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ORIGINAL ARTICLE

Optimal culture conditions for producing conjugated linoleic acid in skim-milk by co-culture of different *Lactobacillus* strains

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Abstract The ability of different *Lactobacillus* strains to produce conjugated linoleic acid (CLA) was determined. Three species—*Lactobacillus plantarum* (Lp), *Lactobacillus acidophilus* (La) and *Streptococcus thermophilus* (St)—were co-cultured in a medium containing skim-milk supplemented with hydrolyzed safflower oil. This study was aimed at future applications in dairy products. The optimal operation parameters were established by response surface methodology. More CLA was produced by co-culture than by single strain culture. The CLA produced by co-culture of La and Lp (La–Lp) was more than that produced by La and St (La–St). Maximum CLA production of 316.52 μg/mL was obtained with La–Lp co-culture using a substrate concentration of 5.0 %, inoculum size of 5.0 %, an initial medium pH of 6.4 and a temperature of 36.4 °C for 48 h. To our knowledge, this is the first report in the literature of the use of co-cultures of La–St and La–Lp to produce CLA.

Keywords Co-culture · *Lactobacillus* · Conjugated linoleic acid · Safflower oil

Introduction

Conjugated linoleic acid (CLA), which possesses a mixture of positional and geometric isomers of octadecadienoic acids (C18:2) with conjugated double bonds, has gained considerable attention due to its health benefits. Double bonds of CLA are found mainly at positions 9 and 11, or 10 and 12, while isomers with double bonds at other positions also have been reported. Among the isomers, the c9, t11-CLA is the predominant one in natural lipids, and constitutes 90 % of the total isomers (Oliveira et al. 2009). Based on studies of in vivo and in vitro models, CLA has been suggested to have potential beneficial effects on health, including anticarcinogenic (Belury 1995; Kimoto et al. 2001; Parodi 1996; Buccioni et al. 2010), antiatherosclerotic (Nicolosi et al. 1997), antidiabetogenic (Houseknecht et al. 1998), body mass enhancing (Park et al. 1999; West et al. 1998), antioxidative (Decker 1995), immunomodulative (Hayek et al. 1999), antibacterial (Sugano et al. 1997), cholesterol depressing (Huang et al. 1994), and growth-promoting (Chin et al. 1994; Oliveira et al. 2009) properties.

CLAs exist widely in many kinds of natural foods, such as meat, seafood, cheese, butter, poultry, milk and vegetable oil. Dairy products and beef in particular are the major dietary sources of CLA for humans (Chin et al. 1992; Fogerty et al. 1998). Ruminant fat is the richest natural source of CLA among muscle food. Many reports have indicated that CLA is formed as an intermediate during the bio-hydrogenation of linoleic acid by rumen microorganisms. This is the primary mechanism of CLA formation (Gangidi and Proctor 2004), which is also formed by endogenous conversion of vaccenic acid (VA; trans-11 C18:1) by Δ9-desaturase in mammary glands (Kim and Liu 2000; Nag et al. 2008). Many studies have reported that microorganisms have the ability to produce CLA from linoleic acid (Lin et al. 2002). Isomerase activity of linoleic acid was observed in several strains of ruminal bacteria (Shorland et al.

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1995), lactic acid bacteria (Coakley et al. 2003; Lin 2006) and propioni bacteria (Jiang et al. 1998). Biosynthesis of CLA using these bacterial strains results in the uniform composition of isomers under mild reaction conditions. In addition, the primary products contain desired biological activities, such as those from c9, t11-CLA and the 10 t, 12c-CLA isomers. Therefore, biosynthesis has received much attention (Christie et al. 1997; Alonso et al. 2003). Linoleic acid is abundant in plant oil, and safflower oil is an economical source of linoleic acid. About 70 % to 80 % of the total fatty acids in safflower oil is linoleic acid. However, CLA was produced from unhydrolyzed safflower oil in an unexpected low yield in one previous study (Yang et al. 2011). Most lactic acid bacteria are found to use only the free form of linoleic acid for CLA production, rather than its triacylglycerol form, which is found mainly in safflower oil.

