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Original Research Paper

A highly selective whole cell biocatalysis method for the production of two major bioactive conjugated linoleic acid isomers

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ABSTRACT

Conjugated linoleic acid (CLA) is well known potential anticarcinogenic, antiatherogenic and a number of other therapeutic qualities bearing substance. In the present study we have isolated *Lactobacillus plantarum* from local dairy buffalo milk and used as a whole cell biocatalyst, for selective CLA isomer production from linoleic acid (LA). Quantitative analysis revealed that the *cis*-9, *trans*-11 CLA comprised of 51% (*w*/*w*), while *trans*-10, *cis*-12 CLA accounted for 49% (*w*/*w*) of total CLA produced by *L. plantarum*. The product formation and quantitation was monitored by the spectrophotometric method and the individual isomers separation was done with GC–FID. After optimization of biotransformation conditions such as pH, LA concentration, effect of preincubation, fermentation time; the highest CLA production (19.07% conversion) was achieved at 120 h in the presence of 1.62 mg ml^{-1} of LA. The results revealed that the bacterium is an efficient biocatalyst for the production of two major bioactive CLA isomers using LA as a substrate.

1. Introduction

Conjugated linoleic acid (CLA) is not a single substance but indeed a mixed group of positional and geometrical isomers of linoleic acid (C18:2), the position of conjugated double bonds may vary from 7 to 14 carbon atom and including all possible geometric configurations of *cis–trans, trans–cis, cis–cis,* and *trans–trans* isomers (Ha et al., 1987). Fats and meat from ruminant species are a rich natural source of CLA, which includes beef (0.43% of total beef lipids) and dairy products (0.40–0.55% of total dairy lipids). *Cis*-9, *trans*-11 CLA, also called rumenic acid, is the main isomer, constituting 90% of the total CLA found in dairy and beef lipids (Sonja and Jan, 1998). Besides anticarcinogenic and antiatherogenic properties, other health benefits of CLA in animal and cell-line studies include the ability to reduce body fat, increase lean body mass, and protect against immune-induced muscle wasting (Herrmann et al., 2009; McGuire and McGuire, 2000)

However, the current human intake of CLA is 10 times less than the 3 g day^{-1} minimum value extrapolated from animal studies for optimal beneficial effects (Gaullier et al., 2007; Ip et al., 1994). To achieve the estimated optimum dietary CLA levels of approximately 3–4 g day^{-1}, it would be necessary to increase dietary animal fat, which would increase saturated fat intake. Therefore, efforts have been made to produce CLA through organic synthesis, microbial fermentation, enzymatic isomerization, or genetic engineering/bioengineering (Gangidi and Proctor, 2004). Traditional organic synthesis is highly capital-intensive and results in an isomeric mixture of CLA. However, low yields, extensive purification steps, and the inseparability of isomers all limit the commercial use of most chemical methods (Dunford, 2001). Both enzyme extracts and whole cells have been used for CLA production, compared with enzyme extracts, whole cell catalysts can be much more readily and inexpensively prepared. Because enzymes in cells are protected from the external environment, they are generally more stable in the long-term than free enzymes (Lin, 2006; Lin et al., 2003). The isomers of CLA are formed during biohydrogenation of linoleic acid in the rumen and also through conversion of vaccenic acid in the mammary gland. In addition, several strains of *Lactobacillus, Propionibacterium, Bifidobacterium* and *Enterococcus* are able to form CLA from linoleic acid and thus could be used to increase the CLA level in fermented dairy products such as yoghurt and cheese (Siebera et al., 2004). Recently we have chemoenzymatically synthesized *cis*-9, *trans*-11 CLA using whole cell of *L. plantarum* starting with linoleic acid leading to an intermediate 10-hydroxy-*cis*-12-octadecaenoic followed by microwave reaction (Ayhan and Farah, 2010). Keeping in view of our previous experience we proposed to develop an efficient direct whole cell biocatalysis method without using any

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[1] This paper is dedicated to the memory of our wonderful colleague, Prof. Ayhan S. Demir, who recently passed away.

