was converted into fatty acid methyl esters (FAMEs) by method described by Raes et al. [23]. The FAMEs were dissolved into hexane and analyzed by the gas chromatography-mass spectrometry carried out with JOEL GC Mate-II fitted with HP5 column. The gas chromatography condition was as follows: injector, 220 °C; helium as a carrier gas; rate of temperature 15 °Cmin^{-1} and range of temperature were 70–250 °C. Injection volume was 1 μl. Peaks were identified by comparing with the corresponding external standard (Sigma-Aldrich). The scan mass range was 80–280 amu.

Statistical Analysis All experiments were carried out in triplicates and expressed with ±SD wherever required. The statistical analysis for each experiment was done by using one-way ANOVA with significance level <0.05.

Results

Biohydrogenation of Linoleic Acid

Pediococcus spp. GS4 showed the ability to biohydrogenate LA to CLA. Biohydrogenation of supplemented LA was estimated (S.1) by using UV scanning (200–400 nm) of the organic layer extracted from cell-free supernatant after 48 h of incubation and major peak appeared at 234 nm, which was attributed to conjugated form of LA (CLA). A standard curve was

drawn in favor of the quantification of CLA (S.2). CLA concentration was calculated considering the dilution factor and by using the linear trend of the standard curve by the equation $y = 5.7x + 0.004$.

Figure 1a, b showed the sustainability of *Pediococcus* spp. GS4 during the experiment along with CLA formation and at different incubation periods. The conjugated bond formation started appearing after 6 h of incubation and produced CLA amounting to 48 μg/ml and increased to 121 μg/ml at 48 h of incubation (Fig. 1b). Decreased trend of CLA production was observed significantly ($p<0.05$) when the incubation period extended beyond 48 h (data not shown). The viability of *Pediococcus* spp. GS4 was found dwindling. Sustainability of cells with LA (0.2 mg/ml) was 8.94 log cfu/ml while 9.04 log cfu/ml without LA (control) at 48 h, demonstrating the survival ability of the *Pediococcus* spp. GS4 with LA. The apparent drop in pH of the medium was observed; dropped from 6.9 to 4.5 after 48 h of incubation.

Biohydrogenation Ability at Different Concentration of Linoleic Acid

Figure 2a showed the hydrogenation ability of *Pediococcus* spp. GS4 at different concentrations of LA. The CLA concentration was increased from 126 to 195 μg/ml in the presence of LA of 0.5 to 2.5 mg/ml, respectively, at 48 h. It was apparent from cell sustainability that there was some inhibitory effect of LA on strain which was found 116 % cell survivability at 0.5 mg/ml while decreased to 85 % at 2.5 mg/ml of LA. The final pH of the medium was found related with cell viability as shown in Fig. 2b.

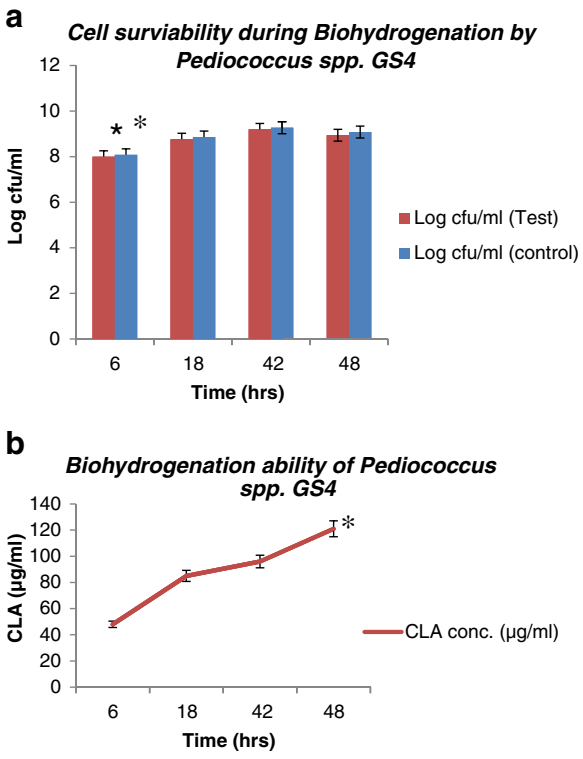

Fig. 1 Assessment of cell viability (**a**) and CLA concentration (**b**) during the biohydrogenation by *Pediococcus* spp. strain GS4 and both "*" and "**" the study was found significant level at <0.05

