CLA Production from Ricinoleic Acid by Lactic Acid Bacteria

Akinori Ando, Jun Ogawa, Shigenobu Kishino, and Sakayu Shimizu*

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

ABSTRACT: The ability to produce CLA from ricinoleic acid is widely distributed in lactic acid bacteria. Washed cells of *Lactobacillus plantarum* JCM 1551 were selected as a potential catalyst for CLA production from ricinoleic acid. Cells cultivated in medium supplemented with a mixture of α-linolenic acid and linoleic acid showed enhanced CLA productivity. Under optimal reaction conditions, with the free acid form of ricinoleic acid as the substrate and washed cells of *L. plantarum* as the catalyst, 2.4 mg/mL CLA was produced from 3.4 mg/mL ricinoleic acid in 90 h, with a molar yield with respect to ricinoleic acid of 71%. The CLA produced, which was obtained in the FFA form, consisted of a mixture of two CLA isomers, *cis*-9,*trans*-11-octadecadienoic acid (21% of total CLA) and *trans*-9,*trans*-11-octadecadienoic acid (79% of total CLA), and accounted for 72% of the total FA obtained.

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KEY WORDS: CLA, castor oil, conjugated linoleic acid, lactic acid bacteria, *Lactobacillus plantarum,* ricinoleic acid.

CLA has attracted much attention as a novel type of biologically beneficial functional lipid. For example, CLA reduces carcinogenesis, atherosclerosis, and body fat (1–9). However, only a few CLA isomers, e.g., *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers, are reported to have such effects. Considering the expected future uses of CLA for medicinal and nutraceutical purposes, an isomer-selective and safe process for its production is required. Today, CLA is produced through chemical isomerization of linoleic acid, which results in by-production of unexpected isomers (10,11). The use of biological reactions for CLA production will solve such problems.

We screened microorganisms for reactions useful in CLA production and found that washed cells of several lactic acid bacterial species are good catalysts for CLA production from linoleic acid (12,13). They produced specific CLA isomers, i.e., *cis*-9,*trans*-11-octadecadienoic acid (CLA1) and *trans*-9,*trans*-11-octadecadienoic acid (CLA2) (14). Further analysis of the reaction using *Lactobacillus* spp. as model strains revealed the involvement of a hydroxy FA, 10-hydroxy-12-octadecenoic acid (HY), as an intermediate (12). Therefore, we investigated the potential of hydroxy FA as alternative substrates for CLA production and found that ricinoleic acid (12-hydroxy-*cis*-9-octadecenoic acid) is transformed to CLA by *L. plantarum* AKU 1009a (15).

*To whom correspondence should be addressed at Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan.
E-mail: sim@kais.kyoto-u.ac.jp

In this study, a wide range of lactic acid bacteria were screened for the ability to produce CLA from ricinoleic acid. Production of CLA from ricinoleic acid with a selected potential strain was also carried out on a preparative scale.

EXPERIMENTAL PROCEDURES

Chemicals. Standard samples of CLA1, CLA2, and HY were prepared as described previously (12). Ricinoleic acid and FA-free (<0.02%) BSA were purchased from Wako Pure Chemical (Osaka, Japan) and Sigma Chemical Co. (St. Louis, MO), respectively. All other chemicals used were of analytical grade and were commercially available.

Microorganisms, cultivation, and preparation of washed cells. Lactic acid bacteria preserved in our laboratory (AKU Culture Collection, Faculty of Agriculture, Kyoto University) and those obtained from other culture collections (IAM, Institute of Molecular and Cellular Bioscience, The University of Tokyo; IFO, Institute for Fermentation, Osaka; and JCM, Japan Collection of Microorganisms, Wako) were subjected to screening. For that purpose, strains were cultivated in MRS medium (12) supplemented with 0.06% α-linolenic acid or linoleic acid, or both. Each strain was inoculated into 15 mL of medium in screw-capped tubes (16.5 × 125 mm) and then incubated under O_2-limited conditions in the sealed tubes for 24–72 h at 28°C with shaking (120 strokes/min). For optimization of the reaction conditions and preparative CLA production, cultivation was carried out microaerobically with 550 mL of MRS medium containing 0.2% (wt/vol) of a mixture of α-linolenic acid and linoleic acid in the ratio of 1:5 (by wt) in 600-mL flasks for 24 h at 28°C with shaking (120 strokes/min). Cells were harvested by centrifugation (12,000 × *g*, 10 min), washed twice with 0.85% NaCl, and then used as washed cells for the reactions.