Co-culture methods have been used for many years in the area of biotechnology for remediation. Co-culture utilizes synergetic effects and alternate functions of mixed bacteria. As a result, the production rate of co-culture is normally better than that of a single strain (Kropp et al. 1997; Boopathy 2000). *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Streptococcus thermophilus* are Gram-positive strains that are fermented under anoxic or anaerobic conditions. Also, they are used extensively in the production of milk and dairy food. It is well documented that they are able to synthesize CLA using linoleic acid under single culture conditions (Yang et al. 2011). However, there is little information about co-culture for CLA production. The objective of this study was to evaluate the feasibility of different *Lactobacillus* co-culture for production of CLA in skim-milk, and to study the effects on the production of CLA of incubation time, incubation temperature, inoculum size, initial medium pH, and of adding hydrolytic safflower oil to skim-milk.

Materials and methods

Microorganisms

Streptococcus thermophilus (St), *Lactobacillus acidophilus* (La) and *Lactobacillus plantarum* (Lp) were maintained in our laboratory.

Lactobacillus plantarum and *Lactobacillus acidophilus* were cultivated under aerobic conditions at 37 °C for 24 h in 12 % (w/v) skim-milk (SKM) towards the end of the logarithmic phase (10^8 CFU/mL). The detailed procedure was reported in Wang et al. (2007). *Streptococcus thermophilus* was cultivated at 42 °C and other conditions were the same as those for Lp and La cultures.

The co-culture was inoculated at 37 °C for 36 h in 12 % (w/v) SKM, with specific strain ratios of La:St=1:1 or La:Lp=1:4 v/v (Yang et al. 2011).

Culture conditions

The reaction mixture contained 10 mL SKM in a glass tube (18×180 mm) with 50 mg emulsion consisting of 5:1 safflower oil and Tween-80 added as substrates. For optimization of reaction conditions, the reactions were carried out essentially under the conditions described above with variation of target parameters. Independent variables and their levels for co-culture are given in Table 1.

The results of all experiments were the average of three separate determinations that were reproducible within ±10 %, in which the mean results of duplicated experiments were presented.

Hydrolysis of safflower oil

Safflower oil (150 g) and potassium hydroxide (35 g) were dissolved in 120 mL ethanol in a water bath at 80 °C for 1 h. The hydrolysis mixture was then adjusted to pH 2.0–3.0 using 4 M sulfuric acid, and then transferred to a separatory funnel. After phase separation, the top layer (organic phase) was collected and washed by water. To remove all aqueous components, anhydrous sodium sulfate was added and filtered out later. Free fatty acids were extracted at 50 °C for 1 h on a rotary evaporator (Xu et al. 2004).

Titratable acidity measurement

Lactic acid is the primary metabolite of lactic acid bacteria, so the growth rate of lactic acid bacteria is assessed according to the content of lactic acid in the culture/product mixture (Oliveira et al. 2009; do Espirito Santo et al. 2010).

Table 1 Independent variables and their levels for central composite design. *La Lactobacillus acidophilus*, *Lp Lactobacillus plantarum, St Streptococcus thermophilus*

Independent variable	Code	Variable level (La–St)			Variable level (La–Lp)		
		−1	0	+1	−1	0	+1
Incubation temperature (°C)	A	37	40	43	34	37	40
Substrate concentration (%)	B	4	5	6	4	5	6
Inoculum size (%)	C	2	3	4	4	5	6
The initial medium pH	D	4.5	5.5	6.5	5.5	6.5	7.5

Titratable acidity was determined by titrating a sample (10 mL sample and 20 mL distilled water) with 0.1 mol/L NaOH to an end point of pH 7.0 with phenolphthalein as a color indicator. Titratable acidity was calculated based on the assumption that lactic acid was the predominant acid produced in the system and was expressed as micrograms of lactic acid per 100 mL product.

Determination of CLA

Lipid extraction and analysis

Bacterial suspensions (1 mL) were mixed with 4 mL chloroform: methanol (2:1, v/v) and centrifuged at 10,000 rpm for 20 min at 4 °C. The organic phase was separated, and dehydrated over anhydrous sodium sulfate, then concentrated under vacuum at 30 °C. The sample was mixed with 10 mL hexane in a volumetric flask for further quantification (Wang et al. 2007).