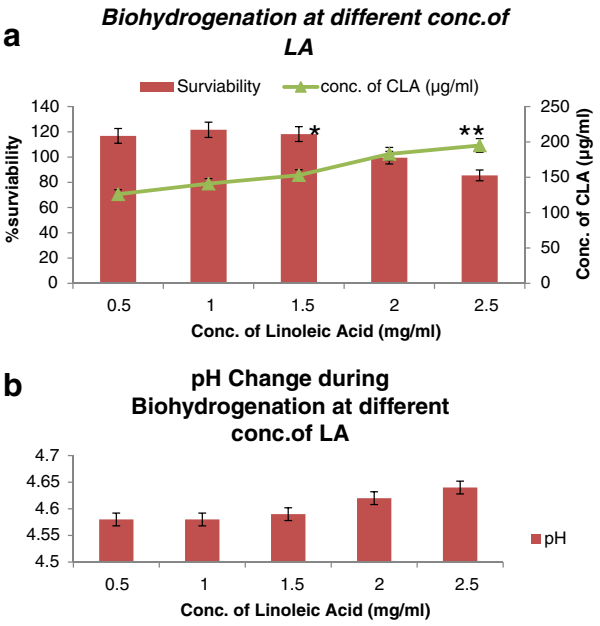

Fig. 2 **a** Graphic representation of relationship between initial concentration of LA and CLA formation while cell viability decreased with increased concentration of LA. Both cell viability (*) and CLA concentration (**) were found significant at <0.05. **b** Graphical representation of pH change of the medium after 48 h of incubation at different concentration of LA during biohydrogenation

Biohydrogenation Ability at Different pH

Figure 3 showed the effect of initial pH of the medium on biohydrogenation ability (Fig. 3a) and cell sustainability (Fig. 3b). CLA produced was found 149 µg/ml at pH6.0 while

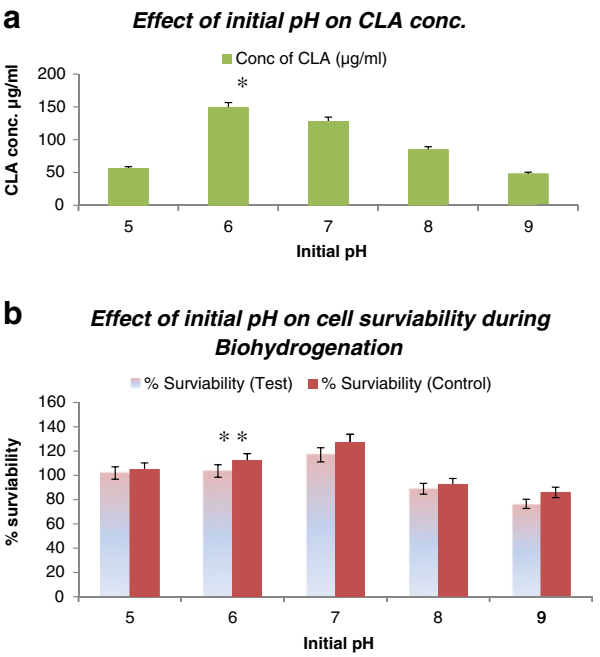

Fig. 3 Effect of initial pH of the medium on the biohydrogenation ability **(a)** and cell sustainability **(b)** of the *Pediococcus* spp. GS4. Both studies (* and **) were found significant at <0.05

128 μg/ml at pH7.0 and 63 μg/ml at pH9.0, after 48 h of incubation. The optimum pH for the production of CLA was found at the range of 6.0–7.0.