Reaction conditions. The standard reaction mixture (1 mL in a 16.5 × 125 mm test tube) contained 0.1 M potassium phosphate buffer (KPB, pH 6.5), 12.5% (wet cell wt/vol) washed cells, 0.08% (wt/vol) BSA, and 4.3 mg/mL ricinoleic acid. Ricinoleic acid (50 mg/mL) was mixed with BSA (1.0 % wt/vol) in 100 mM KPB (pH 6.5) before adding it to the reaction mixture in final concentrations of 4.3 mg/mL (ricinoleic acid) and 0.08% BSA (wt/vol), respectively. BSA is a lipid carrier that disperses the lipid in the reaction mixture. The amount of washed cells (12.5% wet cell wt/vol) corresponded to 1.78% dry cell (wt/vol). The reactions were carried out microaerobically under an O_2-adsorbed atmosphere in a sealed chamber with an O_2-absorbent (Anaeropack "Kenki," Mitsubishi Gas Chemical Co., Ltd., Tokyo, Japan), and gently shaken (120

strokes/min) at 37°C for 24–72 h. For optimization of the reaction conditions, the reactions were carried out essentially under the standard conditions as described above with variation of the targeted parameters, as described in the Results and Discussion section. All experiments were carried out in triplicate, and the averages of three separate experiments, which were reproducible within ±10%, are presented in the figures and tables, except for data in Table 1, in which the mean results of duplicate experiments are presented.

Lipid analyses. Lipids were extracted from the reaction mixtures with chloroform/methanol (1:2 vol/vol) according to the procedure of Bligh and Dyer (16), and transmethylated with 10% methanolic HCl at 50°C for 20 min. The resultant FAME were extracted with *n*-hexane and then analyzed by GC as described previously (12). Extraction and fractionation into lipid classes were carried out as described previously (17,18).

RESULTS AND DISCUSSION

Screening of lactic acid bacteria producing CLA from ricinoleic acid. Two hundred fifty strains of lactic acid bacteria were assayed for their ability to produce CLA from ricinoleic acid. They belonged to various genera, i.e., *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Propionibacterium*, *Bifidobacterium*, *Aquaspirillum*, *Enterococcus*, *Tetragenococcus*, *Aerococcus*, *Butyrivibrio*, *Lactococcus*, and *Weissella*. They were cultivated in MRS medium supplemented with α-linolenic acid or linoleic acid, or both. After harvesting and washing, the resultant cells were examined as the catalyst for CLA production from ricinoleic acid. The ability to produce CLA from ricinoleic acid was found for various kinds of lactic acid bacteria belonging to genera *Lactobacillus, Propionibacterium, Streptococcus, Leuconostoc,* and *Pediococcus*. All of them specifically produced two CLA isomers, CLA1 and CLA2. The strains that produced more than 100 μg/mL CLA (sum of CLA1 and CLA2) under the screening conditions are summarized in Table 1. The *L. plantarum* strains produced relatively high amounts of CLA from ricinoleic acid, especially when they were cultivated in the medium supplemented with α-linolenic acid. *Lactobacillus plantarum* JCM 1551 produced the highest amount of CLA and thus was selected for the following experiments.