Gas chromatography and UV spectrum analysis of CLA

Fatty acids methyl esters (FAMEs) were prepared by esterification according to method n. 15884 ISO (2002). FAMEs were separated on a Supelcowax-10 fused silica capillary column (Supelco, Bellefonte, PA) using a Hewlett Packard model HP5890 gas chromatograph equipped with a flame ionization detector and model HP3392 integrator. The conditions were as follows: helium flow, 2.4 mL/min; injector, 200 °C; detector, 250 °C; column chamber temperature, initially 40 °C (5 min) and then increased to 220 °C at 20 °C/min and held for 30 min.

According to a maximum absorption at 233 nm of the double bond of CLA under the UV light exposure, determination of CLA was carried out by the UV spectrum analysis method described by Rosson and Grund (2001). Absorbance was measured in 1 cm quartz cuvettes at room temperature. The sample was scanned from 200 nm to 300 nm and then the concentrations of CLA (μg/mL) in samples were calculated based on the standard curve of CLA UV absorbance at 233 nm.

Response surface methodology design

Response surface methodology (RSM) is an effective statistical technique for the investigation of complex processes that has been adapted successfully for food science research. It is a faster and cheaper method for gathering research results than the classical one-variable-at-a-time or full-factorial experiments (Wanasundara and Shahidii 1999). In this study, RSM was used to evaluate the effects on CLA production of several variables.

A four-factor central composite design (Box 1954; Cornell 1992) was employed to study the responses, namely the production of CLA. An initial screening step was carried out to select the major response factors and their values (Wang et al. 2010). The effects on CLA biosynthesis (dependent variable) of four independent variables X_1 (incubation temperature, °C), X_2 (substrate concentration,%), X_3 (inoculum size,%) and X_4 (initial medium pH) at three levels, were investigated using central composite design and RSM.

The model proposed for response (Y) was

$$Y = b_0 + \sum_{n=1}^{4} b_n X_n + \sum_{n=1}^{4} b_{nn} X_n^2 + \sum_{n\langle m}^{4} b_{nm} X_n X_m \qquad (1)$$

where b_0 was the value for the fixed response at the central point of the experiment, *and* b_n, b_m, b_{nn} and b_{nm} were the linear, quadratic and cross product coefficients, respectively. The central composite design is shown in Table 1.

In a contour plot, curves of equal response values were drawn on a plane whose coordinates represent the levels of the independent factors. Each contour represents a specific value for the height of the surface above the plane defined for combination of the levels of the factors. Therefore, different surface height values focus attention on the levels of the factors at which changes in the surface height occur (Wanasundara and Shahidii 1999).

Statistical analysis

Three replicates were measured for each sample during the above-mentioned assays. The data was subjected to analysis of variance (ANOVA) using RSM. Canonical analysis involved a mathematical approach, which was used to locate the stationary point of the response surface and to determine whether it represented a maximum, minimum or saddle point. Analysis was performed on the predicted quadratic polynomial models to examine the overall shape of the response surface curves and used to characterize the nature of the stationary points (Wanasundara and Shahidii 1999; Mason et al. 1989; Lee et al. 2003).

Results

Analysis of CLA

Figure 1 shows that the retention times observed in the gas chromatogram of FAMEs of fermented SKM in the medium were 31.24 min of c9 t11-CLA (Fig. 1b) and 31.66 min of t10, c12-CLA, respectively, compared to the gas chromatogram for fatty acids and conjugated linoleic acid isomer standards (Fig. 1a). Seventy percent of linoleic acids (w/w) were added to fermented SKM and the remaining 30 % were other kinds of fatty acids. The non-linoleic acids were