Assessment of the Biohydrogenation Ability of Strain GS4 in Lipolysed Skim Milk

Lipolysed milk made from SSO in skim milk showed the increased acid value from 11.2 to 28.62 after 8 h of incubation at 40 °C indicating the formation of free fatty acid (oleic acid) during lipase-assisted lipolysis. Calculated free fatty acid (oleic acid) was 14.38 (percentage by weight) after 8 h of lipolysis. Figure 4 showed the concentration of formed CLA at 0.2 % of lipolysed oil in skim milk medium was 31 μg/ml and increased to 150 μg/ml at 1.0 %.

Analysis of the Biohydrogenated Product

Since UV spectrophotometric analysis do not differentiate between CLA isomers as all the conjugated bonds have characteristic absorbance at 234 nm, chromatographic analysis by RP-HPLC was carried out to find out the isomers formed during biohydrogenation along with the use of standard CLA (a mixture of c9,t11 and t10,c12 octadecadienoic acid).

In RP-HPLC analysis, chromatogram of test sample showed two major peaks at retention time 20.0 and 23.7 min, respectively, which were found almost similar to peaks found in chromatogram of standard sample at 19.7 and 23.65 min of retention time (Fig. 5a, b). From RP-HPLC analysis, it was found that the strain was able to produce a mixture of C18:2 *cis* 9, *trans* 11, and C18:2 *trans* 10, *cis* 12 octadecadienoic acid.

As RP-HPLC enabled for CLA isomer analysis in the extracted lipid, consequently GC coupled with mass spectrophotometry helped for determination of the mass and structural confirmation of the formed CLA during biohydrogenation. In the gas chromatogram of the extracted organic phase (Fig. 6), one peak was observed at 11.79 min which correspond to the standard peak at the same retention time. The mass spectra of extracted lipid sample were compared with the standard mass spectra. The mass spectra indicated a molecular ion at *m/z* 280.45, which confirmed a carbon chain length of 18 carbon and two double bonds (Fig. 7). The base peak is formed at *m/z*=67(M-213), and was similar to the mass spectra of the sample. The gaps of 12 amu between *m/z* 194.4706 and 207.3989 confirmed the location of double bond at carbon atoms 9 [4]. Conjugation of peaks in the GC-mass spectrum and mass fragmentation pattern confirm the structure of conjugated linoleic acid.

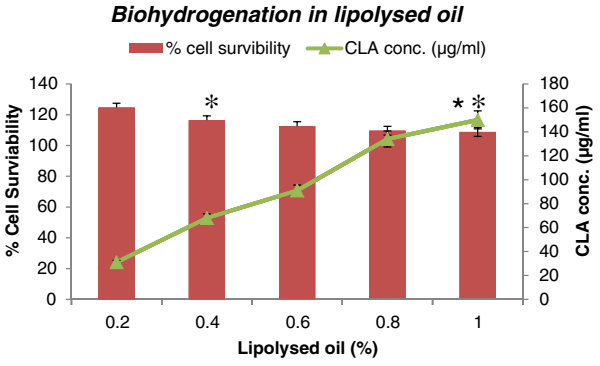

Fig. 4 Graph representing effect of lipolysed oil concentration in skim milk medium on the CLA amount during biohydrogenation by *Pediococcus* spp. GS4. Both cell survivability (*) and CLA concentration (**) found significantly at 0.05