Optimization of culture conditions for L. plantarum *JCM 1551.* The results of screening (Table 1) and preliminary investigations showed that the CLA-producing activity of washed cells of *L. plantarum* JCM 1551 increased when they were cultivated in medium supplemented with α-linolenic acid. However, expression of the activity was limited to the late log-phase only, i.e., not the other growth phases. This resulted in difficulty in the reproducible preparation of active cells. Therefore, a compound that enabled the strain to express the activity stably throughout the cultivation was sought. Ricinoleic acid and linoleic acid were examined. Linoleic acid made stable expression of the activity possible when it was added to the culture medium together with α-linolenic acid. The optimal ratio of α-linolenic acid/linoleic acid was found to be 1:5 by wt. The strain expressed the highest activity when it was cultivated in medium supplemented with 0.2% (wt/vol) of a mixture of α-linolenic acid and linoleic acid in the ratio of 1:5. Figure 1 shows the changes in CLA productivity of washed cells during cultivation under optimal conditions. The activity was highest in the middle logarithmic to early stationary phases and decreased there-

TABLE 1
Potential Strains for CLA Production from Ricinoleic Acid[a]

Additive in medium	Strain	Origin	FA (μg/mL reaction mixture)					
			Other FA	CLA1	CLA2	Total CLA	HY	RA
LA	*Lactobacillus acidophilus*	AKU 1137	275	65.4	216	281	—	436
	Streptococcus glycerinaceus	AKU 1006	313	32.8	72.9	106	158	1360
	Streptococcus liquefaciens	AKU 1009	236	33.9	76.2	110	13.8	492
ALA + LA	*Pediococcus homari*	AKU 1059	68.8	57.4	184	242	—	104
	Lactobacillus acidophilus	AKU 1122	165	16.2	147	163	53.4	89.6
	Lactobacillus arabinosis	AKU 1130	205	38.7	158	197	74.6	292
	Lactobacillus pentosus	AKU 1148	246	37.0	176	213	—	367
ALA	*Lactobacillus plantarum*	AKU 1138	168	37.6	195	233	11.5	457
	Leuconostoc mesenteroides	AKU 1101	95.7	44.9	92.9	138	—	191
	Lactobacillus plantarum	JCM 1551	347	156	979	1140	40.2	947
	Lactobacillus japonicus	IAM 10068	278	178	255	432	60.8	1260
	Lactobacillus plantarum	IAM 1216	306	170	235	405	38.4	1300
	Lactobacillus plantarum	IAM 12477	381	43.4	217	261	—	1800
	Lactobacillus plantarum	AKU 1009a	398	87.9	835	923	101	1230
	Lactobacillus plantarum	JCM 8345	179	99.4	565	664	61.6	12.0
	Lactobacillus plantarum	JCM 8348	305	32.6	249	282	35.0	1890

[a]Reactions were carried out for 24–72 h as described in the Experimental Procedures section. Other FA included myristic acid, palmitic acid, palmitoleic acid, oleic acid, vaccenic acid, and 2-hexylcyclopropanedecanoic acid (lactobacillic acid). LA, linoleic acid, ALA, α-linolenic acid; CLA1, *cis*-9,*trans*-11-octadecadienoic acid; CLA2, *trans*-9,*trans*-11-octadecadienoic acid; total CLA, sum of CLA1 and CLA2; HY, 10-hydroxy-12-octadecenoic acid; RA, ricinoleic acid; —, not detected.

