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Conjugated linoleic acid production by immobilized cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus*

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Abstract

Lactobacillus delbrueckii ssp. *bulgaricus* (CCRC14009) and *L. acidophilus* (CCRC14079), immobilized with chitosan and polyacrylamide, were tested for CLA production. A 10-ml aliquot of *L. delbrueckii* ssp. *bulgaricus* cell suspension (3.59×10^7 CFU/ml) was adsorbed to 0.5 g chitosan and polyacrylamide, mixed with 0.2 ml linoleic acid (0.9 g/ml), and incubated at 37 °C for 24 h at pH 5, 6, 7, and 8 for CLA production. CLA levels, produced by immobilized cells of *L. delbrueckii* ssp. *bulgaricus* and *L. acidophilus* with increasing cell counts to 1.08 and 1.28×10^{10} CFU/ml, respectively, at optimal reaction pHs were evaluated. More CLA was formed at pH 8 of chitosan and pH 7 of polyacrylamide-immobilized *L. delbrueckii* ssp. *bulgaricus* cell treatments. Increase in cell count resulted in higher CLA production. The adsorption of *L. delbrueckii* ssp. *bulgaricus* cells onto polyacrylamide at pH 7 showed significant improvement in total CLA level. Results demonstrated a potential for enhancing CLA production through immobilization.

Keywords: Conjugated linoleic acid; Lactic acid bacteria; Polyacrylamide; Chitosan; Immobilization

1. Introduction

Conjugated linoleic acid (CLA) comprises a mixture of positional and geometric isomers of octadecadienoic fatty acid with conjugated double bonds. These conjugated dienes were found to be responsible for many biological properties that relate to health (Hayek et al., 1999; Park et al., 1999; Houseknecht et al., 1998; Nicolosi, Rogers, Kritchevsky, Scimeca, & Huth, 1997; Decker, 1995).

CLAs occur naturally in a variety of foods, including meat, poultry, seafood, cheese, butter, milk and vegetable oils (Ip, 1994). Ruminant fats are the richest natural sources of CLA among those products (Chin, Liu, Storkson, Ha, & Pariza, 1992; Shantha, Crum, & Decker, 1994). The high CLA levels in ruminant depot fat originate partly from ruminal bacteria (Shorland, Weenink, & Johns, 1995), due to the presence of linoleic acid isomerase, which converts linoleic acid into CLA (Chin, Storkson, Liu, Albright, & Pariza, 1994; Yang and Pariza, 1995). The presence of linoleic acid isomerase activity was also observed in several strains of propionibacteria (Jiang, Bjröck, & Fondön, 1998) and lactic bacteria (Lin, Lin, & Wang, 2002).

Immobilization methods have been used for a considerable time and have become significantly interesting in the area of biotechnology over the past few years. There are five principal methods of immobilization of microbial cells: adsorption, covalent binding, entrapment, encapsulation and crosslinking

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(Bickerstaff, 1997). Among the advantages are eliminations of enzyme purification and extraction, higher yields of enzyme activity, lower effective enzyme cost, lower susceptibility to contamination by undesirable microorganisms (Champagne, Girard, & Gardner, 1989), maintenance of stable and active cells for extended periods (Scott, 1987), reuse of biocatalysts (Hemachander, Bose, & Puvanakrishnan, 2001), shorter fermentation time (Sodini, Lagace, Lacroix, & Corrieu, 1998; Sodini-Gallot, Corrieu, Boquien, & Lacroix, 1995), and higher reproducibility of microbiological composition in cheese making (Sodini et al., 1998).

The applications of immobilization of lactic acid bacteria in dairy fermentations have been studied intensively, and include the production of lactic acid (Norton, Lacroix, & Vuillemard, 1994), propionic acid, diacetyl, and concentrated starters (Champagne, Girard, & Rodrigue, 1993), increase in milk quality and production tests for cream (Champagne & Baillargeon-Cote, 1987), yogurt (Prévost & Diviès, 1988), and fresh cheese (Sodini-Gallot et al., 1995). However, little information is available on CLA production with immobilized lactic cultures.

