1646 J. Agric. Food Chem. 2010, 58, 1646–1652
DOI:10.1021/jf903521b

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Chemoenzymatic Conversion of Linoleic Acid into Conjugated Linoleic Acid

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An efficient chemoenzymatic method for preparing conjugated linoleic acid (CLA) using free linoleic acid (LA) as a substrate is described. In the first step, LA was transformed into 10-hydroxy-*cis*-12-octadecenoic acid (HA) by the whole cells of *Lactobacillus plantarum* after 48 h of incubation. The preincubation of whole cells with 0.03% LA resulted in a better yield of HA (480 mg/g) compared to cells grown without LA. In a second fast microwave step, HA was converted to *cis*-9,*trans*-11-octadecadienoic acid in the presence of iodine as a catalyst over a silica gel surface. The advantage of this method in preparing *cis*-9,*trans*-11 CLA is simple via the whole cell bioconversion of LA into HA via *L. plantarum* followed by the fast microwave-assisted synthesis of *cis*-9,*trans*-11 CLA in higher yields.

KEYWORDS: Chemoenzymatic; CLA; *Lactobacillus plantarum*; microwave irradiation; LA

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term that refers to a mixture of the positional and geometric isomers of linoleic acid (LA) with conjugated double bonds at carbons 10 and 12 or 9 and 11, in all of the possible cis and trans combinations. It was first isolated and identified by Pariza and co-workers from a fraction of grilled beef that possessed antimutagenic activity (*1*). Since then, interest has increased markedly because of its discovered physiological activities, such as the reduction of cancer incidence (*2*), a decrease in body fat content (*3*), profitable effects on atherosclerosis (*4*), and strengthening of the immune system (*5*).

Recently, it was discovered that each isomer has different bioactivities: *cis*-9,*trans*-11 CLA shows anticancer activity (*6*); *trans*-10,*cis*-12 CLA shows activities that decrease body fat content (*6*, *7*), increase energy expenditure (*8*), and suppress the development of hypertension (*9*). The *cis*-9,*trans*-11 isomer is predominantly found in foods sourced from ruminants. The formation of this conjugated polyunsaturated system was catalyzed by ruminal bacteria (*10*). Gaullier et al. (*11*) assessed the effects of the supplementation of 3.4 g/day of CLA (1:1 ratio 9- and 10-CLA in triglyceride form) in healthy, overweight adults and revealed a decrease in body weight and body fat mass along with increased circulating lipoprotein, thrombocytes, and aspartate aminotransferase. Human intake of CLA from natural food sources, such as dairy (0.55% total fat) and beef (0.60% total fat), is approximately 10% of the suggested recommended value (*12*).

To achieve the estimated optimum dietary CLA levels of approximately 3−4 g/day, 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 (*13*). 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 (*14*). Therefore, biotechnology offers new alternatives to traditional lipid manufacturing methods. A rumen bacterium, *Butyrivibrio fibrisolven*, was the first identified for converting LA to *cis*-9,*trans*-11 CLA, due to the presence of linoleic acid isomerase activity (*15*). Since then, there have been numerous reports that a large number of benign bacteria, which are naturally present in the environment and intestinal tracts of ruminants, can convert LA to CLA (*16*−*18*). Further analyses with *Lactobacillus* species washed cells as model strains have revealed the involvement of a hydroxy fatty acid, 10-hydroxy-12-octadecanoic acid, as an intermediate for CLA production (*19*). However, the production of CLA was greatly influenced by aerobic conditions. Because of the growth inhibitory action of LA at low concentrations, the yields of CLA remain poor, as LA is required for any feasible production process. In addition, some of the CLA-producing bacteria transform the wanted isomers further to secondary compounds (*20*, *21*). Therefore, the chemoenzymatic conversion that combines the flexibility of chemical synthesis and the high selectivity of enzymatic conversions is a powerful approach to obtain individually pure isomers (*22*).

Herein we describe a chemoenzymatic method for preparing *cis*-9,*trans*-11 CLA by using a two-step approach. In the first step, linoleic acid is converted into 10-hydroxy-12-octadecenoic acid by the whole cell reaction of *Lactobacillus plantarum* bacteria followed by the fast microwave production of *cis*-9,*trans*-11 CLA via the dehydration of hydroxyoctadecenoic acid in the presence of iodine as a catalyst. Microwave techniques offer a reduction in the amount of solvents and hazardous substances required, along with a more efficient use of energy with minimum time and better yields in synthesis (*23*).