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chemical approach for the production of CLA. We used *L. plantarum* as a whole cell biocatalyst isolated from local dairy milk for direct conversion of linoleic acid into conjugated linoleic acid.

2. Material and Methods

2.1. Chemicals

Linoleic acid, standard CLA, chloroform, bovine serum albumin (BSA) and H_2SO_4 were purchased from Sigma-Aldrich (St. Louis, MO). De Man Rogosa Sharpe (MRS) broth and Agar–Agar were bought from Oxoid Ltd. (Basingstoke Hampshire, ENG). KOH and n-hexane were obtained from Scharlau Chemie S.A (Sentmenat, Spain). Methanol analytical grade was purchased from Fisher Scientific and anhydrous sodium sulfate was purchased from Merck Ltd.

2.2. Isolation and identification of bacteria

Unpasteurized raw buffalo milk samples were collected from the local dairy farm of Jamshoro Sindh, under aseptic conditions in the sterile screw cap tubes, and the milk samples were stored at 4 °C. The samples were plated onto MRS medium for Lactobacillus isolation and the plates were incubated at 37 °C for 24–28 h. Well-isolated colonies with typical physiological characteristics namely white, elongated and round in shape were picked from each plate and transferred to MRS broth. Biochemical characteristics such as Gram reaction, production of catalase, carbohydrate fermentation patterns were performed according to Bergey's Manual of systematic Bacteriology (Kandler and Weiss, 1986). Purified cultures were maintained at −20 °C in MRS broth with 15% glycerol.

Genomic identification of the sample was done by using partial 16S rRNA sequencing. Preparation of genomic DNA was performed using GeneJET Genomic DNA Purification Kit (Fermentas, Lithuania) according to manufacturer's instructions. RNase (Fermentas) was used to digest RNA, and samples were stored at −20 °C until use. The quality of DNA isolations was tested both with A260/A280 ratios and on agarose gels with comparison to decreasing concentrations of Bacteriophage λ (Fermentas). Amplification of 16S rRNA gene was performed as follows, the reaction mixture was 50 ml, and consisted of 1.5 mM $MgCl_2$, 200 mM of each dNTP (Fermentas), 1 mM of each primer (forward primer 5′–AGAGTTTGATCCTGGCTCAG-3′ (Mora et al., 1998), reverse primer U926 5′-CCGTCAATTCCTTTRAGTTT-3′) (Baker et al., 2003), 0.5 U Taq DNA polymerase (Fermentas), and 500 ng DNA. Amplification conditions were as follows, an initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1 min, and final annealing of 72 °C for 10 min.

The PCR products were run on 1.5% agarose gels for 1 h at 80 V in 1× TAE (40 mM-Trisacetate and 1 mM-EDTA, pH 8.0), post-stained with ethidium bromide (1 mg/ml), and visualized under UV light in a gel documentation system (BioRad, Hercules, USA). A DNA ladder of 100 bp (Fermentas) was used as a DNA molecular weight marker. PCR amplicons were recovered from PCR mixtures using DNA Extraction Kit (Fermentas) and further subjected to sequencing analysis (Iontek, Istanbul, Turkey). The sequence of isolate was sequenced twice and trace files were analyzed using MEGA5 (Tamura et al., 2011). For species identification, sequences were submitted to Seqmatch of Ribosomal Database Project II (Cole et al., 2009), revealing that the species belongs to *L. plantarum* (GenBank accession no. KC288535).

2.3. The whole cell reaction conditions for CLA production

The study employed *L. plantarum* as a whole cell biocatalyst for CLA production. For preincubation cells were subcultured twice in the conical flasks (250 ml) containing 100 ml MRS broth (pH-8) with LA (0.09–0.45 mg ml^{-1}) supplemented with BSA (0.5 mg mg^{-1} of LA) for better solubility of the substrate and the cultures were kept in a mechanical shaker (Gallenhamp) at 110 rpm keeping 37 °C for 8–40 h. After preincubation biotransformation of LA to CLA was carried out by adding various amounts of the substrate (0.54–2.25 mg mg^{-1}) with BSA and the fermentation was carried out for different time intervals (8–144 h).The effect of pH was monitored between 4 and 8 for optimum CLA production. The incubation was continued in all set of optimization at 37 °C and 110 rpm under fully aerobic conditions.