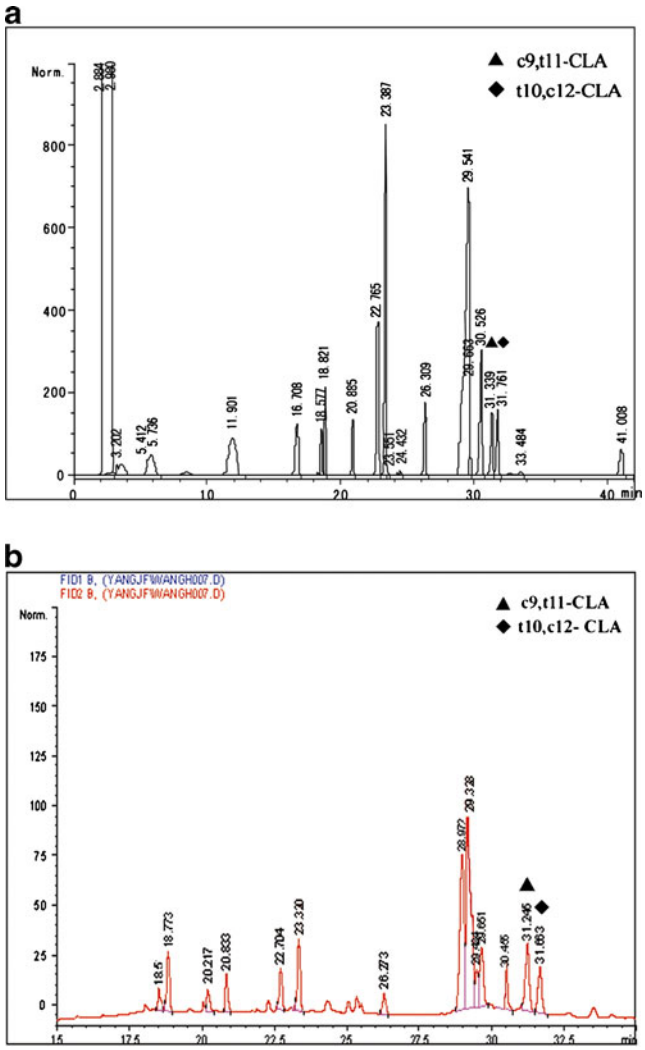

Fig. 1 Gas chromatograms of **a** fatty acids and conjugated linoleic acid (CLA) isomer standards and **b** the fatty acids of fermented skim-milk (SKM)

extracted and methylated, and this decreased the level of fatty acids produced.

The absorbance peak of conjugated double bonds is at 232–234 nm. A linear relationship between absorbance at 233 nm and standard CLA concentration was observed in the concentration range of 0–12 μg/mL. The formula from the standard curve of CLA UV absorbance at 233 nm at room temperature is as follows: $y=0.0791x+0.014$ ($R^2=0.999$). CLA concentration can be analyzed by many methods, such as gas–liquid chromatography (Aldai et al. 2006), silver-ion high-performance liquid chromatography (Muller et al. 2006), nuclear magnetic resonance (NMR), and gas chromatograph-mass spectrometer (GC-MS) (Rodríguez-Alcalá et al. 2011). The GC method is used more commonly than other methods; however, CLA needs to be methylated before analysis by GC. The disadvantage of methylation is that it may isomerize conjugated double bonds. Spectrophotometry has the advantages of avoiding methylation and low analytical cost. Thus, we chose GC as the method of verification, and UV spectrophotometry as the method used to analyze CLA in this study.

CLA production by different culture models

Production of CLA using single strain cultivations under optimized conditions is summarized in Table 2. The production of CLA by co-culture was more than those by single strain culture through the interaction between different strains of *Lactobacillus* (Fig. 2).

Effect of initial medium pH on titratable acidity and CLA production

pH is an important factor affecting the growth of microorganisms not only because it changes surface charges of

Table 2 Conjugated linoleic acid (CLA) production by various microorganisms using a single strain culture method

Microorganism	CLA production (μg/mL)	Reference
Propionibacterium freudenreichii ssp. *shermanii*	78.8	Wang et al. 2007
Bifidobacterium infantis	24.6	Coakley et al. 2003
Bifidobacterium dentium	160	
Bifidobacterium angulatum	1.2	
Lactobacillus acidophilus	131	Alonso et al. 2003
Lactobacillus casei	111	Lee et al. 2003
Lactobacillus reuteri	300	
Lactobacillus delbrueckii ssp. *bulgaricus* (CCRC14009)	209	Lin 2006
Streptreptococcus thermophilus	198.6	Van-Nieuwenhove et al. 2007
Bifidobacterium bifidum	90	
Lactactobacillus rhamnosus	190.2	