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 Published on Web 01/08/2010

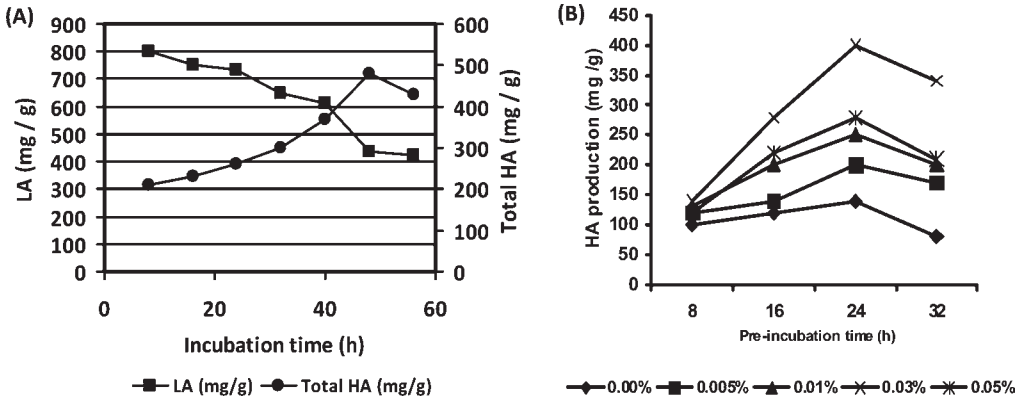


Figure 1. (**A**) Time course for the conversion of linoleic acid into hydroxyoctadecenoic acid by the whole cells of *L. plantarum* under optimized conditions. (**B**) Effect of preincubation time and concentration on hydroxyoctadecenoic acid conversion. The incubations were performed in triplicate, and the values are shown in the figure as an average.

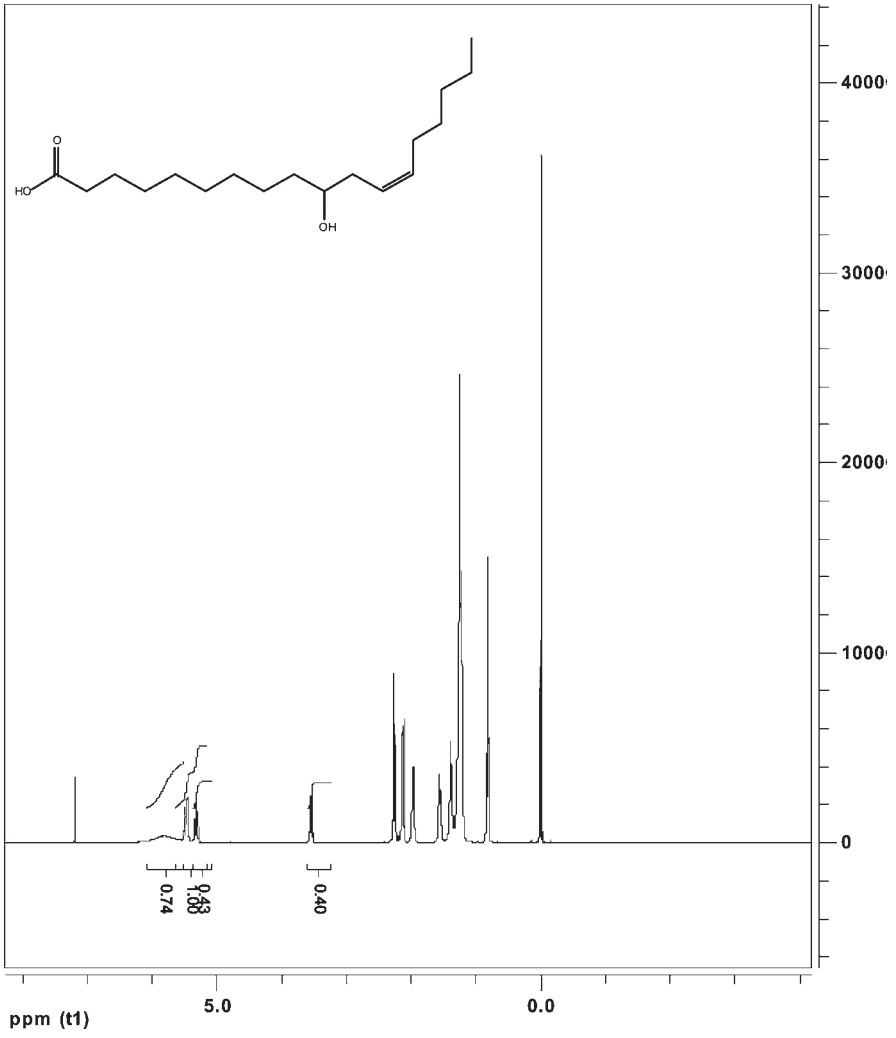


Figure 2. ^{1}H NMR spectrum of 10- hydroxy-*cis*-12-octadecenoic acid (HA) produced from LA by the whole cell incubation of *L. plantarum*.