2.4. Extraction of fatty acids

Fatty acids were extracted from the whole cell reaction mixture with chloroform/methanol (1:2, v/v) according to the procedure of Bligh and Dyer (Bligh and Dyer, 1959).

2.5. Product monitoring and quantification by spectrophotometer

After the extraction, product formation was monitored by UV/visible spectrophotometer (Biochrom Libra S22) at 233 nm, a specific wavelength for CLA confirmation (Alcala et al., 2011; Barrett et al., 2007). Absorbance was measured in 1 cm quartz cuvettes at room temperature. The quantitation of the reaction product was done with straight line equation drawn from the calibration curve, obtained with standard CLA mixture.

2.6. Esterification of the fatty acids for GC analysis

The esterification of free fatty acids was done by the acid catalyzed method (Park et al., 2002). Methanolic H_2SO_4 solution (1 N,3 ml) was added to a screw-cap test tube (15 mL), containing sample with heptadecanoic acid (1 mg) as the internal standard, and vortexed for 30 s. The test tube was heated in a water bath for 55 °C for 5 min. After that the test tube was cooled to room temperature, CLA methyl esters were extracted with hexane (3 mL × 3) by hand shaking for 1 min. The hexane extract was washed with 1.0 N NaOH in 50% ethanol (3 mL × 2) and with distilled water (3 mL × 3). The sample was dried over anhydrous sodium sulfate and analyzed by the GC as described below.

2.7. GC–FID conditions for CLA isomer separation

The separation of CLA isomers was achieved by using RT-2560 (0.25 mm ID) 100 m long polar capillary column, with the gas chromatograph (GC-8700 PERKIN ELMER). Nitrogen was used as a carrier gas, oven temperature was increased from 180 to 200 °C at a ramp rate of 2 °C min^{-1} and then held for 30 min, injection volume was 1 μl, injector and detector temperature were kept 240 °C and 260 °C respectively.

2.8. Statistical analysis

Data were statistically evaluated by one-way ANOVA test using SAS 16.0 (SAS Inst. Inc., Cary, N.C., U.S.A.) at the significance level of $P < 0.05$. The reported results were expressed as mean ± standard deviation (SD) of triplicate samples.

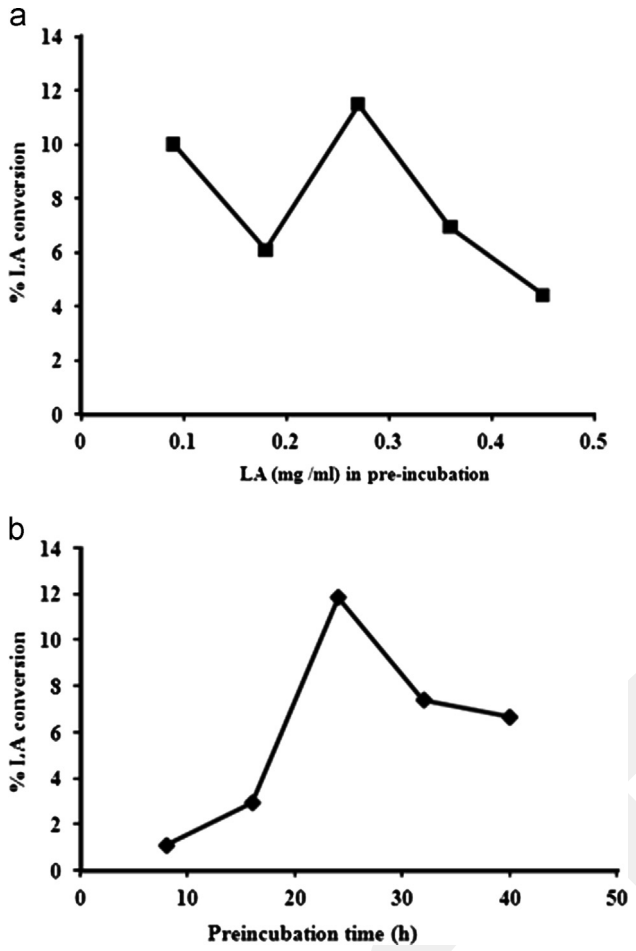

Fig. 1. (a) Effect of LA concentration during preincubation and (b) impact of preincubation time on LA conversion.