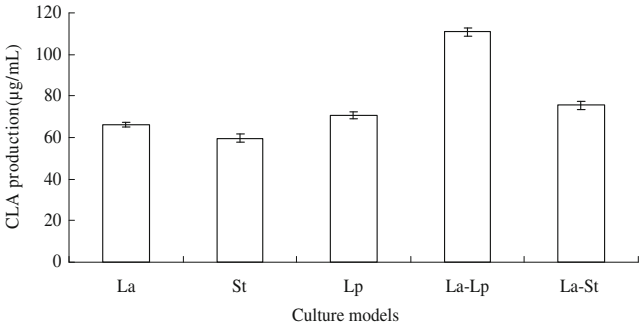

Fig. 2 Production of CLA in single strain and co-culture fermentation. Reactions were carried out at 37 °C for 36 h with an inoculum size of 3 % under aerobic conditions

microorganisms, but also because it can affect the ionization of organic compounds in the medium and alter the pathways of microorganisms absorbing nutrients. The effects of initial medium pH on titratable acidity in the medium and on CLA production are shown in Fig. 3a. CLA production by La–Lp increased from 54.17 μg/mL at pH 4.5 to 110.70 μg/mL at pH 6.5 in SKM and then decreased dramatically with further pH increase. Titratable acidity followed the same trend as CLA production, increasing from 83.30 °T at pH 4.5 to 123.33 °T at pH 6.5, and then reducing with further pH increase. While the peak of CLA production by La–St shifted to a lower pH of 5.5, specifically with an increase from 61.27 μg/mL at pH 4.5 to 95.57 μg/mL at pH 5.5 and then dropped at higher pH ranges, titratable acidity of La–St co-culture was similar to that of La–Lp, increasing from 83.01 °T at pH 4.5 to a peak of 110.30 °T at pH 6.5.

Effect of incubation temperature on titratable acidity and CLA production

Temperature is one of the most important factors affecting growth and survival of microorganisms. The effects of incubation temperature on titratable acidity and CLA production are presented in Fig. 3b. There was a significant elevation ($P<0.05$) in total amount of CLA formation by La–Lp from 71.30 μg/mL at 31 °C to 119.51 μg/mL at 37 °C, but production decreased at higher temperatures. Titratable acidity showed a similar tendency, increasing significantly from 78.83 °T at 31 °C to 111.67 °T at 37 °C. The best temperature for CLA production by La–St was 40 °C, with an increase from 63.21 μg/mL at 31 °C to 104.11 μg/mL at 40 °C; production then dropped to 60–70 μg/mL at 43 °C. Titratable acidity of La–St was similar to that of La–Lp, increasing from 83.31 °T at 31 °C to 116.72 °T at 40 °C.

Effect of incubation time on titratable acidity and CLA production

Another important factor for growth and survival of microorganisms is incubation time. Incubation time also affects the synthesis of LA isomerase. The effects of incubation time on titratable acidity and CLA production are shown in Fig. 3c. For CLA production by both La–Lp and La–St co-cultures, the optimal incubation time was 48 h, and CLA production reached the maximum rates of 140.67 μg/mL and 120.37 μg/mL, respectively. Similarly, titratable acidity reached maximum values of 96.72 °T and 110.04 °T at 48 h, respectively.

Effect of inoculum size on titratable acidity and CLA production

In general, inoculum size affects growth rate and fermentation time, and delays the length of the lag phase during growth of the microorganism. Inoculum size can be used at various levels to improve the production of crude enzymes (Li et al. 2009). Figure 3d shows the effects of the inoculum size on titratable acidity and CLA production. The optimum inoculum size for the La–Lp co-culture was 5 %, with a significant increase ($P<0.05$) in total CLA from 65.19 μg/mL to 149.59 μg/mL when the inoculum size was increased from 2 % to 5 %, while remaining at the same rate when inoculum was added at 6 %. Similarly, titratable acidity had the same tendency in the La–Lp co-culture mixture, increasing from 83.31 °T at 2 % to 116.70 °T at 5 % and staying unchanged with further inoculum size increase. CLA production by the La–St co-culture increased up to 120.02 μg/mL at the optimal inoculum size of 3 %, and then decreased sharply when the inoculum size was increased further. Unsurprisingly, titratable acidity also reached a peak value of 114.51 °T at 3 %.