The CLA levels of various dairy products, determined by GC methods, were 0.55–9.12 mg CLA/g fat, as summarized by Lin and Lee (1997). CLA yields, using lactic cultures with linoleic acid addition, ranged from 1.26 to 2.10 mg in 20 ml of 0.1% LA-treated culture media after 24 h of incubation (Lin, Lin, & Lee, 1999). Such CLA levels are far below the suggested daily intake of 0.35–1 g/day (Fritsche & Steinhart, 1998). Since many advantages are observed when using immobilized bacterial cells, as mentioned previously, immobilization of lactic culture could improve CLA production. The aim of this study, therefore, was to compare two hydrophilic support materials, chitosan and polyacrylamide, for the immobilization of *Lactobacillus delbrueckii* ssp. *bulgaricus* and optimize pH condition for CLA production. The levels of CLA produced by immobilized cells of *L. delbrueckii* ssp. *bulgaricus* and *L. acidophilus* with increasing cell counts at optimal reaction pH's were also evaluated.

2. Materials and methods

2.1. Culture of cells

Lactobacillus delbrueckii ssp. *bulgaricus* (CCRC14009) and *L. acidophilus* (CCRC14079), obtained from the Culture Collection and Research Center (CCRC), Food Industrial Research Institute, Shin Chu, Taiwan, were subcultured twice under aerobic conditions at 37 °C for 24 h in MRS broth (Difco Lab., Detroit, Mich, USA). One percent of the subcultures were then inoculated into 1000 ml MRS broth (v/v) and incubated toward the end of the logarithmic phase at 37 °C.

2.2. Preparation of cell suspension

Following incubation, cells of *L. delbrueckii* ssp. *bulgaricus* were harvested by centrifugation (10000*g* for 10 min at 4 °C) (Parra, Casal, & Gomez, 2000), washed once with 30 ml of 0.85% sodium chloride at 4 °C, and suspended in 100 ml of each of the four 0.1 M buffer solutions: acetate buffer (pH 5), sodium phosphate buffer (pH 6 and 7), and Tris-HCl buffer (pH 8), making up the total cell count to 3.59×10^7 CFU/ml, determined by plating on MRS agar.

2.3. Cell immobilization and CLA producing reaction

Adsorption of cells of *L. delbrueckii* ssp. *bulgaricus* was performed using a modification of the method published by Itozawa and Kise (1995). A 10 ml aliquot of cell suspension at each pH was immobilized by adsorption on 0.5 g ground chitosan and polyacrylamide absorbent gel (Sigma Chemical Co., St. Louis, MO, 63178, USA), respectively, for 2.5 h at 4 °C in a 20 ml test tube. The virtually 100% cross-linked polyacrylamide purchased had an acrylamide polymerized with sodium acrylate solids content of 90.9% with minor amounts of residual acrylamide monomer of 281 ppm. The immobilized cells were then mixed with 0.2 ml linoleic acid (0.9 g/ml), 0.18 g bovine serum albumin, diluted to 60 ml with addition of the same pH buffer solution, giving a final concentration of 3 mg/ml linoleic acid in the reaction mixture, and were incubated at 37 °C for 24 h in an orbital shaker at speed 3 (Heidolph Titramax 1000, Germany) for CLA production.

2.4. Extraction and methylation for CLA analyses

Following incubation, the total volume (60 ml) of each of the three different immobilized mixtures at pH 5–8 was extracted with chloroform: methanol (2:1, v/v) and methylated with 14% BF_3-MeOH according to the method of Lin et al. (2002). The methylated sample was mixed with 2 ml hexane: water (1:1, v/v) and centrifuged at 2000*g* for 5 min at 4 °C, and the organic layer was concentrated to ~1 ml under a stream of nitrogen at room temperature for further quantification of CLA isomers by HPLC.

2.5. CLA quantification by HPLC

Instrumentation used for the analyses was as follows: A Jasco HPLC (Jasco Co., Tokyo, Japan) equipped with two ChromSpher 5 Lipids analytical silver-impregnated columns (4.6 mm i.d. × 250 mm stainless steel; 5 μm particle size; Chrompack, Bridgewater, NJ, USA)

in series (Sehat et al., 1999), a Jasco 870-UV detector operated at 233 nm, and a Jasco PU-980 pump. The mobile phase was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 ml min^{-1} (Sehat et al., 1999). The column head pressure was maintained at 48 atm at this flow rate. Whenever necessary, the column was restored by flushing with 1% acetonitrile in hexane for 2–4 h followed by 1–2 h with 0.1% acetonitrile in hexane. A Rheodyne 7725i injector (Rheodyne, L.P. Cotati, CA, USA) with a 50-μl injection loop was used and the injection volume was 10 μl. The results were analyzed by a SISC32 Chromatography Data Station (SISC, Taipei, Taiwan).

