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RESEARCH ARTICLE

PROFILING OF CONJUGATED LINOLEIC ACID PRODUCED BY PROBIOTIC LACTIC ACID BACTERIA ISOLATED FROM DAIRY PRODUCTS

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ABSTRACT

The fortification of dairy products with prebiotic and probiotic ingredients is gaining a huge impact in the food industry. A quick screening methodology was developed to isolate existing lactic acid bacteria from dairy products and determine compatible carbon and nitrogen sources using Phenotypic screening for production of conjugated linoleic acid. Three isolates were selected from different dairy products based on their acid production potential when grown on sugar sources and/or fatty acids. The microorganisms were able to produce conjugated linoleic acid from castor oil hydrolysate in MRS broth and also in fat rich milk during fermentation under the given experimental conditions. The conjugated linoleic acid profiles and contents were determined and compared with *Lactobacillus delbrueckii*. An average CLA content of 0.1g L^{-1} was observed in fermented fat rich milk with the isolates while 0.5g L^{-1} was seen with *L. delbrueckii*.

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INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of naturally occurring, conjugated, geometric and positional isomers of linoleic acid (Kelly, 2001; Aydin, 2005; Gorissen *et al*., 2013). As a functional element in dairy products, positional isomers of CLA are those with double bonds located at positions 9-11 and 10-12 along the carbon chain (Hosseini *et al*., 2014). Depending upon the position of double bonds, isomers of CLA have innumerable health benefits (Yang *et al*., 2015).

Commercial production of CLA is mainly though chemical means by isomerisation of linoleic acid using an alkali as a catalyst (Salamon *et al*., 2015). However, due to less selectivity, the product obtained is a mixture of many positional isomers (He *et al*., 2015). Various groups of scientists have synthesized CLA using microorganisms as biocatalysts in de Man, Rogosa and Sharpe (MRS) broth, milk, yogurt and cream (Kim and Liu, 2002; Van Nieuwenhove *et al*., 2007; Ye *et al*., 2013; Villar-Tajadura *et al*., 2014; Trigueros *et al*., 2015).

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CLA uptake in milk could be improvised by employing dairy based lactic acid bacteria (LAB) which are capable of enhancing CLA levels in dairy products. Additionally, fortification of milk with certain prebiotics can lead to enhanced CLA production, thus increasing the nutritional value of milk.

The present study was focused on isolation of LAB from dairy products. A rapid screening protocol was employed to select organisms from milk, curd and Yakult® (a commercially available probiotic drink). The isolates were identified using Biolog GEN III MicroPlate™ technology (www.biolog.com). The CLA production capability of the isolates along with the distribution of isomers, in both MRS broth and milk, was compared with *L. delbrueckii*.

MATERIALS AND METHODS

Materials

Unprocessed curd, milk, Yakult® (probiotic drink) and Amul Gold (homogenized standardized milk with high fat content) were procured from local dairy shop and stored at 4°C before

use. The strain, *L. delbrueckii* (NCIM 2025), was procured from National Collection of Industrial Microorganisms, NCL, Pune. Castor Oil and Linseed Oil were provided as a kind gift from Acme Synthetic Chemicals Ltd., India. These oil samples were hydrolyzed, to obtain free fatty acids with removal of glycerol, according to the protocol optimized using immobilized lipase (Vadgama, 2014).

Isolation of lactic acid bacteria (LAB)

Serial dilution of all samples was performed in saline followed by spread plate technique on MRS-BCP plates, with composition (g L^{-1}): 10g peptone, 10g beef extract, 5g yeast extract, 20g glucose, 1g Tween 80, 5g sodium acetate, 2g ammonium citrate, 2g K_2HPO_4, 0.1g $MgSO_4.7H_2O$, 0.05g $MnSO_4.H_2O$, supplemented with 0.2g bromocresol purple (BCP), 20g agar with final pH 6.8 to 7.0. These MRS-BCP plates were then over laid with 10g L^{-1} agar to provide microaerophilic atmosphere and incubated at 28±2°C for 48h. The colonies with a surrounding yellow zone (indicating acid production) were picked and inoculated in a microtiter plate containing 200µL MRS-BCP broth in each well. After incubation for 48h at 28±2°C, isolates showing an intense yellow colour in the broth were first tested for catalase, by placing 10µL of broth in a drop of 3% hydrogen peroxide solution. Only catalase-negative cultures were purified on fresh MRS-BCP agar plates. The purified cultures and *L. delbrueckii* were maintained on MRS agar slants at 4°C. All growth studies were carried out on a shaker (80 rev min^{-1}) in batch cultures at 37°C for isolates Y1, Y2, *L. delbrueckii* and 30±2°C for M1. Microbial growth was determined at 580nm using a colorimeter.