MATERIALS AND METHODS

Chemicals. LA (*cis*-9,*cis*-12-octadecadienoic acid) and CLA mixture of *cis*-9*trans*-11 and *trans*-10,*cis*-12 CLA was purchased from Sigma-Aldrich (St. Louis, MO). The other chemicals and reagents (analytical grade, J. T. Baker, Deventer, The Netherlands) that were used in the present study are commercially available.

Culture of Cells. *L. plantarum* isolated from tulum cheese, as reported earlier (*19*), was obtained from Kafka's University in Kars, Turkey. The cells were grown twice under aerobic conditions at 30 °C in MRS broth, as described previously (*24*).

Whole Cell Reaction Conditions. A pure active culture was prepared by picking a single colony from the MRS agar plate and growing it in 100 mL of MRS broth for 24 h, supplemented with 0.03% LA. After 24 h of preincubation, a 250 μg mL^{-1} LA complex with BSA (0.5 mg of BSA/mg of LA) was added to the MRS culture. The whole cell reaction was further continued for 48 h at 30 °C with gentle shaking (120 strokes/min) under aerobic conditions. All of the incubations were performed in triplicate.

Lipid Extraction and Isolation of the Reaction Products. Lipids were extracted from the reaction mixture with chloroform/methanol (1:2, v/v) according to the procedure of Bligh and Dyer (*25*). The reaction product isolation was carried out by column chromatography on silica gel 60 (40−63 μm) with petroleum ether/diethyl ether/acetic acid (70:30:1) as eluting solvents. TLC was conducted on aluminum sheets that were precoated with silica gel 60F 254, in which any possible spots were visualized by phosphomolybdic acid (Sigma).

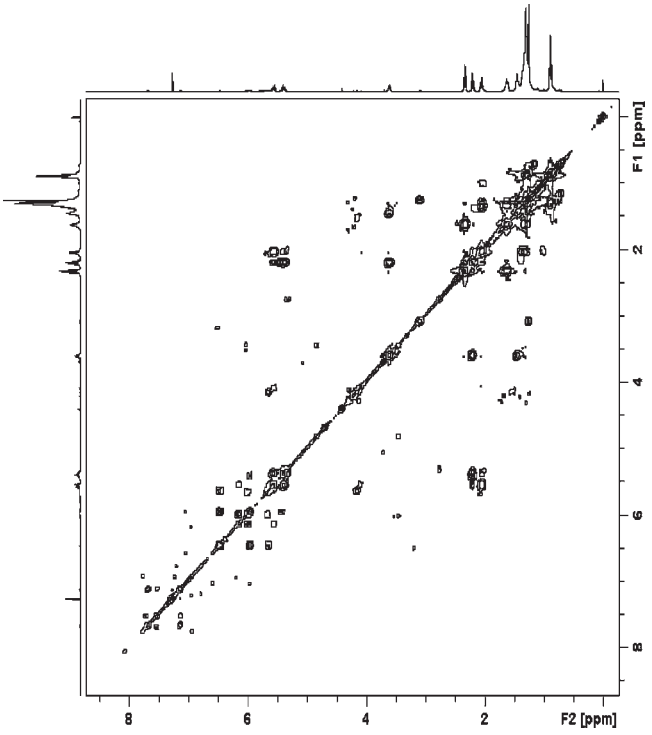


Figure 3. $^{1}H-^{1}H$ COSY spectrum of 10- hydroxy-*cis*-12-octadecenoic acid (HA) produced from LA by the whole cell incubation of *L. plantarum*.

Table 1. Effect of the Amount of Iodine and Reaction Conditions on the Conversion of 10-Hydroxy-*cis*-12-octadecenoic Acid into CLA

entry	solvent[a]	I_2 (%)	time (min)	temperature[b] (°C)	conversion[c] (%)
1	CH_3COOH	1	60	30	–
2		5	60	30	43
3	CH_3CN	1	60	50	–
4		5	60	50	38
5	CH_3Cl	1	60	50	–
6		5	60	50	25
7		5	120	50	37
8	neat	1	60	50	–
9		5	60	50	40

[a] One milliliter of each solvent was used. [b] MW temperature monitored and controlled with Terminal 320 from MLS-Milestone. [c] Conversion determined from the ^{1}H NMR spectra.

^{1}H NMR and $^{1}H-^{1}H$ COSY Analysis. All of the NMR experiments were performed on a Bruker DPX 400 NMR instrument. The chemical shifts δ are reported in parts per million relative to $CDCl_3$ (^{1}H, δ 7.27), (^{13}C, δ 77.0), and CCl_4 (^{13}C, δ 96.4) as internal standard.