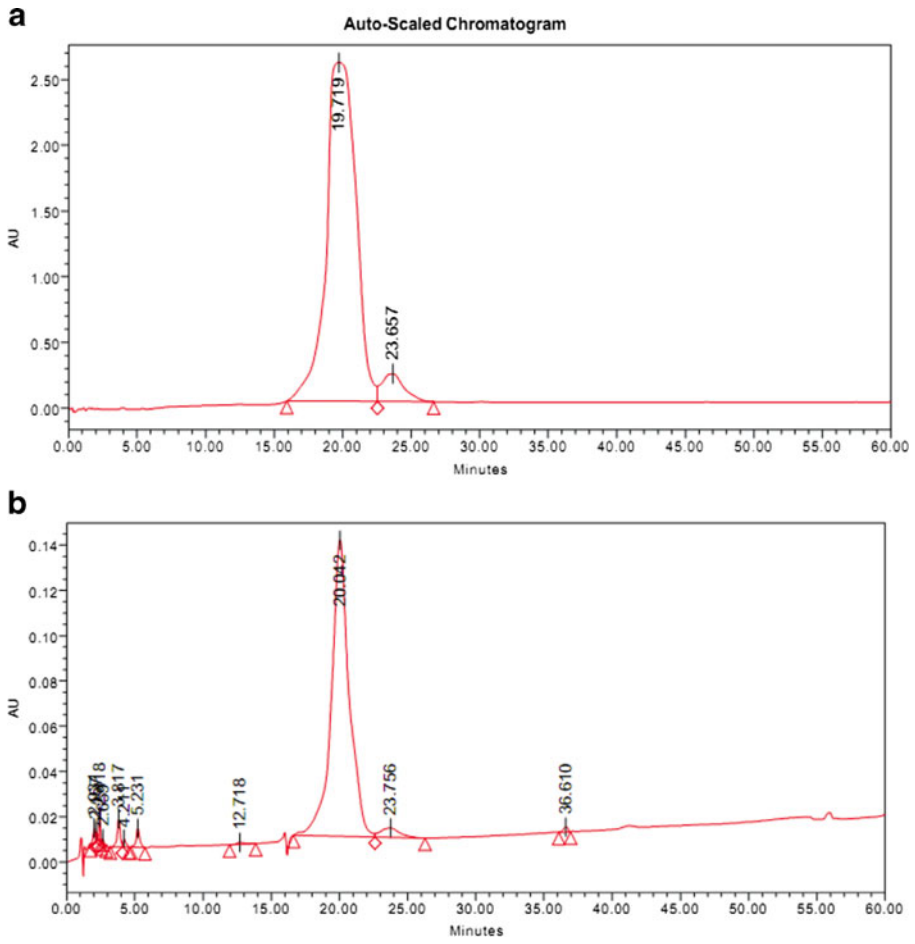

Fig. 5 Chromatogram of RP-HPLC of CLA using standard (**a**) and test sample lipid extracted from cell free supernatant from *Pediococcus* spp. strain GS4 inoculated in MRS broth supplemented with LA (**b**)

Discussion

Probiotic LAB has several health benefit properties through which they are considered as an important component of functional foods [10]. Researchers suggested that they exhibit anti-cancer activity by the formation of CLA which is a natural ligand for the activation of PPARγ, known for its inhibitory role in different types of cancers [19]. Therefore the probiotic bacteria with ability to hydrogenate free fatty acid to conjugated form could be an additional health beneficial property of probiotics. Different LAB strains are able to produce CLA when LA is supplemented in medium as reported elsewhere [7]. In this study, the biohydrogenation ability of probiotic *Pediococcus* spp. strain GS4 to convert LA into CLA was assessed. An increase in CLA production from 121 to 195 μg/ml was found with 0.2 to 2.5 mg/ml, respectively, of LA at 48 h of incubation. However, no significant increase ($p<0.05$) in CLA level was observed as incubation time increased after 48 h, this might be due to further conversion of produced CLA into saturated form like stearic acid [2, 13]. This

