Biotechnology and Bioprocess Engineering **2005**, 10: 357-361 ©KSBB

Bioconversion of Linoleic Acid to Conjugated Linoleic Acid by *Bifidobacterium breve*

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Abstract The bioconversion of linoleic acid (LA) to conjugated linoleic acid (CLA) was investigated to examine LA-adaptation of *Bifidobacterium breve* KCTC 3461 to additions of 1 to 5 mg/mL of LA overtime. To induce LA-adaptation, *B. breve* KCTC 3461 was treated with LA, according to three schemes. For LA-adapted *B. breve* the maximum concentration of CLA, 300~350 μg/mL, was obtained in cys-MRS medium containing 1 mg/mL of LA. The CLA production significantly increased with increasing LA concentration, from 1 to 4 mg/mL, but the conversion of LA to CLA gradually decreased. The CLA production capability of *B. breve*, and its tolerance, improved significantly with LA-adaptation. The addition of LA (1 mg/mL) into the culture broth after 24 h of cultivation in a 100-mL media bottle was most effective at promoting CLA production. In a 2.5-L stirred-tank bioreactor, the observed conversion and productivity of 56.6% and 35.4 $\mu gmL^{-1}h^{-1}$, respectively, by LA-adapted *B. breve* were approximately 6.6 and 9.8 times higher than those of LA-unadapted *B. breve*.

Keywords: *Bifidobacterium breve*, bioconversion, conjugated linoleic acid (CLA), linoleic acid (LA), linoleic acid-adaptation

INTRODUCTION

Bifidobacterium species are Gram-positive anaerobes, fermentative rods, often Y-shaped or clubbed at the ends, and asporogenous bacteria [1], which are recognized as constituting one of the major organisms in the colonic flora of breast-feed infants and healthy adults [2]. And *Bifidobacterium* species are known for their health promoting effects in both human and animal intestinal tracts [3].

Conjugated linoleic acid (CLA), the generic name of geometric and positional isomers of linoleic acid (LA; *cis*-9, *cis*-12 octadecadienoic acid), is receiving attention because of its many potential and beneficial biological effects [4]. In animal models, CLA has been shown to enhance the immune system and reduce body fat, and also has growth-promoting, anticarcinogenic and antiatherogenic activities [5].

CLA isomers are produced in the rumen during the microbial biohydrogenation of LA through an enzymatic conversion mechanism [6]. However, CLA cannot be produced by the human body, although low concentrations of CLA are found in human blood and tissues. Its presence may be accounted for *via* two possible pathways. Firstly, CLA may be produced *in vivo* from the free radical-mediated oxidation of LA; or secondly, CLA in human tissues may be derived from dietary sources, such as whole milk, dairy products and the meat of ruminants. Blood CLA levels are known to increase in humans receiving a CLA-rich diet [7,8].

Research on CLA, which is currently produced chemically, is actively directed towards its use as an additive in animal feed and as a food supplement. Free LA is known to inhibit the growth of CLA-producing bacteria [9], and it has been suggested that fatty acids exert their antimicrobial action at the cytoplasmic membrane [10]. Therefore, the anti-microbial activity of LA is one of the difficulties in developing large-scale CLA production processes [11].

The CLA production of microorganisms has been studied using anaerobic bacteria, such as *Butyrivibrio fibrisolvens*, *Clostridium* sp., *Bifidobacterium* sp., *Propionibacterium* sp., *Lactobacillus* sp., *Lactococcus* sp. and *Streptococcus* sp. [5,12-14]. However, the productivities and amounts of CLA produced remain low. Moreover, the mechanism of CLA formation has yet to be elucidated, but it has been suggested that conversion of free LA to CLA might function as a detoxification mechanism in bacteria [15].

The purposes of this study were to improve the conversion of LA to CLA, and thus, the productivity of CLA by LA-adapted *Bifidobacterium breve*.