LC-MS Analysis of Fatty Acid Methyl Esters (FAME). The FAME derivative of free CLA was prepared as reported earlier (*26*), followed by analysis with Theromo Finnigan LC-MS equipped with a Surveyor MSQ Mass detector (San Jose, CA). The mass analysis was performed in atmospheric pressure chemical ionization (APCI) mode with a probe temperature of 450 °C, corona current of 5 μA, and cone voltage of 30 V, and the sheath gas was high-purity nitrogen operated at 55 psi. The mass spectrometer was set to scan positive ions over a range of *m*/*z* 140−350 at 200 spans. Two Chromsphere Lipid 5 columns (250 × 4.6 mm.) connected in series were used for the separation. The mobile phase was hexane and isopropanol (90:10) at a flow rate of 0.7 mL/min. The photodiode array detector was set at a primary wavelength of 233 nm to detect conjugated dienes.

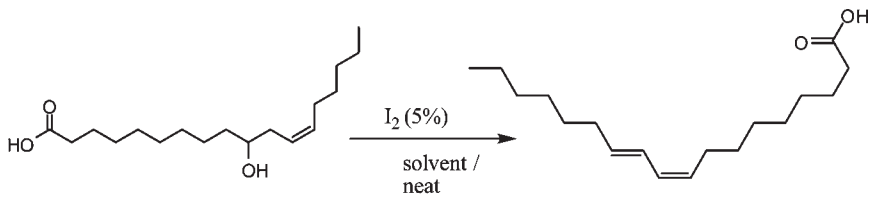
Microwave Reaction Conditions. One millimole of isolated and purified 10-hydroxy-*cis*-12-octadecenoic acid was placed in a vial containing 200 mg of silica and iodine crystals (1−5%). Then, the vial was heated for 60−120 min at 30−50 °C and 800 W by using a Milestone-Start microwave apparatus. Subsequently, the sample was dissolved in 10 mL of chloroform, vortexed for 1 min, and filtered. For the removal of iodine, the sample was shaken for 10 s with 5 mL of 0.01 N $Na_2S_2O_3$, in which this step was repeated until a transparent solution was obtained. The solvent was removed under vacuum, and prepared TLC with petroleum ether/diethyl ether/acetic acid (70:30:1) as the eluent system yielded the product.

RESULTS AND DISCUSSION

Transformation of LA by the Whole Cells of *L. plantarum*. The whole cells of *L. plantarum* cultivated in an MRS medium containing LA was investigated for the production of 10-hydroxy-*cis*-12-octadecenoic acid (HA). This was conducted because whole-cell conversion has advantages, such as greater resistance to environmental perturbations and a lower effective enzyme cost, all while eliminating the enzyme purification and extraction steps (*27*). The inhibitory effect of LA on bacterial growth has been reported by many authors (*20*, *28*) who have demonstrated that there are different tolerances according to strain. In the present paper, the LA tolerance was evaluated by the addition of increasing LA concentrations (0, 50, 100 150, 200, 250, and 300 μg mL^{-1}) in MRS agar plates where the bacteria were spread. Although previous studies (*28*) have reported bacterial growth inhibition by using a lower fatty acid level (25 μg mL^{-1}), in the present study *L. plantarum* was able to grow in the presence of higher LA concentrations (250 μg mL^{-1}). This fact is supported by previous explanations (*29*) wherein *L. plantarum* strains were able to adapt to different environments and could grow to high cell densities. However, a higher concentration of LA (300 μg mL^{-1}) inhibited the growth of the cells and decreased HA production.

To determine the optimal incubation time for HA conversion, HA formation was monitored from 0 to 56 h of incubation at an interval of 8 h in MRS broth supplemented with 250 μg mL^{-1}. As can be seen in **Figure 1A**, the cells that incubated for 48 h formed