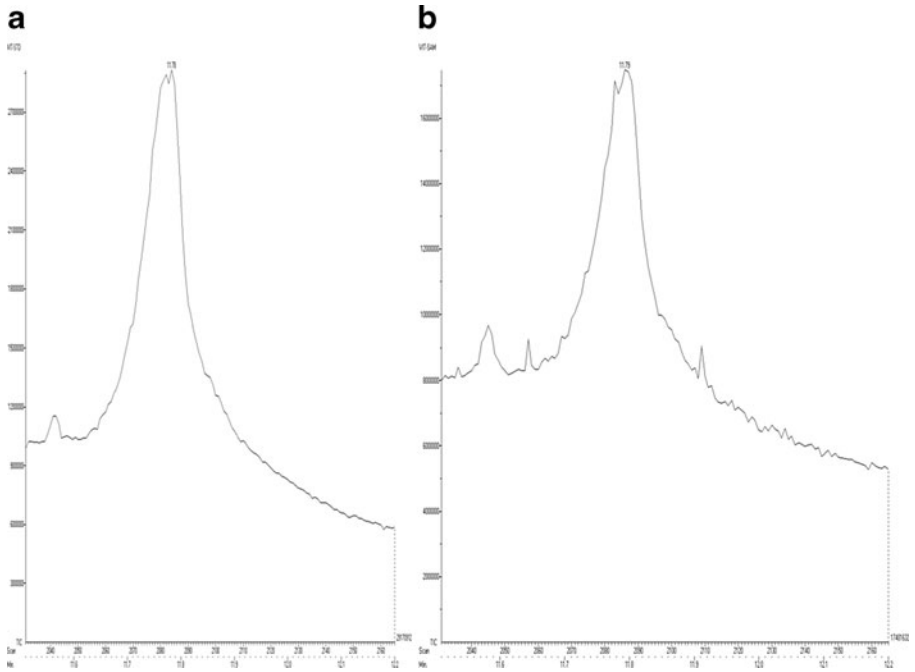

Fig. 6 GC chromatogram of standard (**a**) and lipid sample extracted from cell-free supernatant from *Pediococcus* spp. strain GS4 inoculated in MRS broth supplemented with LA (**b**)

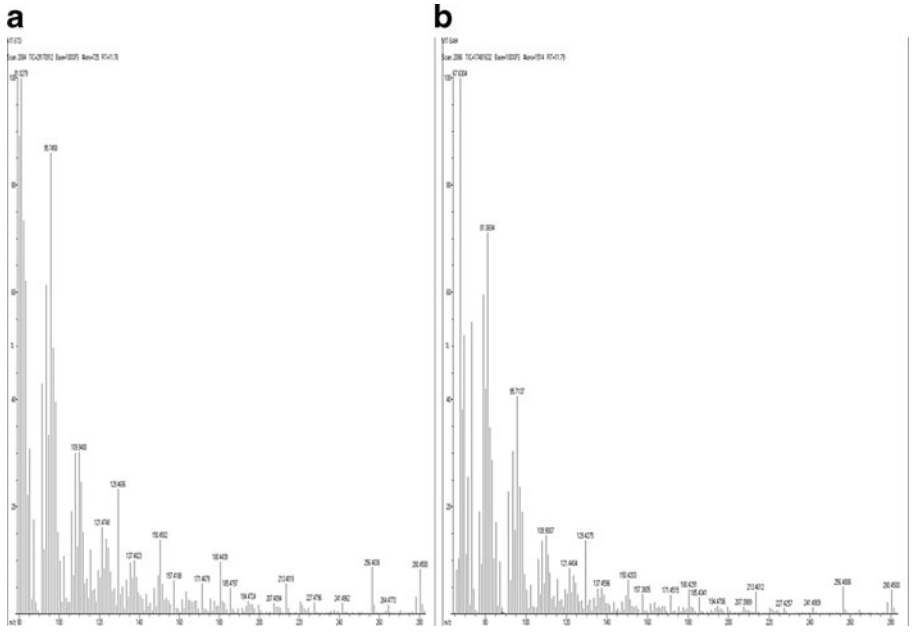

Fig. 7 Mass spectrum of the standard (**a**) and lipid extracted from cell-free supernatant from *Pediococcus* spp. strain GS4 inoculated in MRS broth supplemented with LA (**b**)

suggested the importance of incubation time for CLA production as it is an intermediate product during bioconversion of unsaturated fatty acids [26].