Effect of substrate concentration on titratable acidity and CLA production

As mentioned above, safflower oil was hydrolyzed and added as a lipid substrate to determine the influence of a natural source of LA on CLA production in the co-culture of lactic acid bacteria. The hydrolyzed safflower oil was added to pasteurized milk at a concentration of 2 %–6 %. Tween-80 was used as a surfactant to aid the homogeneity of the oil–milk matrix. The effects of substrate concentration on titratable acidity and CLA production were then examined and the results are presented in Fig. 3e. CLA production by both co-cultures of La–Lp and La–St increased from 2 % to

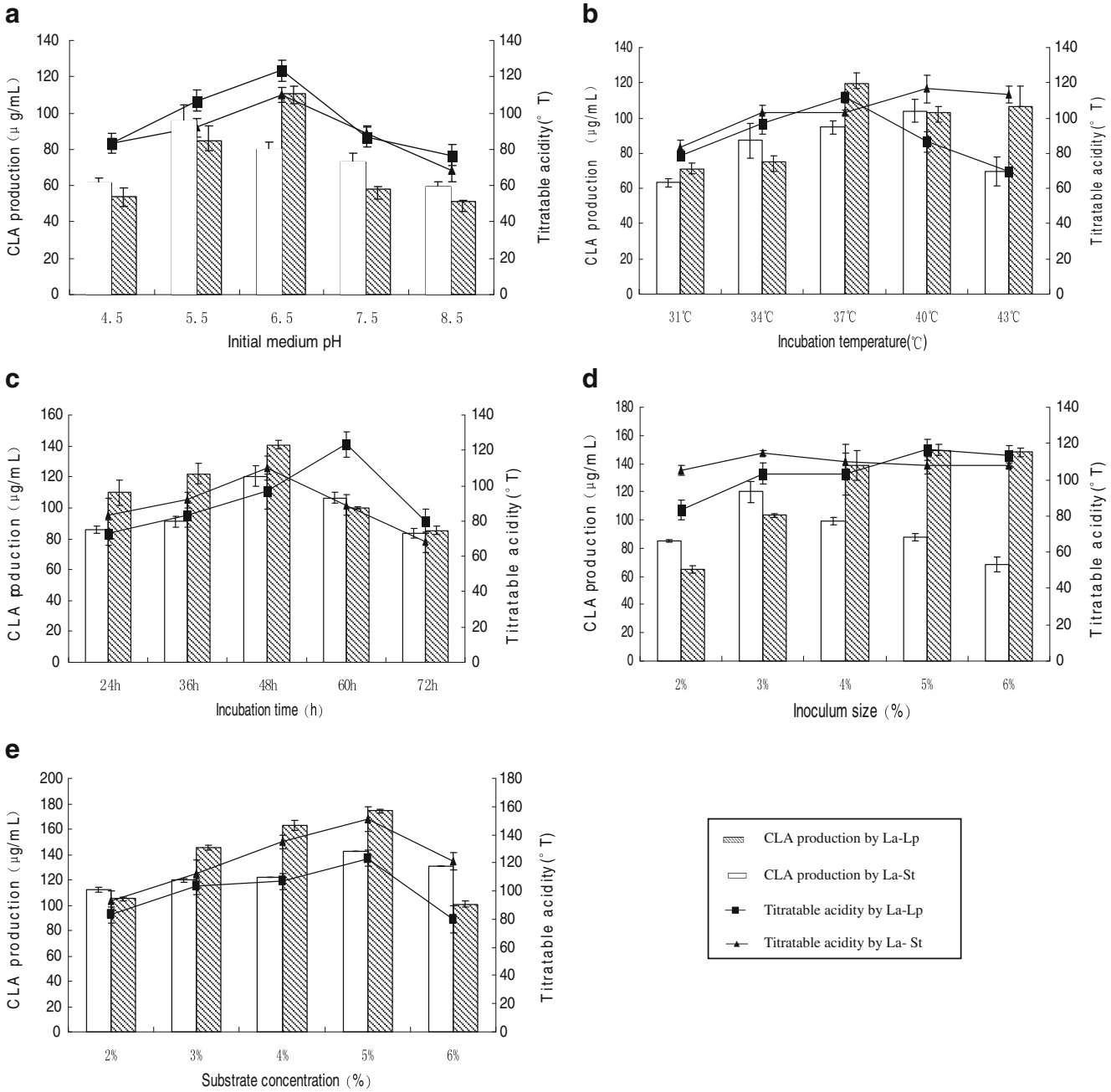

Fig. 3a–e Effect of culture conditions on titratable acidity and CLA production. **a** Effect of initial medium pH. Inoculum size was 3 % for La–St and 4 % for La–Lp. **b** Effect of incubation temperature. Initial medium pH values were pH 6.5 and pH 5.5 for La–Lp and La–St, respectively, in a 36-h fermentation. **c** Effect of incubation time. Initial medium pH 6.5 for La–Lp at 37 °C and pH 5.5 for La–St at 40 °C. **d** Effect of inoculum size. Initial medium pH 6.5 for La–Lp at 37 °C and pH 5.5 for La–St at 40 °C, for 48 h fermentation. **e** Effect of substrate concentration. Reaction system: 5 % (v/v) inoculation size of La–Lp at initial medium pH 6.5, 37 °C for 48 h fermentation, and 3 % (v/v) inoculation of La–St at initial medium pH 5.5, 40 °C for 48 h fermentation

5 % with the addition of the lipid substrates, and then decreased upon over-addition of 6 %. At the lipid addition level of 5 %, production of CLA reached maximum values of 174.21 μg/mL and 142.08 μg/mL for La–Lp and La–St, respectively. The tendency of titratable acidity was the same as that of CLA production.

Optimization of co-culture condition using RSM and statistical analysis

Model fitting

The response and variables were fitted to each other by multiple regressions; a good fit was obtained. The quadratic

regression coefficients obtained by employing a least squares method technique to predict quadratic polynomial models for the production of CLA by La–Lp (Y_1) and La–St (Y_2) are given in Table 3.