Scheme 1



10-hydroxy-*cis*-12-octadecenoic acid

cis-9, *trans*-11-octadecadienoic acid

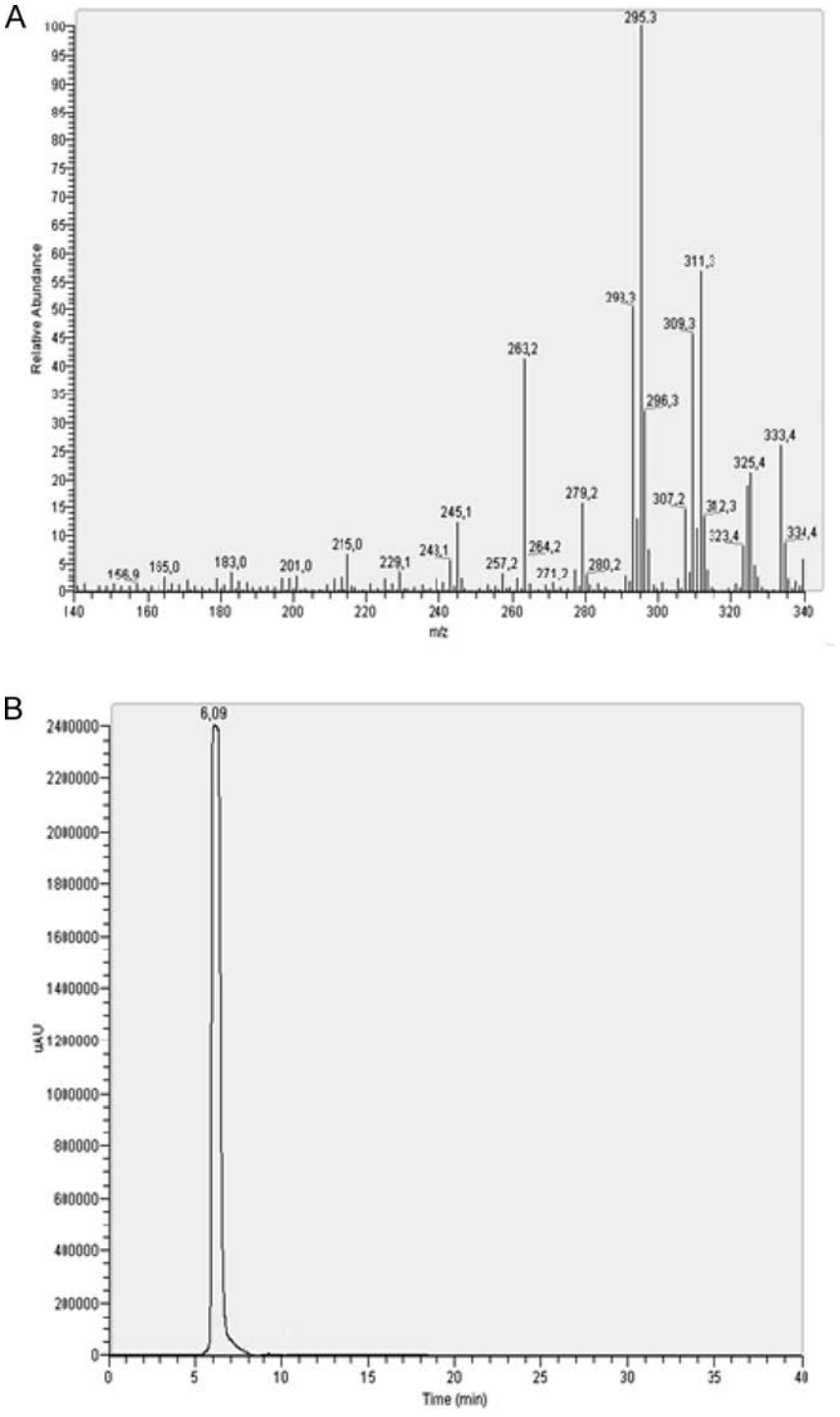


Figure 4. (**A**) Mass spectrum of *cis*-9,*trans*-11 CLA produced via 10-hydroxy-*cis*-12-octadecenoic acid (HA) by microwave irradiation under optimized conditions. (**B**) HPLC−diode array detection of *cis*-9,*trans*-11 CLA produced under microwave irradiation. For MS and HPLC conditions, see Materials and Methods.

more HA (480 mg/g of LA) than the cells of other incubation times, followed by an insignificant decrease in 56 h. Recently, Yu et al. (*28*) have also described an LA conversion into 10-hydroxy-12-octadecenoic acid by using the whole cells of *Stenophomonas nitireducens* in a bioreactor.

The higher productivity for HA conversion has already been reported (*17*) for the washed cells of *L. acidophilus* when preincubated with LA (0.05 wt %/vol) versus those cells that are obtained by cultivation without LA. With such findings in mind, the effect of the preincubation time on the production of HA was investigated in the present study by varying the incubation time from 8 to 32 h and the LA concentration from 0.005 to 0.05% at 30 °C. As shown in **Figure 1B**, the highest conversion was obtained after 24 h of incubation in broth supplemented with 0.03% LA. It was found that the addition of a greater amount of LA exerted an inhibitory effect on HA production, as less HA was produced in the presence of 0.05% LA than 0.03% LA, which is in agreement with the studies of the antimicrobial effect of free LA on propionic acid bacteria as reported by Kishino et al. (*17*) and Jiang et al. (*29*).