Cell viability of the strain was observed with and without LA (0.2 mg/ml) in the MRS broth. Growth inhibition was seen in increased concentration of LA during study. This might be due to antibacterial activity of LA as was demonstrated in several studies [18]. It had been shown that gram-positive bacteria used this biohydrogenation ability as a detoxification strategy to reduce the antibacterial effect of LA [22]. The strain GS4 effectively utilized this ability for the conversion of LA into CLA. It was apparent that with increase in concentration of LA, concentration of CLA was increased, and simultaneously cell viability was decreased. The biohydrogenation of LA is a multiple-step process in which CLA is found as an intermediate product. In this process, unsaturated fatty acid is first converted into conjugated form and further hydrogenated into its monoenoic form depending on the linoleate isomerase (LI) and reductases, respectively (R) [16]. This phenomenon may be explained that increasing concentration of LA to form biohydrogenated CLA to a critical point of concentration, both LA and CLA play a negative impact on cell growth. These results showed consistent with the previous study conducted by Kim et al. on *Butyrivibrio fibisolvens* A38 and suggested that CLA was accumulated when the hydrogenation step was inhibited by increasing concentration of LA and simultaneously the cell viability declined [16]. Harfoot et al. also explained that it might be due to competitive inhibition of the hydrogenation of CLA into its monoenoic form [13]. Additionally, it was also found that membrane fraction from the dead or lysed cells retained their ability to convert LA into CLA [16] and thus supports the results found and it might be the reason why the CLA was increased while the cell survivability declined.

It was observed that initial pH of the media of 8.0–9.0 exerted an inhibitory effect on CLA production; this might be due to inhibition of enzymatic activity of linoleate isomerase. The optimum pH for LI for the production of CLA was found to be 6.0–7.0, which was in agreement with a study reported elsewhere [24].

SSO is Ayurvedic oil and had been in use since time immemorial in India [20]. It is rich in LA and thus could be a good source for CLA production using probiotic strains. On lipolysis of SSO, the acid value increased from 11.20 to 28.62 representing the formation of free fatty acid (oleic acid) [14]. For acid value 28.62, the calculated free fatty acid (oleic acid) was 14.38 (percentage by weight) after 8 h of lipolysis. This was due to lipase-assisted lipolysis of triacylglycerol. The CLA concentration was found statistically significant ($p<0.05$) as the concentration of lipolysed sesame oil increased in skim milk medium. The highest amount of CLA was found at 150 μg/ml at 1 % concentration of sesame oil in the medium. *Pediococcus* spp. GS4 was capable of producing CLA in both MRS broth and in skim milk medium supplemented with lipolysed sesame oil. Sunflower oil had been used to demonstrate for the production of CLA using *Propiobacterium freudenreichii* in a separate study [26]. This showed that the quantity of CLA produced was 78.8 μg/ml at 36 h. In contrast, *Pediococcus* spp. GS4 bioconjugated 150 μg/ml at 48 h using lipolysed sesame oil. Thus the use of sesame oil over sunflower oil is found to be effective and economical.

Incubation period is very critical for the formation of CLA, as it is produced by LAB as an intermediate product which is further changed into saturated fatty acids [2, 13]. The optimum duration for CLA formation by *Pediococcus* spp. GS4 was 48 h in the present study while 36 h by *P. freudenreichii* [26]. This suggests that the biohydrogenation of free fatty acid to conjugated form is unique and specific to strains used. There might be different pathways for such bioconversion. Future work is influenced to discover such pathway(s).

Several combinations of methods were used to provide a complete confirmation of the CLA-forming ability of the *Pediococcus* spp. strain GS4. CLA is positional and geometric

conjugated isomers of LA with double bond at 9,11; 10,12; 8,10; and 7,9 positions [8]. UV spectrophotometry has its limitation to show only absorbance of all dienoic bonds at 234 nm without specific differentiation among isomers. For the specific identification of isomer(s) produced, RP-HPLC analysis was carried out on the basis of retention time in comparison with the commercially available standard CLA (Sigma, USA). Thus RP-HPLC analysis enabled to find out the isomers formed in the reaction mixture. The RP-HPLC analysis of extracted lipid showed two similar major peaks with standard at same retention time and identified isomers as mixture of *cis* and *trans* 9,11 and 10,12 octadecadienoic acids.