3. Results and discussion

3.1. Optimization of factors effecting CLA production

3.1.1. Effect of LA concentration and time on preincubation

Preincubation is a period before the transformation process starts or initiated by adding substrate, and microorganism adapt the environment or reaches certain growth stage to be ready for biotransformation. Therefore LA concentration has been optimized for preincubation as previous studies (Ayhan and Farah, 2010; Dong and Qi, 2006) has shown that the substrate could enhance or inhibit the cell growth and enzymatic activity for CLA production. As depicted in Fig. 1(a) there was an increasing current of LA conversion from 0.09–0.27 mg ml^{-1} LA and reached at maximum 0.27 mg ml^{-1}. However, when the amount of LA increased from 0.27–0.45 mg ml^{-1}, the % LA conversion decreased from 11% to 4%, which indicates that LA isomerase from *L. plantarum* might be inhibited by increasing the LA concentration. Infact unsaturated fatty acids including LA are generally toxic to a wide spectrum of Bacteria. The mechanism of LA preincubation is not clearly established, it is probably related to a difference in the adaptability to the inhibitory substrate. The effect of LA preincubation may not only be related to biochemical metabolism but also to more complex processes, such as expression levels of enzymes involved in CLA production (Park et al., 2009).

The effect of preincubation time on the LA conversion was investigated by varying preincubation time from 8–40 h, as depicted in Fig. 2. The highest conversion was obtained when preincubation time was 24 h. However, increase in LA conversion

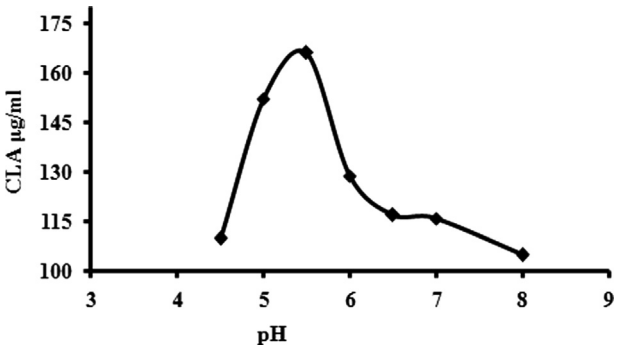

Fig. 2. Influence of initial pH on CLA formation.

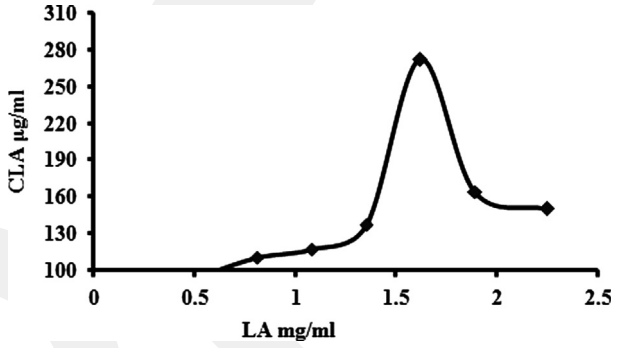

Fig. 3. CLA production as a function of LA concentration.

was not evident from 24–40 h; it indicates that too long time may cause counter-reaction for the production of LA isomerase which may affect the conversion negatively (Dong and Qi, 2006).

3.1.2. Effect of initial pH

During the culture of *L. plantarum*, pH can affect the growth rate and the fatty acid profile (Nikkila et al., 1996). The significant effect of initial pH on the formation of CLA is shown in Fig. 2 the range of pH was from 4 to 8 and the other conditions were as described above. The amount of CLA was increased from pH 4.5 to 5.5 and reached a maximum (166 µg ml^{-1}) at pH 5.5 then decreased. CLA production mainly depends on the enzymatic conversion of LA, which may be sensitive to the pH of the system. From the results it can be concluded that initial pH 5.5 was beneficial for LA isomerase activity in the whole cell reaction, further incubations were performed at pH 5.5.