Identification of HA. ^{1}H NMR and ^{1}H−^{1}H COSY analyses were carried out to identify the positions and geometric configurations of double bonds in HA. The ^{1}H NMR spectrrum of HA is shown in **Figure 2**. The signal at 3.5 ppm suggested the existence

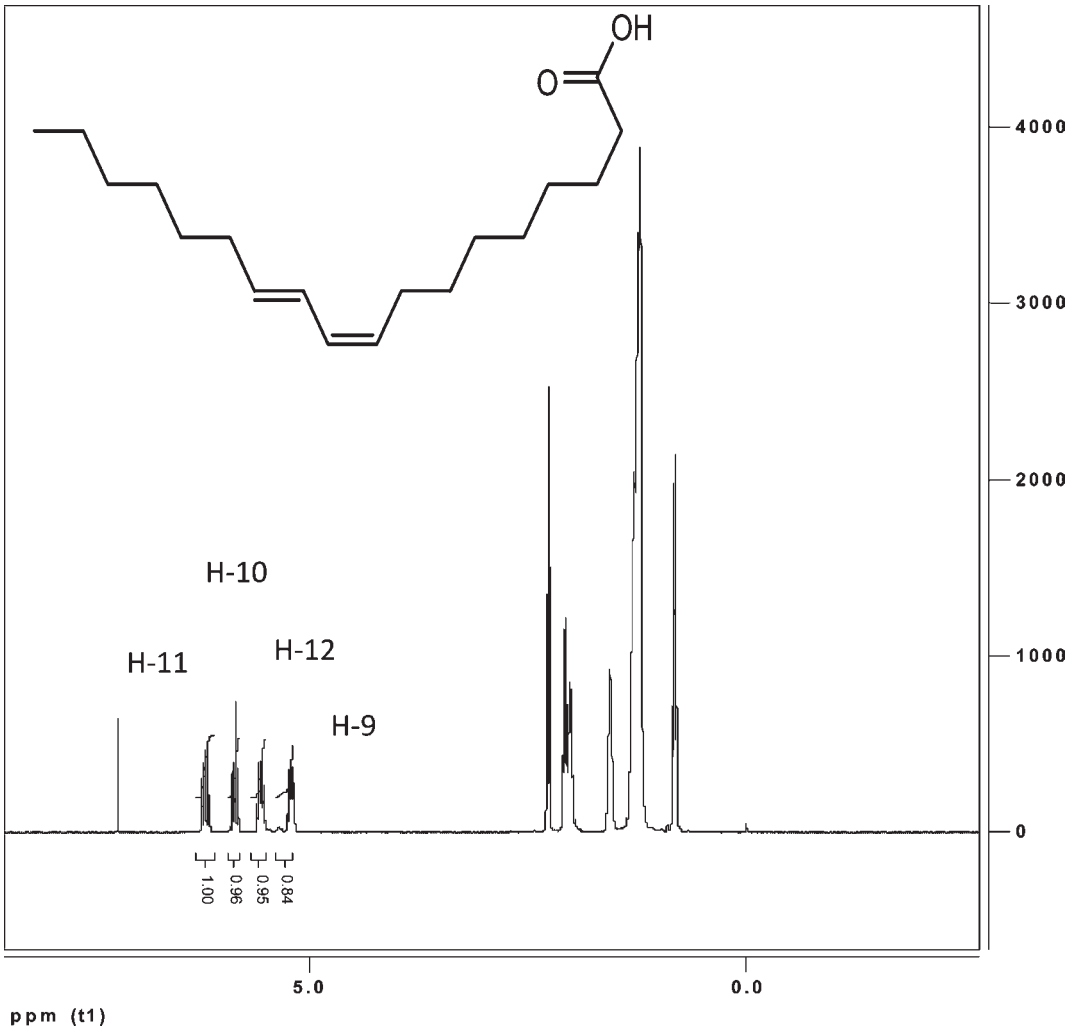


Figure 5. ^{1}H NMR spectrum and structure of *cis*-9,*trans*-11-octadecadienoic acid (CLA) produced from 10-hydroxy-*cis*-12-octadecenoic acid (HA) by microwave irradiation under optimized conditions.

of a hydroxyl group, and the signals H-12 (5.4 ppm, m, 1H) and H-13 (5.5 ppm, m, 1H) were identified as the protons on the double bond. The ^{1}H−^{1}H COSY spectrum showed that there is one methylene group between the double bond and the carbon bonding to the hydroxyl group (**Figure 3**). Therefore, a double bond was thought to be located at the Δ12-position. The coupling constants between H-12 and H-13 were calculated as 10.90 Hz, as described for AB spin proton coupling (*30*), in turn indicating that the double bonds are in the cis configuration. From these results, HA was identified as 10- hydroxy-*cis*-12-octadecenoic acid.