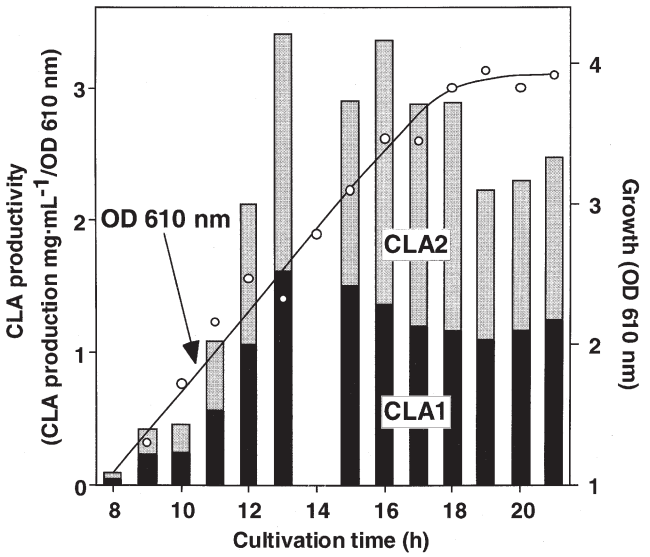

FIG. 1. Time courses of growth and CLA-producing activity of *Lactobacillus plantarum* JCM 1551. Cultivation was carried out in MRS medium containing 0.2% (wt/vol) of a mixture of α-linolenic acid and linoleic acid in the ratio of 1:5. Reactions were carried out with 4.3 mg/mL ricinoleic acid as the substrate for 24 h as described in the Experimental Procedures section. CLA1, *cis*-9,*trans*-11-octadecadienoic acid; CLA2, *trans*-9,*trans*-11-octadecadienoic acid. (○) Growth OD 610 nm; (■) CLA1; (▩) CLA2.

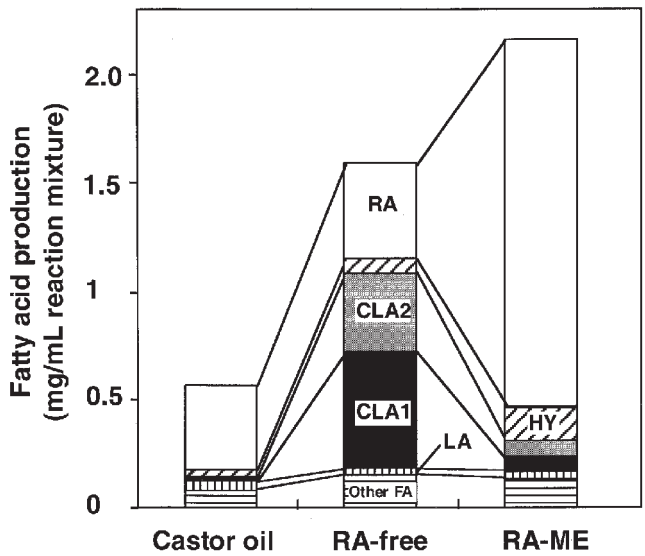

FIG. 2. Effect of substrate form on CLA production. Cultivation, reactions, and analysis were carried out under the conditions given in the Experimental Procedures section. The FA compositions were analyzed after transmethylation of lipids. RA-free, FFA form of ricinoleic acid; RA-ME, ricinoleic acid methyl ester; RA, ricinoleic acid; LA, linoleic acid; HY, 10-hydroxy-12-octadecenoic acid; other FA, see legend to Table 1. For other abbreviations see Figure 1.

after. Washed cells obtained at the late log-phase (16 h cultivation) were used for optimization of the reaction conditions.

Optimization of reaction conditions. The reaction conditions for CLA production from ricinoleic acid were investigated with washed cells of *L. plantarum* JCM 1551 as the catalyst.

(i) Effect of reaction pH. Reactions were carried out for 24 h in different buffer systems, i.e., 0.1, 0.5, or 1.0 M sodium citrate buffer (pH 4.0, 5.0, 6.0, and 6.5), KPB (pH 6.0, 6.5, 7.0, and 8.0), Tris/HCl buffer (pH 7.0, 8.0, and 9.0), or NH_4Cl/NH_4OH buffer (pH 9.0, and 10.0). CLA was most efficiently produced with 0.5 M sodium citrate buffer, pH 6.0.

(ii) Effect of reaction temperature. Reactions were carried out for 24 h at different temperatures in the range of 24–50°C. CLA production increased with increasing temperature from 20 to 42°C but decreased with higher temperature.