3.1.3. Effect of substrate (LA) concentration

LA concentration was a main factor that could impact the conversion rate of CLA. Thus, this factor was also taken into consideration in the experiments. The substrate concentration was studied between 0.54–2.25 mg ml^{-1}. As shown in Fig. 3 that initially there was a slow trend in CLA formation from 0.05 to 0.13% LA, after that a significant change in CLA production was noted between 0.13 to 0.16% substrate concentration and maximum yield of CLA was achieved in the mixture with 1.62 mg ml^{-1} linoleic acid and reached 272 µg ml^{-1} and then the amounts of CLA started to decrease when more substrate was added. The reason for this might be that high level of LA concentration could change the structure of enzyme and reduce the contact areas between enzyme and substrate (Troegeler-Meynadier et al., 2003). The rate of the formation of CLA reached 19.07% when the substrate concentration was 0.16% at which the total yield of CLA was 272 µg ml^{-1}. Therefore 1.62 mg ml^{-1} LA was chosen as the optimal substrate concentration.

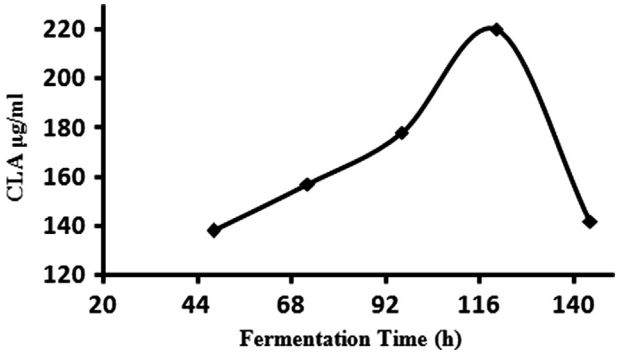

Fig. 4. Effect of fermentation time on CLA yield.

3.1.4. Effect of fermentation time

In the present study, incubation time was closely related to CLA production. The fermentation time period was kept from 48 to 144 h at an interval of 24 h. There was a gradual enhancement in the CLA formation from 48 to 120 h of time period and reaching at maximum value of 220 µg ml^{-1} at 120 h as shown in Fig. 4. However, prolonged incubation time did not yield a further increase in CLA synthesis, the decrease in CLA formation with prolonged time may be attributed to drop-off in pH value below 3 at 144 h. The optimal pH range for LA isomerase activity has been reported between pH 4.5 to 7.5 (Miao et al., 2005). Therefore 120 h reaction time was optimized.

All the incubations were performed under aerobic conditions for CLA production. Aerobic incubations with LA might be more favorable for the production of CLA isomers by inactivating the CLA reduction steps, which are active only when energy metabolism is normal (Kim, 2003).

3.2. CLA quantification by UV spectrophotometry

The samples were scanned from 200–400 nm and the strong absorption band at 233 nm indicates the presence of conjugated double bond chromophoric group. The standard UV absorption curve of CLA was obtained in the range of 0.35–17.39 µg ml^{-1}. Quantitation was done by putting the absorbance value in straight line equation (y=0.1015x+0.0547), which gives an excellent regression coefficient value of 0.998. Besides UV other methods could also be used for CLA detection such as gas-liquid chromatography, silver-ion high performance liquid chromatography, gas chromatography mass spectrometry (26) and reversed-phase high-performance liquid chromatography (Mossoba, 2001). But all these methods are time consuming and need derivatization steps for CLA determination so UV spectroscopy offers the fast and most economical method to regularly monitor the CLA formation in biocatalytic reactions. Similarly use of UV spectroscopy for rapid detection of CLA formation by bacterial culture has been reported (Barrett et al., 2007).