$$Y_1 = 314.45 - 33.39 * X_1 - 3.15 * X_2 + 9.29 * X_3 - 25.88 * X_4 - 43.63 * X_1 * X_2 + 21.03 * X_1 * X_3 - 3.71 * X_1 * X_4 + 13.67 * X_2 * X_3 - 1.11 * X_2 * X_4 + 26.12 * X_3 * X_4 - 77.25 * X_1^2 - 92.59 * X_2^2 - 56.72 * X_3^2 - 150.57 * X_4^2 \quad (2)$$

$$Y_2 = 174.07 + 24.24 * X_1 - 18.37 * X_2 + 20.24 * X_3 + 5.31 * X_4 + 2.31 * X_1 * X_2 + 25.48 * X_1 * X_3 + 23.60 * X_1 * X_4 - 19.84 * X_2 * X_3 - 4.27 * X_2 * X_4 + 21.33 * X_3 * X_4 - 38.95 * X_1^2 - 63.01 * X_2^2 - 16.41 * X_3^2 - 15.88 * X_4^2 \quad (3)$$

ANOVAs for the fitted models are summarized in Table 3. The regression models for data on the production of CLA were highly significant ($P<0.0001$) with satisfactory regression

Table 3 Analysis of variance, showing the effect of the variables interactions on the response Y_1 and Y_2 of the central composite design

Source	Sum of squares	Degree of freedom	Mean square	F value	Prob > F
La–Lp (Y_1); R^2=0.9119. Adeq precision=10.339. CV(%)=24.69					
Model	2.216E+005	14	15,828.22	10.35	<0.0001***
A	13,380.04	1	13,380.04	8.75	0.0104*
B	119.13	1	119.13	0.078	0.7843
C	1,034.53	1	1,034.53	0.68	0.4246
D	8,034.71	1	8,034.71	5.25	0.0397*
AB	7,165.18	1	7,165.18	4.98	0.0425*
AC	1,769.88	1	1,769.88	1.16	0.3002
AD	55.13	1	55.13	0.036	0.8521
BC	747.20	1	747.20	0.49	0.4960
BD	4.95	1	4.95	3.237E-003	0.9554
CD	2,728.50	1	2,728.50	1.78	0.2030
A^2	38,707.43	1	38,707.43	25.31	0.0002**
B^2	55,602.25	1	55,602.25	36.36	<0.0001***
C^2	20,870.94	1	20,870.94	13.65	0.0024**
D^2	1.471E+005	1	1.471E+005	96.15	<0.0001***
Lack of fit	21,267.14	10	2,126.71	58.91	0.0007
Pure error	144.41	4	36.10		
Cor total	2.430 E+005	28			
La–St (Y_2); R^2=0.8556. Adeq precision=8.691. CV(%)=21.80					
Model	55,338.32	14	3,952.74	5.92	0.0010**
A	7,049.48	1	7,049.48	10.56	0.0058**
B	4,049.48	1	4,049.48	6.07	0.0273*
C	4,915.49	1	4,915.49	7.37	0.0168*
D	338.99	1	338.99	0.51	0.4877
AB	21.34	1	21.34	0.032	0.8606
AC	2,596.92	1	2,596.92	3.89	0.0686
AD	2,228.31	1	2,228.31	3.34	0.0890
BC	1,574.50	1	1,574.50	2.36	0.1468
BD	72.93	1	72.93	0.11	0.7459
CD	1,820.30	1	1,820.30	2.73	0.1209
A^2	9,842.64	1	9,842.64	14.75	0.0018**
B^2	25,753.17	1	25,753.17	38.59	<0.0001***
C^2	1,747.57	1	1,747.57	2.62	0.1279
D^2	1,636.28	1	1,636.28	2.45	0.1397
Lack of fit	9,028.51	10	902.85	11.50	0.0155
Pure error	314.17	4	78.54		
Cor total	64,681.00	28			