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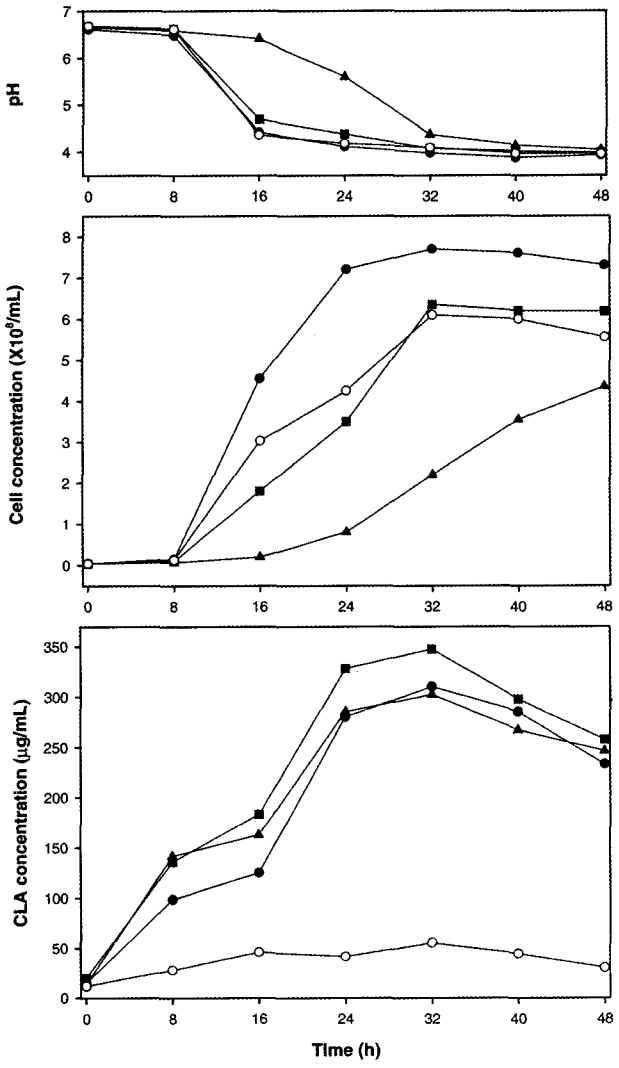

Fig. 1. The time course of the pH, and the cell and CLA concentrations of LA-unadapted and LA-adapted *B. breve* in cys-MRS medium containing 1 mg/mL LA. LA-unadapted *B. breve* KCTC 3461; ○, LA-adapted *B. breve* 1; ●, LA-adapted *B. breve* 2; ■, LA-adapted *B. breve* 3; ▲.

MATERIALS AND METHODS

Microorganism

Bifidobacterium breve KCTC 3461, purchased from the Korean Collection for Type Cultures (KCTC), used for CLA production, was subcultured on MRS agar plate (Difco, USA), containing 0.05% L-cysteine, at 37°C for 5 days in an anaerobic cabinet (AnaeroPack, Mitsubishi Gas Chemical Co. Inc., Japan).

Medium and Culture Conditions

B. breve was anaerobically grown in a 100-mL media bottle, containing 50 mL cys-MRS medium, at 37°C for 24 h, with shaking at 100 rpm. The composition of the cys-MRS medium was as follows: 1.0% tryptone, 1.0% beef extract, 0.5% yeast extract, 2.0% glucose, 0.05% L-cysteine, 0.1% Tween 80, 0.2% K_2HPO_4, 0.5% sodium acetate, 0.2% ammonium citrate, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.005% $MnSO_4 \cdot H_2O$ [16], but the pH was not adjusted. This seed culture (1.0%) was used as the inoculum for the main cultures, which were carried out in 100-mL media bottles containing 50 mL of individual test media. Linoleic acid (99%, Sigma, USA), as the substrate, was supplemented to the main cultures at different concentrations, with 2% (v/v) Tween 80 added as a surfactant. Inoculated media bottles were incubated in a rotary shaking incubator at 100 rpm for 3 days at 37°C. A 2.5-L stirred-tank bioreactor (Korea Fermentation Co. Ltd., Korea), with a working volume of 1 L, was used for CLA production. Cultivation in the bioreactor was carried out at 50 rpm for 3 days at 37°C. Nitrogen gas was used to remove oxygen during the culture medium preparation stage, but no nitrogen sparging was conducted during cultivation.