Eleven CLA methyl esters eluted between 15 and 30 min were identified by comparing the retention times with the methylated CLA standard (Sigma Chemical Co., St. Louis, MO, 63178, USA). After computing the amounts of all the standard CLA isomers using area %, the areas of the sample peaks were further calculated as μg CLA, using heptadecanoic acid as the internal standard. Total CLA was obtained by summing the levels of those isomers calculated.

2.6. Determination of calculated cell count and immobilization efficiency

A 10 ml aliquot of cell suspension of *L. delbrueckii* ssp. *bulgaricus* was immobilized with 0.5 g ground chitosan and polyacrylamide, respectively, at the pH of the highest CLA yield, diluted to 60 ml with addition of the same pH buffer solution, and shaken in an orbital shaker at speed 3 for 30 s. After filtering through a Whatman #1 filter paper, a 1 ml aliquot of the filtrate was serially diluted with peptone-water, and plate count was conducted in triplicate at each dilution on MRS agar. The calculated count of the cells retained in the support materials after immobilization was obtained by subtracting the cell count of the filtrate from the initial total cell count of 3.59×10^7 CFU/ml, and the immobilization efficiency, the ratio of cells immobilized (Leu, 1994), was calculated.

2.7. CLA production by two immobilized lactic cultures with higher cell counts at optimal pHs

In order to compare immobilized *L. delbrueckii* ssp. *bulgaricus* with *L. acidophilus* for further improving CLA production, cells of those two strains were cultured and cell suspensions were prepared at the optimal pH's, according to the procedures described previously, making up the total cell count to 1.08×10^{10} and 1.28×10^{10} CFU/ml, respectively. The obtained optimal reaction pH's of polyacrylamide and chitosan-immobilized *L. delbrueckii* ssp. *bulgaricus* cells and free cells for CLA production were pH 7, 8, and 7, respectively, as discussed in Section 3.

An aliquot of 10 ml of each cell suspension was immobilized with 0.5 g polyacrylamide at pH 7 and ground chitosan at pH 8, respectively, for 2.5 h at 4 °C in a 20 ml test tube, and was mixed with 0.2 ml linoleic acid (0.9 g/ml) for CLA production at each optimal pH and the same conditions as described previously. The reaction of CLA production by free cells was performed at pH 7.

Following the CLA-producing reaction, fatty acids were extracted from the reaction mixture and were methylated for CLA quantification by HPLC, according to the procedures described previously.

2.8. Statistical analysis

All data were subjected to general MANOVA and Duncan's multiple range test and critical ranges using STATISTICA (StatSoft, 1998) and a significance level of 0.05 was used. Each immobilization for CLA production was performed in three replications.

3. Results and discussion

3.1. CLA produced by polyacrylamide- and chitosan-immobilized cells of L. delbrueckii ssp. bulgaricus at pH 5, 6, 7, and 8

The highest total CLA level of 121 μg was produced ($P < 0.05$) by polyacrylamide-immobilized *L. delbrueckii* ssp. *bulgaricus* cells at pH 7, followed, in descending order, by 84.3 μg of chitosan immobilized cells at pH 8 and 29.4 μg of free cells at pH 7 (Table 1). Higher CLA production by polyacrylamide than chitosan was probably due to the better cell adsorption capacity of polyacrylamide (Brodelius, 1985) and the stronger interaction of this solid support (Itozawa & Kise, 1995) with linoleic acid. The finding corresponded to those observed by Itozawa and Kise (1995) who found a better catalytic activity of HLADH enzyme immobilized onto polyacrylamide than chitosan using a simple adsorption method. A similar result, regarding a higher lipase production by polyacrylamide-immobilized *Ralstonia*