As RP-HPLC enabled for CLA isomer analysis in the extracted lipid so GC coupled with mass spectrophotometry helped the detection of the mass of the CLA during biohydrogenation. From the results of GC-MS analysis, it was found that the methyl esters of extracted lipid contained double bond containing CLA with (18:C) based on the retention time of methylated standard. Mass spectra of both standard as well as sample showed the same ionization fragments at different m/z. Results from the current study confirmed the formation of CLA during biohydrogenation.

Considering probiotics as *future panacea for all diseases*, the advantage of biohydrogenation ability to form CLA could contribute to the enhanced probiotic properties. The present study was contributed to investigate the biohydrogenation ability of *Pediococcus* spp. strain GS4 and extended to understand the role of medium and initial pH for the biohydrogenation ability. The concentration of sesame oil were used in the skim milk was found to be a cheap and natural source for the CLA production. Therefore, the present study shows that *Pediococcus* spp. strain GS4 is able to bioconvert LA as well as free fatty acid in lipolysed sesame oil in skim milk medium which could be selected as a potential organism for the industrial production of CLA.

Acknowledgments Authors would like to thank VIT University, Vellore, India for supporting and performing the study. VD is obliged to the management of VIT for partial completion of doctoral works.

References

1. Akalin, A. S., Tokusoglu, O., Gonc, S., & Aycan, S. (2007). Occurrence of conjugated linoleic acid in probiotic yoghurts supplemented with fructooligosaccharides. *International Dairy Journal, 17*, 1089–1095.
2. Alonso, L., Cuesta, E. P., & Gilliland, S. E. (2003). Production of free conjugated linoleic acid by *Lactobacillus acidophilus* and *Lactobacillus casei* of human intestinal origin. *Journal of Dairy Science, 86*, 1941–1946.
3. Bassaganya-Riera, J., Hontecillas, R., & Beitz, D. C. (2002). Colonic anti-inflammatory mechanisms of conjugated linoleic acid. *Clinical Nutrition, 21*, 451–459.
4. Christie, W. W. (1998). Gas chromatography mass spectrometry methods for structural analysis of fatty acids. *Lipids, 33*, 343–353.
5. Coakley, M., Ross, R. P., Nordgren, M., Fitzgerald, G., Devery, R., & Stanton, C. (2003). Conjugated linoleic acid biosynthesis by human-derived *Bifidobacterium* species. *Journal of Applied Microbiology, 94*, 138–145.
6. Coakley, M., Banni, S., Johnson, M. C., Mills, S., Devery, R., Fitzgerald, G., et al. (2009). Inhibitory effect of conjugated α-linolenic acid from bifidobacteria of intestinal origin on SW480 cancer cells. *Lipids, 44*, 249–256.
7. El-Salam, M. H. A., El-Shafei, K., Sharaf, O. M., Effat, B. A., Asem, F. M., & El-Aasar, M. (2010). Screening of some potentially probiotic lactic acid bacteria for their ability to synthesis conjugated linoleic acid. *International Journal of Dairy Technology, 63*(1), 62–69.
8. Ewaschuk, J. B., Walker, J. W., Diaz, H., & Madsen, K. L. (2006). Bioproduction of conjugated linoleic acid by probiotic bacteria occurs in vitro and in vivo in mice. *Journal of Nutrition, 136*, 1483–1487.