3.3. CLA isomer separation by GC–FID

Fig. 5(a) shows the gas chromatogram of CLA produced by whole cells of *L. plantarum* in MRS medium after 120 h of incubation supplemented with 1.47 µg ml^{-1} LA as a substrate at pH 5.5. The two isomers of CLA *cis*-9, *trans*-11 and *trans*-10, *cis*-12 were produced and separated with the retention times 23.90 min and 24.31 min respectively. The confirmation of these two isomers was done by matching the retention times with standard conjugated linoleic acid isomers Fig. 5(b). Quantitative analysis revealed that the *cis*-9, *trans*-11 CLA comprised of 51% (w/w), while *trans*-10, *cis*-12 CLA accounted for 49% (w/w) of total CLA produced by *L. plantarum* in the present study. Production of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers has been evidenced by four different species of *L. Plantarum* IMAU60042, IMAU60171, IMAU70089, and P8, and the maximum conversion of free LA was between 3 and 4% (Li et al., 2012). In another study carried out on *L. plantarum* ATCC 8014 using LA as a substrate the maximum conversion of CLA (4.6%) has been reported (Gorissen et al., 2011), however we have obtained a higher CLA conversion 19.07% using *L. Plantatrum*.

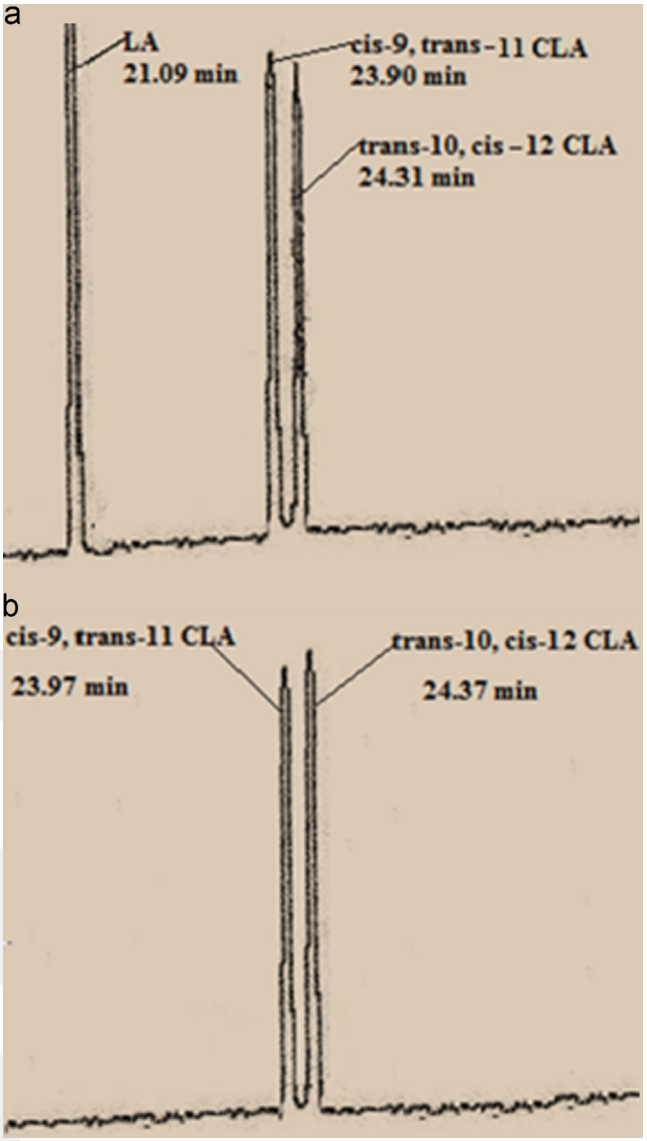

Fig. 5. Partial Gas chromatogram of (a) LA cultivated with *L. plantarum* and (b) standard CLA mixture.

Moreover the *L. plantarum* strain exploited in this work has a strong LA tolerance (2.25 mg ml^{-1}), which shows its potency to be used in the dairy industry; in order to produce fermented dairy products with increased CLA content.

4. Conclusions

In conclusion, the capability of *L. plantarum* to produce major bioactive CLA isomers (*cis*-9, *trans*-11; *trans*-10, *cis*-12) is dependent upon different culture conditions, such as pH, substrate concentration, preincubation and fermentation time. The maximum CLA production attained by *L. plantarum* at the optimized parameters was 272 µg ml^{-1}. The strain could be used

as a biocatalyst to increase the CLA content in varieties of functional food products.

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