Conversion of 10-Hydroxy-*cis*-12-octadecenoic Acid into CLA. The transformation of LA to CLA is not the one-step isomerization of a nonconjugated diene to a conjugated diene. The transformation rather involves the production of hydroxy fatty acids, that is, 10-hydroxy-*trans*-12-octadecaenoic acid and 10-hydroxy-*cis*-12-octadecaenoic acid (*17*). Such a conversion of CLA was reported within the past decade by using several bacteria, including *Bifibobacteria*, *Lactobacillus*, and *Propionibacterium* (*31*). However, the potential factors being conducted for the production of CLA highlighted two critical variables to be tested. First, it was considered that the growth stage might be a determinant factor. A second potential factor affecting CLA production was considered to be oxygen availability, in which these factors markedly affect CLA production (*32*). Therefore, the present work aimed to use a simple, clean, and convenient microwave procedure for CLA production. In the current study, 10-hydroxy-*cis*-12-octadecenoic acid was treated in catalytic quantities of iodine under microwave irradiation (MW) on silica gel. The MW irradiation in 60 min results in the production of CLA in 43% yield. First, we investigated the effect of the reaction conditions (**Table 1**) on the transformation of 10-hydroxy-*cis*-12-octadecenoic acid that was induced by various amounts of iodine. We found that 1% of iodine was insufficient to induce dehydration (entries 1, 3, 5, and 8), whereas the solvent importantly influenced the elimination process when 5% of iodine was used. After 60 min in CH_3CN or CH_3COOH, maximum conversion occurred (entries 2 and 4). Transformation in $CHCl_3$ was observed within 120 min, whereas a mild temperature of 30 °C was found to be sufficient for maximum conversion with CH_3COOH.

Finally, we performed the reaction under solvent-free conditions, and in a typical experiment (**Scheme 1**), 10-hydroxy-*cis*-12-octadecenoic acid (1 mmol) and various amounts of iodine (1 and 5%) were left for different times at 50 °C. In the presence of 1% iodine, no transformation took place after 60 min, whereas a maximum yield of CLA (40 mg/g) was achieved when 5% iodine was used (entry 9). Moreover, no CLA product formation was observed when the samples were conventionally heated in the presence of iodine (1 and 5%) in CH_3CN, CH_3COOH, and $CHCl_3$ for 60 min. The acceleration of reactions by microwave exposure results from material-wave interactions leading to thermal effects and specific (nonpurely thermal) effects. Clearly, the combination of these two contributions could be responsible

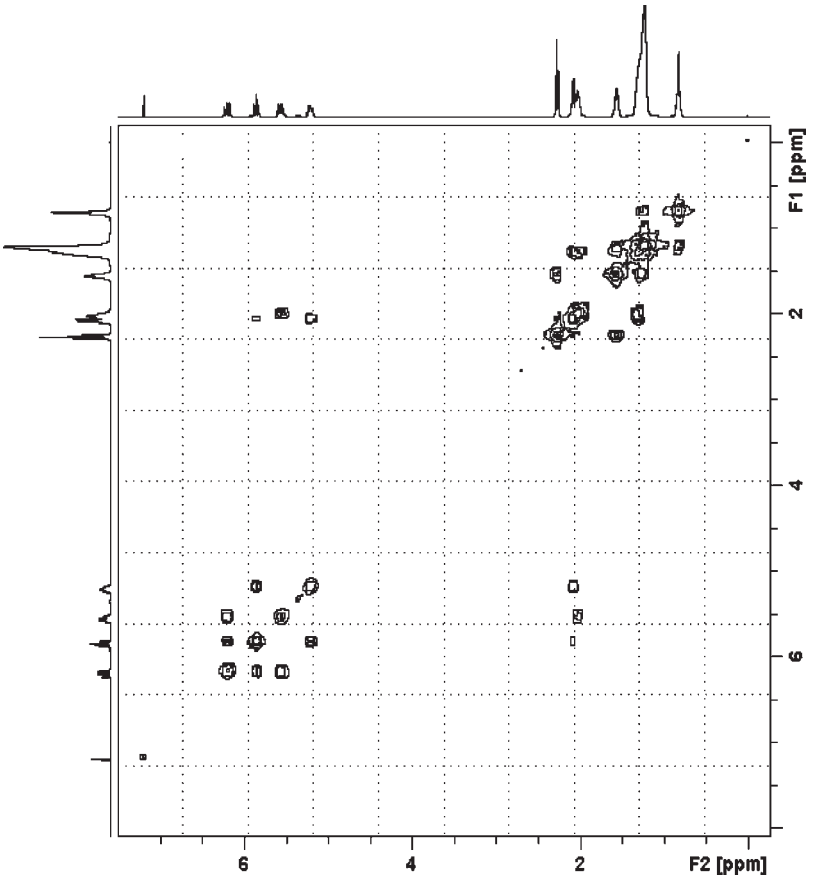


Figure 6. $^1H-^1H$ COSY spectrum of *cis*-9,*trans*-11-octadecadienoic acid (CLA) produced from 10-hydroxy-*cis*-12-octadecenoic acid (HA) by microwave irradiation under optimized conditions.

for the observed effects. It has been shown that higher boiling points could be observed when polar solvents are submitted to microwave irradiation conditions when compared with conventional heating, in which this effect is called "superheating". In the case of solid-state reactions in MW irradiation, atomic level excitation has been reported, which results in the generation of surface defects in solid particles. The generation of these surface defects enables increased ion motion and thereby helps in the easy excitation of these ions to higher energy levels (*33*).