Adaptation of the isolates on fatty acids for growth

The catalase-negative isolates were acclimatized to grow in the presence of linseed oil hydrolysate (α-linolenic acid, linoleic acid, oleic acid and traces of palmitic acid and stearic acid) by gradually reducing the concentration of glucose and increasing the concentration of the hydrolysate. Glucose concentration was gradually decreased from 20g L^{-1} to nil with simultaneous increase in linseed oil hydrolysate up to 20g L^{-1}. The colonies were inoculated in 200µL of MRS-BCP broth in a microtiter plate and incubated at 28 ±2°C.

Identification and Biochemical characterization using OmniLog Phenotype Microarray system

Identification and biochemical characterization of the isolates was performed using Biolog GEN III MicroPlate™ (Biolog Inc., Hayward, CA, USA) according to manufacturer's instructions. The phenotypic fingerprint obtained was used for identification of isolates from Microbial Identification Databases for Biolog Systems (Biolog).

Screening of isolates for CLA production profile and content

Positive cultures from the above screening were further studied for their CLA profiles and content. The isolates were inoculated into 50mL of MRS broth supplemented with 0.6g L^{-1} linseed oil hydrolysate for activation. After 12h of incubation at 37°C, the cells were harvested by centrifuging at 12,857g for 10min in an Eppendorf 5810 R centrifuge. Washed cells (0.5g) were transferred in 25mL of production medium with composition (g L^{-1}): 0.05g $MnSO_4$, 5g sodium acetate, 2g ammonium sulphate, 5g yeast extract, 4g castor oil hydrolysate and 0.2g Tween-80. Similarly 0.5g wet biomass was inoculated in 25mL milk and incubated at 28±2°C on a shaker at 150 rev min^{-1}. CLA yield was monitored at different time intervals for isolate Y1, M1 and *L. delbrueckii*. After 12h of incubation, the culture was centrifuged at 12,857g for 10min, to remove the cells, while the supernatant was subjected to extraction of fatty acids. The lipids in the supernatant were extracted using hexane as solvent.

Analysis of fatty acids

For GC analysis of the fatty acids, the hexane layer was evaporated using vacuum distillation (Heidolph Laborota 4002 control). The fatty acids were subjected to derivatization (Shantha and Napolitano, 1992) and the resulting fatty acid methyl esters (FAME) were analysed on a Agilent 7890A GC system equipped with flame ionisation detector system (Agilent technologies Canada, Kirkland, QC, Canada) and high cyanopropyl-containing polysiloxane HP88 column (100m × 0.25mm i.d. × 0.20µm of thickness). The flow rate of the carrier gas (Nitrogen) was 0.5mL min^{-1}, the injector and the detector temperature was 250˚C. The injection volume was 2µL (split). The temperature program was as follows: initial temperature 80°C, temperature from 80 to 150°C at 20°C min^{-1}, held isothermally at 150°C for 1min, from 150 to 200°C at 5°C min^{-1}, held isothermally at 200°C for 10min and from 200 to 230°C at 2°C min^{-1} held isothermally for 10min. The concentration of CLA was calculated as compared to the standard CLA (Sigma Aldrich, USA).