(iii) Effect of the substrate form. The free and methyl ester forms of ricinoleic acid and castor oil, in which the main FA component is ricinoleic acid, were examined as substrates (4.3 mg/mL) in the presence of BSA (0.8 mg/mL). Each substrate (50 mg/mL) was mixed with BSA (1.0% wt/vol) in 100 mM KPB (pH 6.5) before adding it to the reaction mixture in final concentrations of 4.3 mg/mL (substrate) and 0.08% BSA (wt/vol), respectively. After 24 h reaction, the free form of ricinoleic acid had been well converted to CLA, whereas neither the methyl ester nor castor oil was (Fig. 2). Ricinoleic acid methyl ester and castor oil might have remained unhydrolyzed, for once they were transformed to free ricinoleic acid they could act as substrate.

(iv) Effect of oxygen. Reactions were carried out under an O_2-adsorbed atmosphere in test tubes in a sealed chamber with an O_2-absorbent, or under air in open test tubes. The amounts of CLA produced and FA compositions of the lipids produced in 24-h reactions under both conditions are presented in Figure 3. Under aerobic conditions, both CLA production and the total FA amount obtained after the reaction decreased. These results were the same as those obtained for CLA production from linoleic acid with *L. acidophilus* AKU 1137 in our previous study (12). The presence of oxygen pro-

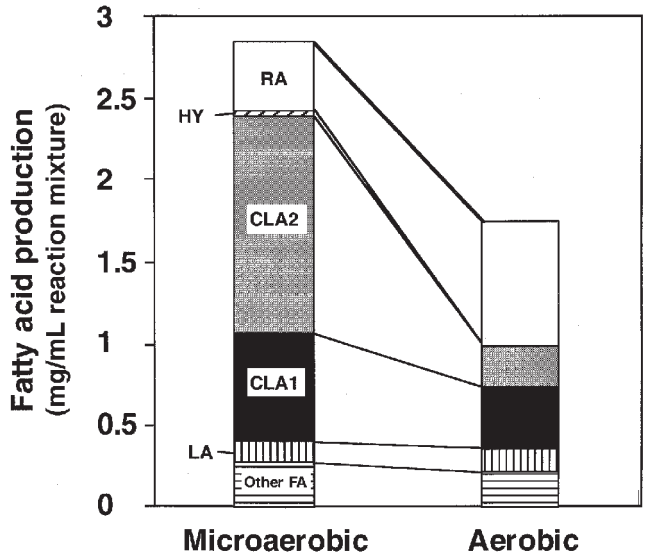

FIG. 3. Effect of oxygen on CLA production. Reactions were carried out for 24 h as described in the Experimental Procedures section under an O_2-adsorbed atmosphere or air. For other abbreviations see Figures 1 and 2.

moted FA metabolism, resulting in lower CLA production and a lower FA yield.

(v) Effects of concentrations of ricinoleic acid and washed cells. Reactions were carried out for 24 h with 20% (wet cell wt/vol) washed cells and different concentrations of ricinoleic acid in 1 mL reaction mixtures with a fixed ratio of ricinoleic acid/BSA, 5:1 (by wt). CLA production increased with increasing concentration of ricinoleic acid up to 3.4 mg/mL but decreased at higher ricinoleic acid concentrations (Fig. 4). Reactions were carried out for 24 h with 3.4 mg/mL ricinoleic acid and different amounts of washed cells in 1 mL reaction mixtures. CLA production increased with increasing amounts of washed cells up to 12% (wet cell wt/vol), which corresponded to 1.7% dry cells (wt/vol), but decreased with greater amounts of washed cells.