Linoleic Acid Adaptation

B. breve was adapted using three different schemes in cys-MRS medium containing LA. In scheme 1, *B. breve* was cultured with 50 mL cys-MRS medium containing LA (0.5 mg/mL) for 12 h. The culture broth was centrifuged at 3,000 × g for 10 min, and the cell pellets suspended in 5 mL of saline. This suspension (1.0 mL) was then inoculated into fresh cys-MRS medium, containing a higher concentration of LA, and cultivated for 12 h at 37°C. This cell transfer procedure was repeated five times, at 12 h intervals, until the concentration of LA in the cys-MRS medium had been increased to 5.0 mg/mL. Finally, the culture broth cultivated with cys-MRS medium containing 5.0 mg/mL LA was diluted and spread on cys-MRS medium. In scheme 2, *B. breve* was cultured with 50 mL cys-MRS medium for 6 h, which was then fed with LA (5.0 mg/mL) and culture for a further 2 days. In scheme 3, *B. breve* was cultivated for 4 days on a cys-MRS agar plate containing 5 mg/mL LA.

Analysis

Cell Concentrations

The culture broth was diluted and vigorously mixed by vortexing. The cell concentrations were measured using a hemocytometer under a light microscope, which was equipped with a CCD camera.

Extraction and Methylation of Fatty Acid

The culture broth (20 mL) was centrifuged at 3,000 × g for 10 min at room temperature. The supernatant (15 mL) was then vigorously shaken with organic solvents (30 mL *n*-hexane and 22 mL isopropanol) in a separating funnel, the upper layer collected, and then concentrated by evaporation to 3 mL [15]. The concentrated lipids were hydrolyzed with 2.0 mL of 1.0 M sodium hydroxide in methanol for 10 min in a water bath at 70°C [17].

Table 1. The effect of the linoleic acid concentration on the growth and CLA production of LA-adapted *B. breve* 2

Linoleic acid concentration (μg/mL)	Cell concentration ($\times 10^8$/mL)	CLA concentration (μg/mL)	Conversion (%)	Productivity ($\mu gmL^{-1}h^{-1}$)
0	6.4	0	0	0
1000	6.6	366	36.6	11.4
2000	6.8	427	21.4	13.4
3000	5.1	397	13.2	12.4
4000	4.5	690[a]	17.3	17.3
5000	3.0	577[a]	11.5	12.0

All results were obtained after cultivation for 32 h

[a] CLA concentration was obtained after cultivation for 40 h

Thereafter, the free fatty acids in the mixture were methylated by the addition of 2 mL 14% boron trifluoride in methanol in a water bath at 60°C for 10 min [18].

Gas Chromatography

The fatty acid methyl esters were analyzed by gas chromatography (DS 6200, Donam Instruments, Inc., Korea), fitted with a flame ionization detector and a split injection system (split ratio set at 1:50), using a HP-FFAP column (30 m × 0.25 mm, 0.25-μm film thickness, Hewlett-Packard Co., USA). The temperatures of the oven, injector and detector were 210, 250 and 270°C, respectively. The flow rate of the make-up gas was 30 mL/min, with helium as the carrier gas, at a flow rate of 1 mL/min [12]. The CLA was identified by comparing the relative retention times with those of standard CLA methyl esters (a mixture of *cis*-9, *trans*-11 and *cis*-10, *trans*-12-octadecadienoic acid methyl esters, Sigma, USA).