RESULTS

Screening of Lactic Acid Bacteria (LAB)

A rapid screening procedure using a microtiter plate based assay was used for the screening of LAB. Acid producers were selected using bromocresol purple (BCP) dye incorporated in MRS medium, which turned yellow with acid producing bacteria. Colonies showing yellow coloration around them were selected after 48h of incubation. Six colonies, one from curd (C1), three from milk (M1, M2 and M3) and two from Yakult® (Y1, Y2) were selected based on morphology and acid production. All the isolates were analysed for Gram nature and catalase production. Out of these, C1, M2, Y1 and Y2 were Gram-positive rods and catalase negative exception being M3, which was catalase positive, and M1, which was Gram-positive cocci.

Screening of the isolates for fatty acid utilization

The selected colonies along with *L. delbrueckii* were grown on MRS-BCP medium with glucose as carbon source and were adapted to assimilate fatty acids from linseed oil hydrolysate, an unsaturated fatty acid source, as sole carbon source. On the basis of acid production it was observed that all six isolates were growing luxuriantly in the presence of glucose as the sole source of carbon. After incubating the isolates with glucose and linseed oil hydrolysate, isolates M1, Y1, Y2 and *L. delbrueckii* all showed luxuriant growth. The isolate M2

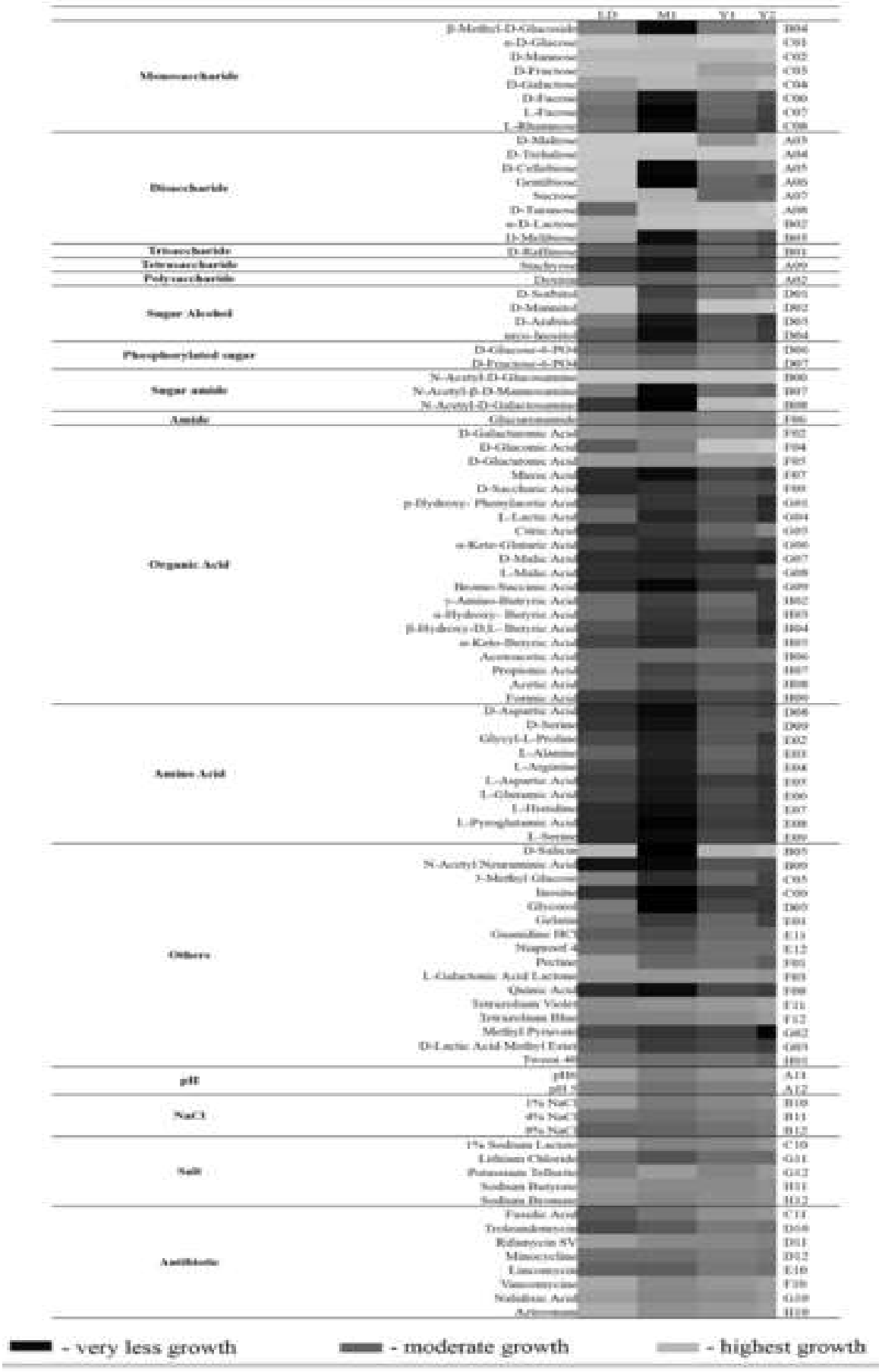

Figure 1. Summed map of the comparison of the four lactic acid bacteria for identification using Biolog GEN III MicroPlate™. The strains were identified as, LD, *L. delbrueckii*, M1, *Leuconostoc lactis*, Y1, *Weissella viridescenses* and Y2, *Weissella viridescenses*. Differences in colour shade intensities represent differences in metabolic activity for different sources tested. The units are increase in Omnilog Units from 0h to 120h incubation, which represent the calculated difference between the values for a specific well and the control well.