Time course of preparative CLA production from ricinoleic acid. The time course of CLA production from ricinoleic acid was monitored (Fig. 5). With 3.4 mg/mL ricinoleic acid as the substrate and 12% (wet cell wt/vol) washed cells as the catalyst, the production of CLA was maximal (2.4 mg/mL) at 48 h and was maintained thereafter. The amounts of CLA1 and CLA2 in a 48-h reaction were 0.8 and 1.6 mg/mL, respectively. The isomer compositions of the produced CLA are also presented in Figure 5. The proportions of CLA isomers changed with the reaction time. A longer reaction tended to increase CLA2 production.

Distribution and lipid classes of FA produced. The reaction mixture with 3.4 mg/mL ricinoleic acid as the substrate and 12% (wet cell wt/vol) washed cells as the catalyst was centrifuged after 90 h of reaction and separated into supernatant and cells. The distribution and lipid classes of the FA produced in both the supernatant and cells were determined (Table 2). Sixty percent of the FA were found in the cells (or associated with them), of which CLA was found to be the most abundant FA; the other 40% was in the supernatant. The unreacted ricinoleic acid was mainly found in the supernatant. Almost all the lipids were obtained as FFA forms.

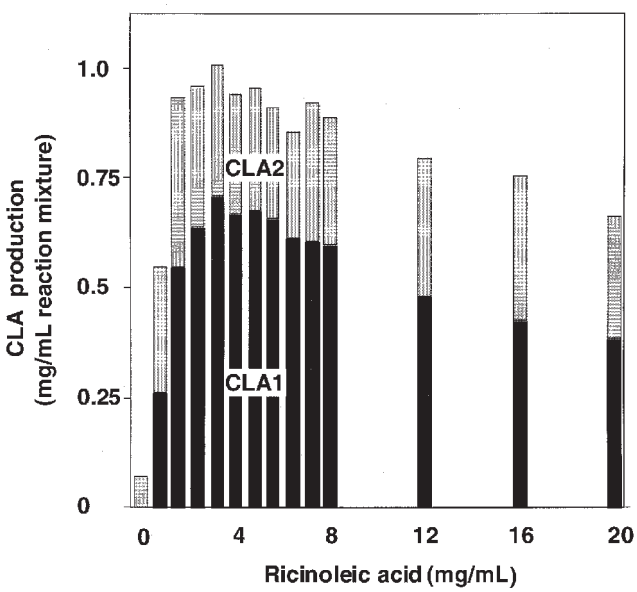

FIG. 4. Effect of substrate concentration on CLA production. Cultivation, reactions, and analysis were carried out under the conditions given in the Experimental Procedures section, except that the washed cell concentration was 20% (wet cell wt/vol) and the substrate (ricinoleic acid) concentration was changed in the range of 0.2 to 20 mg/mL. For abbreviations see Figure 1.

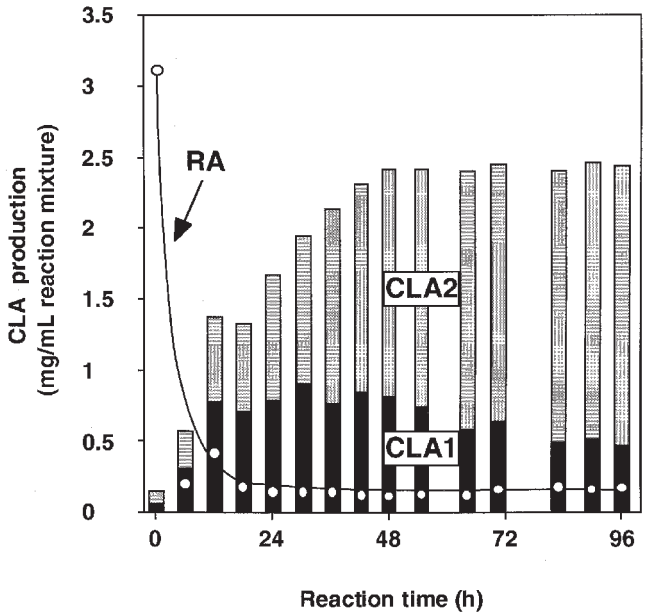

FIG. 5. Time course for preparative CLA production. The reaction was carried out with 3.4 mg/mL ricinoleic acid and 12% (wet cell wt/vol) washed cells as the substrate and catalyst, respectively, under the conditions given in the Experimental Procedures section. For abbreviations see Figures 1 and 2. (○) RA; (■) CLA1; (▩) CLA2.