Table 1
Total CLA production by polyacrylamide- and chitosan-immobilized cells and free cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* at pH 5, 6, 7, and 8

Immobilization	Total CLA level (μg)			
	pH 5	pH 6	pH 7	pH 8
Polyacrylamide	26.6^{a}	30.7^{a}	121^{b}	48.0^{a}
Chitosan	22.2^{a}	0.89^{b}	51.2^{c}	84.3^{d}
Free cells	23.6^{a}	19.4^{a}	29.4^{b}	1.75^{c}

^{abcd} Means in the same row followed by the same superscripts are not significantly different ($P > 0.05$).

pickettii cells, was also found by Hemachander et al. (2001). Polyacrylamide was non-ionic (Trevan, 1980) and the cell adsorption capacity and the activity of CLA formation might be less affected by the pH, which probably resulted in the same optimal reaction pH of 7 observed in both polyacrylamide and free cell treatments. The sharp increase in total CLA production in the treatments of polyacrylamide at pH 7 and chitosan at pH 8, compared with the free cell treatment, suggested that cell immobilization could be effectively used for improving CLA production.

3.2. Calculated cell count and immobilization efficiency

The calculated count of *L. delbrueckii* ssp. *bulgaricus* cells retained in the chitosan beads at pH 8 and the immobilization efficiency were 2.10×10^7 CFU/ml and 58.5%, respectively, whereas those of polyacrylamide at pH 7 were 2.77×10^7 CFU/ml and 77.20%, respectively. Both calculated cell count and immobilization efficiency were higher ($P < 0.05$) in polyacrylamide treatment at pH 7, compared with chitosan at pH 8, which corresponded to the larger total CLA yield observed in the polyacrylamide treatment. More cells being retained by the polyacrylamide was possibly due to the formation of stronger binding force between the cells and polyacrylamide than chitosan (Nomoto, 1999).

3.3. CLA produced by immobilized cells of L. delbrueckii ssp. bulgaricus with increasing cell counts at optimal pHs

A sharp increase in total CLA level to 2211 μg was observed ($P < 0.05$) in polyacrylamide immobilized *L. delbrueckii* ssp. *bulgaricus* cells at pH 7 when the immobilized cell count increased to 1.08×10^{10} CFU/ml, followed in descending order by chitosan immobilized cells (283.76 μg) at pH 8 and the free cell control (9.73 μg) at pH 7, as shown in Table 2. The result further substantiates the improvement of CLA production by immobilization of *L. delbrueckii* ssp. *bulgaricus* cells onto polyacrylamide. Eleven CLA isomers were detected in these treatments, and t9,t11-, and c9,t11-CLA were the major CLA isomers produced in the polyacrylamide treatment.

3.4. CLA produced by immobilized cells of L. acidophilus with increasing cell counts at optimal pHs

Similar results were observed in the immobilized *L. acidophilus* treatments. The highest total CLA level of 218 μg was produced ($P < 0.05$) by polyacrylamide-immobilized *L. acidophilus* cells at pH 7, followed in descending order by chitosan-immobilized cells (55.5 μg) at pH 8 and the free cell control (22.0 μg) at pH 7, as shown in Table 3. The result indicated the improvement of CLA production by immobilization of *L. acidophilus* onto polyacrylamide. C9,t11- and t9,t11-CLA were the major CLA isomers ($P < 0.05$) produced in *L. acidophilus* treatments, which was in accordance with the higher c9,t11- and t9,t11-CLA yields in the reaction of *L. acidophilus* cells with free linoleic acid observed by Kishino, Ogawa, Omura, Matsumura, and Shimizy (2002).