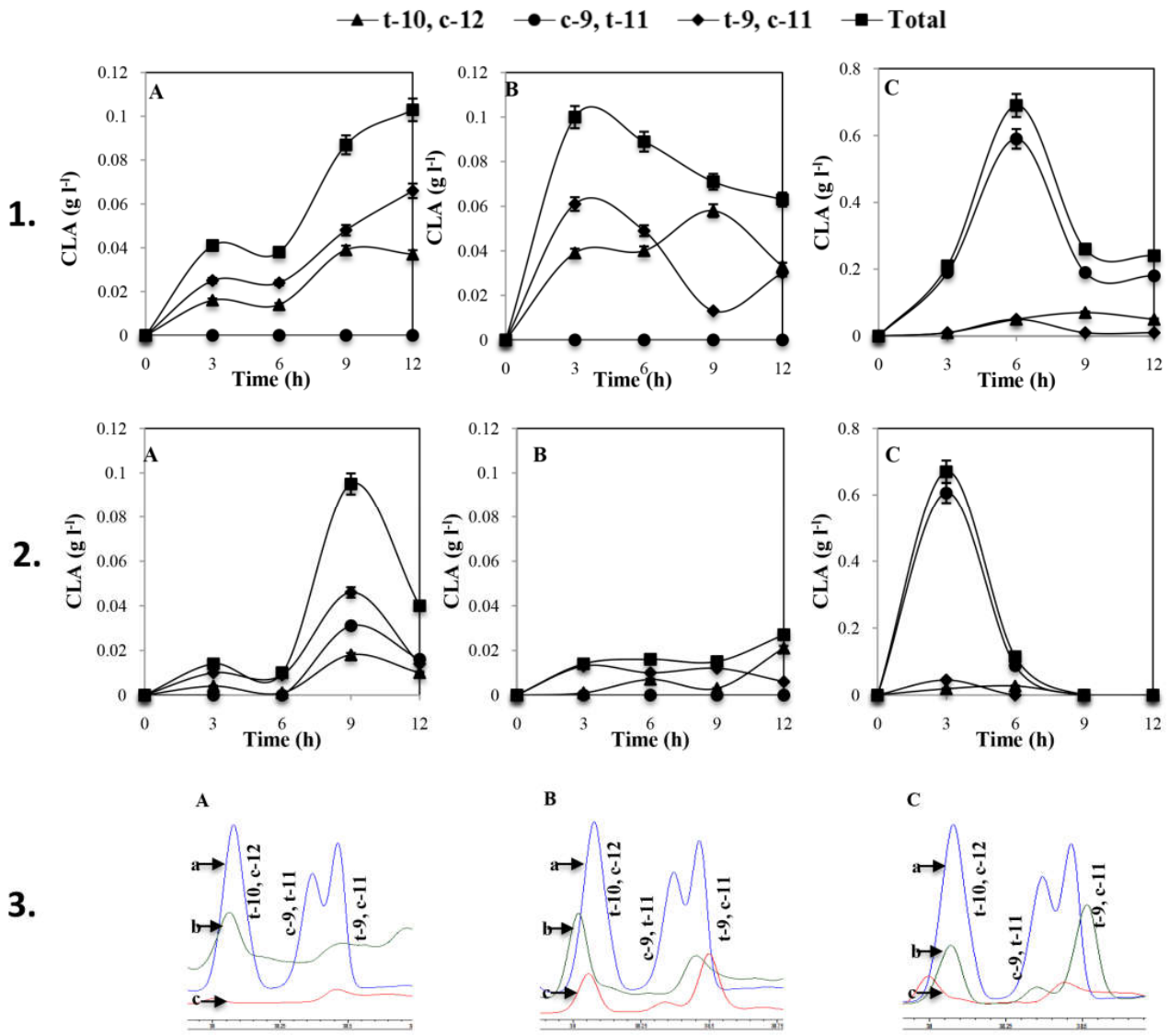

Figure 2. CLA production profile with respect to different isomers vs time. CLA production from castor oil hydrolysate (1) and milk (2) with GC chromatogram of CLA isomers (3) by M1-*Leuconostoc lactis* (A), Y1-*Weissella viridescenses* (B) and *L. delbrueckii* (C). The GC chromatogram shows CLA isomer standard (a), CLA production in castor oil hydrolysate (b) and in milk (c).

showed least acid production, while C1 and M3 did not show any growth and acid production in the medium, indicating that the organisms were unable to metabolize glucose in the presence of fatty acid. When the carbon source was altered completely from glucose to linseed oil hydrolysate, the isolates M1 and *L. delbrueckii* produced negligible amounts of acid, visualized by a faint yellow color. However the isolates C1, M2, M3, Y1 and Y2 did not show any turbidity and acid production. Therefore, based on the growth of the organism on MRS media containing glucose and linseed oil hydrolysate isolates M1, Y1, Y2 along with *L. delbrueckii* were selected for biochemical characterization, identification and CLA production.

Identification and biochemical characterization of the isolates using Biolog system

Biochemical characterization of the unknown isolates was performed by Biolog GEN III Identification system using the GEN III MicroPlate™ (Biolog) with *L. delbrueckii* as a standard organism and positive control. Figure 1 shows the sustained dye reduction heat maps corresponding to various metabolites**.** When the phenotypic fingerprint of the unknown organisms was compared to the GEN III Database using the Microbial Identification Databases for Biolog Systems (Biolog), the identity of the organisms was found to be, M1-*Leuconostoc lactis*, Y1- *Weissella viridescenses* and Y2-*Weissella viridescenses.*

Biochemical characterization also helped to identify various sets of biomolecules, which support or retard the growth of strains. The monosaccharides, disaccharides, sugar alcohols, phosphorylated sugars, sugar amides were found to enhance the growth of the strains with an exception of M1-*Leuconostoc lactis* whose growth was retarded by most of them. The strains were observed to be stable till pH 5. Lower pH was observed to be inhibitory for the growth of all the isolates as observed with all the organic acids and amino acids.

The isolates were tolerant to halophilic condition (8% sodium chloride) and were stable in presence of all salts tested. Even, the presence of antibiotics did not inhibit the growth of the strains. These isolates were able to utilize plant protein (like wheat and soy) hydrolysates as nitrogen source.