Some lactic acid bacteria are well known to produce CLA from linoleic acid (13). Our previous work revealed that ricinoleic acid is an alternative substrate for CLA production by *L. plantarum* AKU 1009a (15). In the present work, lactic acid bacteria were screened for their ability to produce CLA from ricinoleic acid. Our results indicated that this catalytic activity is widely distributed in these bacteria. With *L. plantarum* JCM 1551 as a model strain, the characteristics of ricinoleic acid transformation to CLA by washed cells were studied. The activity increased when the strain was cultivated in a medium containing α-linolenic acid and linoleic acid. α-Linolenic acid and linoleic acid probably induced detoxification systems for PUFA in lactic acid bacteria (19). One of the detoxification systems is the partial hydrogenation of linoleic acid to octadecenoic acid involving CLA as an intermediate (19). We have already proved that a hydroxy FA, HY, is an intermediate in the production of CLA from linoleic acid by lactic acid bacteria (12). This suggests that other hydroxy FA might also be intermediates in PUFA detoxification. Ricinoleic acid is a hydroxy FA, and might be transformed as well as HY to CLA by partial reactions of these detoxification systems induced by α-linolenic acid and linoleic acid.

The CLA isomers produced from ricinoleic acid by lactic acid bacteria were CLA1 and CLA2. The ratio of these iso-

TABLE 2
Distribution and Lipid Classes of FA Produced from Ricinoleic Acid by Washed Cells of *L. plantarum* JCM 1551[a]

FA	FA concentration (mg/mL reaction mixture) after reaction	Distribution of FA in indicated lipid classes (mol%)						
		Supernatant			Cells			
		FFA	NL	PL	FFA	NL	PL	Total
Ricinoleic acid	0.60	13.5	—	—	7.3	0.3	—	21.1
CLA1	0.45	4.9	0.1	—	10.9	Trace	—	15.9
CLA2	1.40	15.3	0.5	—	33.6	0.1	—	49.5
HY	0.01	Trace	0.4	—	Trace	Trace	—	0.5
Linoleic acid	0.05	0.2	0.4	—	1.0	Trace	—	1.7
Other FA	0.32	3.3	0.8	—	7.0	0.1	—	11.3
Total	2.83	37.2	2.3	—	60.0	0.5	—	100

[a]*Lactobacillus plantarum* was cultivated in MRS medium containing 0.2% (wt/vol) of a mixture of α-linolenic acid and linoleic acid in the ratio of 1:5 for 16 h. The reaction was carried out with 3.4 mg/mL ricinoleic acid and 12% (wet cell wt/vol) washed cells as the substrate and catalyst, respectively, under the conditions given in the Experimental Procedures section. NL, nonpolar lipids; PL, polar lipids; for other abbreviations, see Table 1. —, not detected; Trace, <0.05 mol%.

mers was influenced by reaction conditions such as substrate concentration and reaction time (Figs. 4, 5). Lower substrate concentrations and shorter incubations resulted in higher proportions of CLA1, which is a bioactive isomer.

The present strain used only the free form of ricinoleic acid as a substrate for CLA. Ricinoleic acid is abundantly present in castor oil as TAG. Castor oil is an economical natural oil obtained from the seed of the castor plant. Castor oil has a long history of use as a laxative and, aside from these effects, it has been used apparently without harm. Recently, castor oil has been used as a supplement to reduce body weight. TAG is not a suitable substrate for CLA production with lactic acid bacteria. However, with the assistance of a lipase, the TAG can be hydrolyzed to produce FFA that in turn become available to the lactic acid bacteria for subsequent biotransformation (15).

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