The present MW method is faster compared to the earlier reported methods involving microorganisms, photoirradiation, or chemical isomerization techniques for CLA production (*13*, *15*, *28*). As is evident from the literature, the use of microwaves offers improvements, such as a reduced time of reaction, a cleaner product due to fewer side reactions, and the use of minimal quantities of solvent. Thus, microwave-assisted synthesis is more economical and environmentally friendly (*34*).

Recently, Staver et al. (*35*) reported iodine as an efficient catalysis for the transformation of alcohols into corresponding alkenes by undergoing a dehydration reaction under solvent-free conditions. Furthermore, iodine has long been recognized as one of the extremely effective catalysts for the cis−trans isomerization of olefins. It has been suggested that the isomerization process itself involves rotation around the σ-bond, after the reaction of $I^{\bullet}$ with the π-bond. According to this mechanism, the rate-determining step is either the formation of the carbon radical or the rotation around the σ-carbon−carbon bond (*36*).

Identification of CLA. The mass spectra of the isolated methyl ester of CLA lead to the formation of highly abundant $[M + H]^+$ ions (m/z 295, 100%) followed by the main fragment of $[M - 32]^+$ m/z 263 due to methanol loss (**Figure 4A**). The gap of 18 mass units between the ions from m/z 263 to 245 shows the loss of a water molecule. After the removal of the water molecules, a series for methylene-interrupted dienes was detected (m/z 243, 229, 215, 201, 183, and 165). The LC diode array chromatogram (**Figure 4B**) equipped with two Chromsphere Lipid 5 columns in series showed a single peak at a wavelength of 233 nm, which is specific for diene determination. Moreover, ^1H NMR analysis was carried out to identify the geometric configurations of CLA (**Figure 5**). The deduced structure was further confirmed by $^1H-^1H$ COSY analysis (**Figure 6**). The signals for H-9 (5.20 ppm), H-12 (5.57 ppm), H-10 (5.86 ppm), and H- 11 (6.20 ppm) suggested the existence of double bonds. Other signals were identified as shown in **Figure 4**. NMR δ_H ($CDCl_3$) 6.20 ppm (1H, dd, J = 15.03, 10.97 Hz, =CH—CH=), 5.86 (1H, t, J = 10.90 Hz, =CH—CH=), 5.57 (1H, dt, J = 14.79, 7.29, −CH=CH−), 5.20 (1H, dt, J = 10.87, 6.98 z, −CH=CH−), 2.30 (2H, t, J = 7.50 Hz, $-COCH_3$), 2.13 (2H, dt, J = 7.71, 6.89 Hz, $-CH_2$—CH=), 2.08 (2H, dt, J = 7.71, 6.73 Hz, =CH—CH_2−), 1.62 (2H, m, $-CH_2CH_2CH_2-$), 1.39 (4H, m, $-CH_2CH_2CH_2-$), 1.30 (12H, m, $-CH_2CH_2CH_2-$), 0.90 (3H, t, J = 6.99 Hz, $-CH_3$). These ^1H spin couplings are in agreement with the literature (*37*).

The spin−spin coupling constant between H-9 and H-10 was 10.9 Hz, which suggests that the double bond between H-9 and H-10 is in the cis configuration. The coupling constant between H-11 and H-12 was 15.00 Hz, which indicates the trans configuration. These results show that CLA is a cis/trans-conjugated octadecadienoic acid. The IR analysis of CLA also showed characteristic peaks at 990 and 945 cm^{-1}, which suggested that CLA is a cis/trans isomer and, from these results, CLA was identified as *cis*-9, *trans*-11-octadecadienoic acid.

In conclusion, a highly selective, fast, economical, and environmentally friendly chemoenzymatic method for the preparation of *cis*-9,*trans*-11 CLA is described via the simple whole cell incubation of *L. plantarum* of LA into 10-hydroxy-*cis*-12-octadecenoic followed by the microwave production of *cis*-9,*trans*-11 CLA in higher yields.

ACKNOWLEDGMENT

We are grateful to Dr. Abamüslüm Güven from Kafka's University in Kars, Turkey, for a generous donation of the *Lactobacillus plantarum* species.

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Received for review October 8, 2009. Revised manuscript received December 8, 2009. Accepted December 21, 2009. Financial support from The Science and Technology Research Council of Turkey (TUBITAK), The Turkish Academy of Sciences (TUBA), and the Higher Education Commission of Pakistan (HEC) is greatly acknowledged.