CLA production using isolates from castor oil hydrolysate and milk

Amongst the three isolates, only M1 and Y1 were selected for studying production of CLA based on their growth in combination media (linseed oil hydrolysate + glucose), In this study castor oil hydrolysate containing free ricinoleic acid was used as a substrate for production of CLA. The isolate M1 showed linear increase in CLA production after 6h of incubation. The highest concentration of CLA (0.1g L^{-1}) was produced at 12h of incubation (Figure 2.1A). Isolate Y1 showed maximum CLA yield (0.1g L^{-1}) at 3h of incubation, following which the CLA levels dropped gradually with prolonged time, giving very low levels at 12h (Figure 2.1B). *L. delbrueckii* achieved highest concentration of CLA (0.69g L^{-1}) after 6h of incubation, which was also the highest as compared to the isolates. The concentration of CLA was found to decline as the incubation time progressed (Figure 2.1C). The substrate, castor oil hydrolysate did not contain any inherent isomer of CLA. Both the isolates M1 and Y1 were observed to produce *trans*-10, *cis*-12 isomer and *trans*-9, *cis*-11 isomer (Figure 2.3). The distribution of these isomers kept varying with the incubation time. *L. delbrueckii* produced all three isomers *trans*-10, *cis*-12 isomer, *cis*-9, *trans*-11 isomer and *trans*-9, *cis*-11 isomer of CLA. Compared to all the other isomers, the *cis*-9, *trans*-11 isomer was by far the most abundant isomer produced, reaching 0.59g L^{-1} of production medium after 6h of incubation.

CLA production by the isolates was also demonstrated in fat rich milk and compared with that produced by *L. delbrueckii*. Isolate M1 produced 0.09g L^{-1} of CLA at 9h of incubation (Figure 2.2A), while the isolate Y1 was observed to produce maximum CLA of 0.03g L^{-1} at 12h (Figure 2.2B). *L. delbrueckii* was observed to produce the highest concentration of CLA in milk (0.6g L^{-1}) at 3h of incubation, which was found to decrease on further incubation (Figure 2.2C). When compared to the other isolates (M1 and Y1), the standard culture of *L. delbrueckii* was the best producer of CLA in milk. *L. delbrueckii* and M1 transformed fatty acids to all three isomers of CLA, *trans*-10, *cis*-12 isomer, *cis*-9, *trans*-11 isomer and *trans*-9, *cis*-11 isomer. The isolate Y1 was observed to produce only *trans*-10, *cis*-12 isomer and *trans*-9, *cis*-11 isomer (Figure 2.3).

DISCUSSION

Utilization of BCP during the screening procedure, helped to narrow down the selection for LAB at the very first screening step by selecting only those colonies that were capable of producing acid, marked by a yellow zone around the colony. The gradual adaptation of the isolates for growth from glucose to linseed oil hydrolysate helped to activate the cells for CLA production, during pre-inoculum preparation. However the isolates were inhibited in presence of linseed oil hydrolysate. It has been reported that *Lactobacillus sp.* has inhibited growth when the linoleic acid concentration is higher than 2g L^{-1} (Kishino *et al*., 2002; Ogawa *et al*., 2014). Similarly, the isolates were facing metabolic stress when they were grown in media containing linseed oil hydrolysate. The enzymes required for the breakdown of these fatty acids were not inherently synthesized by the organism instead were triggered when there was a supply of fatty acids. For this purpose, our strategy to gradually adapt the culture from glucose to linseed oil hydrolysate, whose unsaturated fatty acid content induces the activation of detoxification pathway for CLA production, was very helpful.

Along with activation of cells certain biomolecules can enhance the growth and CLA production ability of the LAB. With this objective, the biochemical characterization helped to identify certain biomolecules, which were observed to support growth of the isolates. These biomolecules can find applications as prebiotics and thus can be utilized as growth stimulants in various production media or milk, which can help to improve CLA production. After following the activation protocol, all the selected strains were able to produce CLA in both MRS broth and milk. Although production of different isomers of CLA varied among the strains, all showed CLA production in milk without any additives. Similar results have been obtained by employing *Lactobacillus* strains in milk supplemented with safflower oil hydrolysate (Ye *et al*., 2013). An intermittent increase and decrease in the CLA isomer concentration was observed with all the isolates, which may be due to the gradual conversion of all the CLA to hydrogenated products like C18:1 and C18:0 during the detoxification pathway. Therefore, further studies are required for optimization of the fermentation system for maximal production of CLA.

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Competing Interests

The authors declare that they have no competing interests.

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