RESULTS AND DISCUSSION

Effect of LA-Adaptation on the Conversion of LA to CLA

To investigate the effect of LA-adaptation on CLA production, *B. breve* KCTC 3461 was adapted in cys-MRS medium, containing LA, using three schemes. The strains selected for each scheme were named LA-adapted *B. breve* 1, 2 or 3. Comparison of the growths and CLA productions of LA-unadapted and LA-adapted *B. breve* were carried out in cys-MRS medium containing 1 mg/mL LA, the time courses of which are shown in Fig. 1. Compared with LA-unadapted *B. breve,* the lag phase of LA-adapted *B. breve* 3 was maintained for longer, with a slower decrease in the pH. However, the LA-adapted *B. breve* 1 and 2, and the LA-unadapted *B. breve* reached the exponential phase at 8 h of cultivation. The cell concentration of LA-adapted *B. breve* 1 was highest, at 7.7×10^8 cells/mL. In all LA-adapted *B. breve,* the maximum CLA productions were obtained between the late exponential and early stationary phases. These results differed from those found by Alonso *et al.* [19] and Lin *et al.* [17], who reported most CLA was formed after the cultures reach the stationary phase. Of the three LA-adapted *B. breve,* LA-adapted *B. breve* 2 produced the highest level of CLA, 348 μg/mL, with a conversion and productivity of 34.8% and 10.9 $\mu gmL^{-1}h^{-1}$, respectively. These values were 6.3 and 4.7 times higher than those of LA-unadapted *B. breve.* Therefore, the LA-adapted *B. breve* 2 was used in further experiments.

From the above results, the production of CLA by LA-adapted *B. breve* was more efficient than that of LA-unadapted *B. breve.* Thus, the CLA production capability of *B. breve* was markedly improved by LA-adaptation.

Effect of LA Concentration

To determine the optimal concentration of LA, LA-adapted *B. breve* 2 was cultivated in cys-MRS medium containing various concentrations (1~5 mg/mL) of LA, the results of which are shown in Table 1. As the LA concentration was increased, the growth of LA-adapted *B. breve* 2 was retarded; that is, the lag phase and time required to reach the maximum cell concentration was extended. The concentration of CLA gradually increased with increasing LA concentration, from 1 to 4 mg/mL, but the conversion of LA to CLA decreased gradually. Jiang *et al.* [15] reported that the concentration of CLA produced by *Propionibacterium* sp. (PFF) decreased significantly when the LA concentration was increased to 1 mg/mL LA. Lin *et al.* [17] also reported that increasing the LA concentration, from 1 to 5 mg/mL, produced no significant increase in the CLA productions of six lactic cultures. This strong inhibitory effect of LA on the CLA production originates from the antimicrobial effect of LA, as reported by Boyaval *et al.* [10] and Jiang *et al.* [15]. In the present study, although the conversion decreased with increasing LA concentration, the CLA produced at 5 mg/mL LA was approximately 58% higher than that produced at 1 mg/mL LA. This result suggests that the tolerance of *B. breve* to LA was improved by LA-adaptation. The highest conversion (36.6%) and productivity (17.3 $\mu gmL^{-1}h^{-1}$) were obtained at 1 and 4 mg/mL LA, respectively. Therefore, the optimal LA concentration for CLA production was determined to be 1 mg/mL, because at this level the conversion was highest, with no growth inhibition observed.

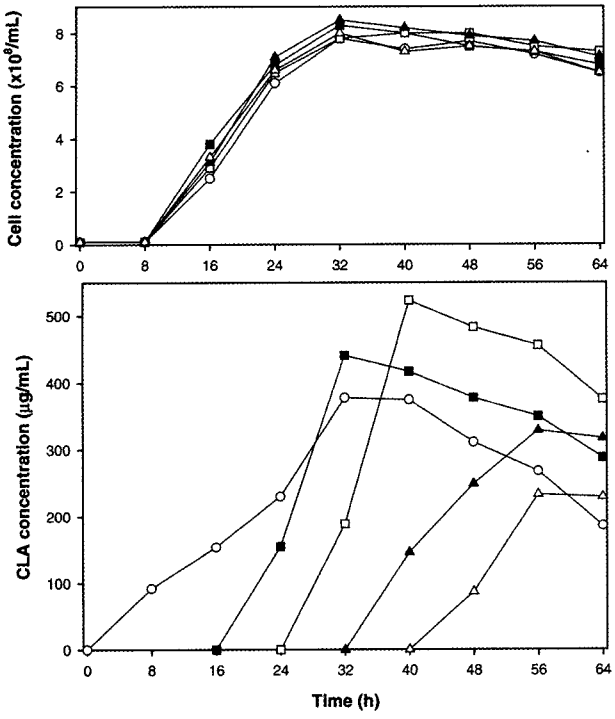

Fig. 2. The effect of the addition time of LA on the growth and CLA production of LA-adapted *B. breve* 2. Addition time of LA (1 mg/mL): 0 h; ○, 16 h; ■, 24 h; □, 32 h; ▲, 40 h; △.