Table 2
CLA production by polyacrylamide- (pH 7) and chitosan- (pH 8) immobilized cells and free cells (pH 7) of *Lactobacillus delbrueckii* ssp. *bulgaricus*

CLA isomers	CLA level (μg)		
	Polyacrylamide	Chitosan	Free cells
t8,t10-	$181^{a,y}$	$72.0^{b,wx}$	$0.32^{c,y}$
t9,t11-	$583^{a,x}$	$91.90^{b,w}$	$2.60^{c,x}$
t10,t12-	$13.4^{a,k}$	$11.2^{a,z}$	$0.55^{b,y}$
t11,t13-	$12.8^{a,k}$	$1.16^{b,z}$	$0.74^{b,y}$
t8,c10-	$82.7^{a,z}$	$13.1^{b,z}$	$0.90^{c,y}$
c9,t11-	$1230^{a,w}$	$52.8^{b,xy}$	$3.20^{c,w}$
t10,12c-	$35.9^{a,k}$	$23.6^{b,yz}$	$0.59^{c,y}$
c11,t13-	$19.6^{a,k}$	$7.81^{b,z}$	$0.79^{c,y}$
c9,c11-	$15.6^{a,k}$	$1.95^{b,z}$	ND[a]
c10,c12-	$15.9^{a,k}$	$1.93^{b,z}$	ND
c11,c13-	$21.9^{a,k}$	$5.30^{b,z}$	ND
Total	2211^{a}	283^{b}	9.73^{c}

[abc] Means in the same row followed by the same superscripts are not significantly different ($P > 0.05$).
[wxyzk] Means in the same column followed by the same superscripts are not significantly different ($P > 0.05$).
[a] Not detected.

Table 3
CLA production by polyacrylamide- (pH 7) and chitosan- (pH 8) immobilized cells and free cells (pH 7) of *Lactobacillus acidophilus*

CLA isomers	CLA level (μg)		
	Polyacrylamide	Chitosan	Free cells
t8,t10-	$10.5^{a,y}$	$6.72^{ab,xy}$	$2.49^{b,y}$
t9,t11-	$40.6^{a,x}$	$11.9^{b,x}$	$3.58^{b,y}$
t10,t12-	$0.52^{a,z}$	$0.04^{b,z}$	$0.02^{b,z}$
t11,t13-	$0.49^{a,z}$	$0.08^{a,z}$	$0.79^{a,z}$
t8,c10-	$5.26^{a,y}$	$3.28^{ab,y}$	$2.15^{b,y}$
c9,t11-	$99.5^{a,w}$	$15.3^{b,w}$	$5.77^{b,xy}$
t10,c12-	$3.67^{a,y}$	$4.50^{a,y}$	$0.52^{b,z}$
c11,t13-	$0.17^{b,z}$	$0.68^{b,z}$	$6.63^{a,x}$
c9,c11-	$13.1^{a,y}$	$4.12^{b,y}$	ND[a]
c10,c12-	$24.6^{a,xy}$	$5.23^{b,y}$	ND
c11,c13-	$19.7^{a,y}$	$3.64^{b,y}$	ND
Total	218^{a}	55.5^{b}	22^{c}

[abc] Means in the same row followed by the same superscripts are not significantly different ($P > 0.05$).
[wxyz] Means in the same column followed by the same superscripts are not significantly different ($P > 0.05$).
[a] Not detected.

3.5. Comparison between L. delbrueckii ssp. bulgaricus and L. acidophilus

By comparing the support materials and lactic cultures regarding CLA yield, total CLA levels produced

by polyacrylamide-immobilized *L. delbrueckii* ssp. *bulgaricus* and *L. acidophilus* cells were significantly higher ($P < 0.05$) than those produced by chitosan immobilized cells. In addition, *L. delbrueckii* ssp. *bulgaricus*, immobilized by either polyacrylamide or chitosan, was significantly higher ($P < 0.05$) than *L. acidophilus* in total CLA yield. Since linoleic isomerase activity, detected in *L. acidophilus* (Lin et al., 2002), could be different between cultures, due to the differences in protein and ion compositions (Price & Stevens, 1989), larger CLA production by immobilized *L. delbrueckii* ssp. *bulgaricus* cells could be attributed to the higher enzyme activity in this culture strain. These results demonstrated that *L. delbrueckii* ssp. *bulgaricus* immobilized with polyacrylamide at pH 7, was most effective in promoting CLA formation.

4. Conclusions

Immobilization of *L. delbrueckii* ssp. *bulgaricus* and *L. acidophilus* cells improved CLA production. C9,t11- and t9,t11-CLA were the major CLA isomers produced by the immobilized cells. The adsorption of *L. delbrueckii* ssp. *bulgaricus* cells by polyacrylamide at pH 7 produced the highest level of CLA, and is suggested for CLA production.

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