9. Gaullier, J. M., Halse, J., Hoye, K., Kristiansen, K., Fagertun, H., Vik, H., et al. (2004). Conjugated linoleic acid supplementation for 1 y reduces body fat mass in healthy overweight humans. *American Journal of Clinical Nutrition, 79*, 1118–1125.
10. Gibson, G. R. (2007). Functional foods: probiotics and prebiotics. *Culture, 28*(2), 965–989.
11. Gorissen, L., Raes, K., Weekx, S., Dannenberger, D., Leroy, F., Vuyst, L. D., et al. (2010). Production of conjugated linoleic acid and conjugated linolenic acid isomers by *Bifidobacterium* species. *Applied Microbiology and Biotechnology, 87*, 2257–2266.
12. Gowri, S., & Ghosh, A. R. (2010). *Pediococcus* spp.—a potential probiotic isolated from Khadi (an Indian fermented food) and identified by 16S rDNA sequence analysis. *African Journal of Food Science, 4*(9), 597–602.
13. Harfoot, C. G., Noble, R. C., & Moore, J. H. (1973). Factors influencing the extent of biohydrogenation of linoleic acid by rumen micro-organisms in vitro. *Journal of the Science of Food and Agriculture, 24*, 961–970.
14. I.S.I. Handbook of Food Analysis (Part XIII)-1984 Page 67/ IUPAC 2.201(1979)/I.S: 548 (Part 1)-1964, Methods of sampling and test for oils and fats).
15. Kim, Y. J., & Liu, R. H. (2002). Increase of conjugated linoleic acid content in milk by fermentation with lactic acid bacteria. *Journal of Food Science, 67*, 1731–1737.
16. Kim, Y. J., Liu, R. H., Bond, D. R., & Russell, J. B. (2000). Effect of linoleic acid concentration on conjugated linoleic acid production by *Butyrivibrio fibrisolvens* A38. *Applied and Environmental Microbiology, 66*(12), 5226–5230.
17. Kritchevsky, D. (2000). Antimutagenic and some other effects of conjugated linoleic acid. *British Journal of Nutrition, 83*, 459–465.
18. Lin, T. Y. (2000). Conjugated linoleic acid concentration as affected by lactic cultures and additives. *Food Chemistry, 69*, 27–31.
19. Luu, H. H., Zhang, R., Haydon, R. C., Rayburn, E., Kang, Q., Weike, S., Sainis, I., Vareli, K., Karavasilis, V., & Briasoulis, E. (2008). PPARγ: The portrait of a target ally to cancer chemopreventive agents. *PPAR Research,* 2008, Article ID 436489.
20. Reshma, M. V., Balachandran, C., Arumughan, C., Sunderasan, A., Sukumaran, D., Thomas, S., et al. (2010). Extraction, separation and characterisation of sesame oil lignin for nutraceutical applications. *Food Chemistry, 120*, 1041–1046.
21. Maggiora, M., Bologna, M., Ceru, M. P., Possati, L., Angelucci, A., Cimini, A., et al. (2004). An overview of the effect of linoleic acid and conjugated linoleic acids on the growth of several human tumor cell lines. *International Journal of Cancer, 112*, 909–919.
22. Nieman, C. (1954). Influence of trace amounts of fatty acids on the growth of microorganisms. *Bacteriology Review, 18*, 147–163.
23. Raes, K., De Smet, S., & Demeyer, D. (2001). Effect of double-muscling in Belgian blue young bulls on the intramuscular fatty acid composition with emphasis on conjugated linoleic acid and polyunsaturated fatty acids. *Animal Science, 73*, 253–260.
24. Rosson, R. A., & Grund, A. D. (2001). Linoleate isomerase. *World Patent, 100846*, 30.
25. Sieber, R., Collomb, M., Aeschlimann, A., Jelen, P., & Eyer, H. (2004). Impact of microbial cultures on conjugated linoleic acid in dairy products—a review. *International Dairy Journal, 14*, 1–15.
26. Wang, L. M., Lv, J. P., Chu, Z. Q., Cui, Y. Y., & Ren, X. H. (2007). Production of conjugated linoleic acid by *Propionibacterium freudenreichii*. *Food Chemistry, 103*, 313–318.
27. Yadav, H., Jain, S., & Sinha, P. R. (2007). Production of free fatty acids and conjugated linoleic acid in probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* during fermentation and storage. *International Dairy Journal, 17*, 1006–1010.