Effect of LA Addition Time in cys-MRS Medium

To increase the conversion of LA to CLA, 1 mg/mL LA was added to culture broths, containing cys-MRS medium, after cultivation for 0, 16, 24, 32 or 40 h, the results of which are shown in Fig. 2. Although LA was added at different culture times, the growth pattern and cell concentrations (7.8~8.5 × 10^8 cells/mL) in all cultures showed similar trends. The production of CLA was highly increased when LA was added during the exponential phase (16 and 24 h) compared with that of control (0 h). These results were similar to those found by Lee *et al.* [11], for the production of CLA using washed cells of *Lactobacillus reuteri* prepared at various cell growth phases. The highest CLA concentration (523 μg/mL) and conversion (52.3%) were obtained when LA was added after 24 h of cultivation, which were approximately 40% higher than those of the control. After 24 h, the CLA productivity was 32.7 $\mu gmL^{-1}h^{-1}$, which was higher than that previously reported for *B. breve* [5,13]. The above results show that proper activation of LA-adapted *B. breve* 2 is essential for the conversion of LA to CLA.

Production of CLA in a 2.5-L Stirred-tank Bioreactor

The lag phases of LA-adapted *B. breve* 2 and LA-unadapted *B. breve* were not observed in a 2.5-L stirred-tank bioreactor (Fig. 3). The growth of LA-unadapted *B. breve* was a little faster than that of LA-adapted *B. breve* 2. The cell concentration of the culture broth was maintained at about 9.0 × 10^8 cells/mL during the stationary phase and the pH rapidly decreased, from 6.5 to 4.3~4.4, between 0 and 8 h after inoculation. The CLA production by LA-adapted *B. breve* 2 rapidly increased after the addition of LA. The maximum CLA concentration (566 μg/mL), conversion (56.6%) and productivity (35.4 $\mu gmL^{-1}h^{-1}$) by LA-adapted *B. breve* 2 were approximately 6.6, 6.6 and 9.8 times greater, respectively, than those of LA-unadapted *B. breve*.

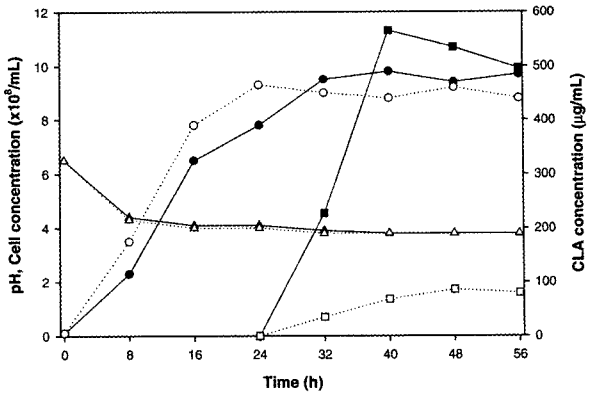

Fig. 3. The time course of the pH, growth and CLA production of LA-adapted *B. breve* 2 (closed) and LA-unadapted *B. breve* KCTC3481 (open) in a 2.5-L stirred-tank bioreactor. LA (1 mg/mL) was added to the bioreactor after 24 h of cultivation. pH; △, cell concentration; ○, CLA concentration; □.

CONCLUSION

The CLA production capability of *B. breve* and its tolerance to LA were significantly improved by LA-adaptation. The conversion of LA to CLA was dependent on the culture time prior to LA addition, and was best with addition during the exponential phase. The highest productivity and conversion were obtained by the addition of LA after 24 h of culturing. This study shows that LA-adaptation and the timing of LA addition are useful in enhancing the productivity of CLA and the conversion of LA to CLA.

Acknowledgements This study was supported by research grants from the Korea Science and Engineering Foundation (KOSEF) through the Applied Rheology Center (ARC), an official KOSEF-created engineering research center (ERC) at Korea University, Seoul, Korea.

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[Received April 13, 2005; accepted August 11, 2005]