*$P<0.05$, **$P<0.01$, ***$P<0.0001$

coefficients (R^2) of 0.91 and 0.86, respectively. These indicated that the generated models adequately explained the data variation and represented the actual relationships among the reaction parameters.

Effects of parameters

Equations (2) and (3) show that the biosynthesis of CLA has a complex relationship with independent variables that encompass both first- and second-order polynomials. RSM is one of the best ways of evaluating the relationships among responses, variables and interactions. The relationships between independent and dependent variables can be shown in a three-dimensional representation as response surfaces. The response surfaces for CLA production are presented in Fig. 4. The plots in Fig. 4 showed similar relationships with respect to the effects of variables. The response obtained was convex in nature, suggesting that there were well-defined optimum operating conditions. However, the convexity may not be high enough, as the surfaces are rather symmetrical and a little flat near the optimum. Examination of these coefficients with tests showed that, for production of CLA by La–Lp (Y_1), the impacts of incubation temperature (X_1) and initial medium pH (X_4) were significant different ($P<0.05$), while for production of CLA by La–St (Y_2) the linear terms of incubation temperature (X_1) were significant different at $P<0.01$ and substrate concentration (X_2), inoculum size (X_3) were significant different at $P<0.05$. Also, in Fig. 4, evaporating incubation temperature(X_1) showed significant variation both above and below the optimum values. So incubation temperature (X_1) had greater impact on the production of CLA compared to substrate concentration (X_2) and inoculum size (X_3). The effects of substrate concentration (X_2), inoculum size (X_3) and the initial medium pH (X_4) were examined.

The production of CLA decreased with less substrate. On the other hand, production of CLA was also low with more substrates due to their toxicities to the cells. LA isomerase did not recycle like a normal enzyme to catalyze more substrate, and CLA production was highly cell-density-dependent (Kim et al. 2000). A high or low initial pH would affect strain growth.

The results of canonical analysis of the response surfaces are displayed in Table 3. The stationary point for the production of CLA by La–Lp predicted a maximum of 319.26 μg/mL at an incubation temperature of 36.34 °C, substrate concentration of 5.04 %, inoculum size of 5.03 %, initial medium pH 6.4 and incubation time of 48 h. While for La–St, the stationary point predicted a maximum of 229.61 μg/mL at an incubation temperature of 42.79 °C, substrate concentration of 4.68 %, inoculum size of 4 %, the initial medium pH 6.5 and incubation time of 48 h. Compared with observed values (316.52 μg/mL and 210.51 μg/mL), the similarity between the estimated and real values validitated Eqs. 1, 2 and the existence of the maximum value.

Discussion

In this study, *Lactobacillus acidophilus* with *Streptococcus thermophilus* and *Lactobacillus acidophilus* with *Lactobacillus plantarum* were co-cultured to produce CLA; *Lactobacillus acidophilus* with *Lactobacillus plantarum* were reported to produce CLA for the first time. The mixed *lactobacillus* yielded a better result due to their synergetic effects, in agreement with previous reports (Boopathy 2000; Puniya et al. 2009). In comparison with these latter studies, CLA production was low in our preliminary study (Yang et al. 2011). This optimization study was therefore carried out to identify optimal cultivation conditions for La–Lp and La–St for the production of CLA.

It is well documented that pH affects the activities of enzymes, which is crucial during various biochemical

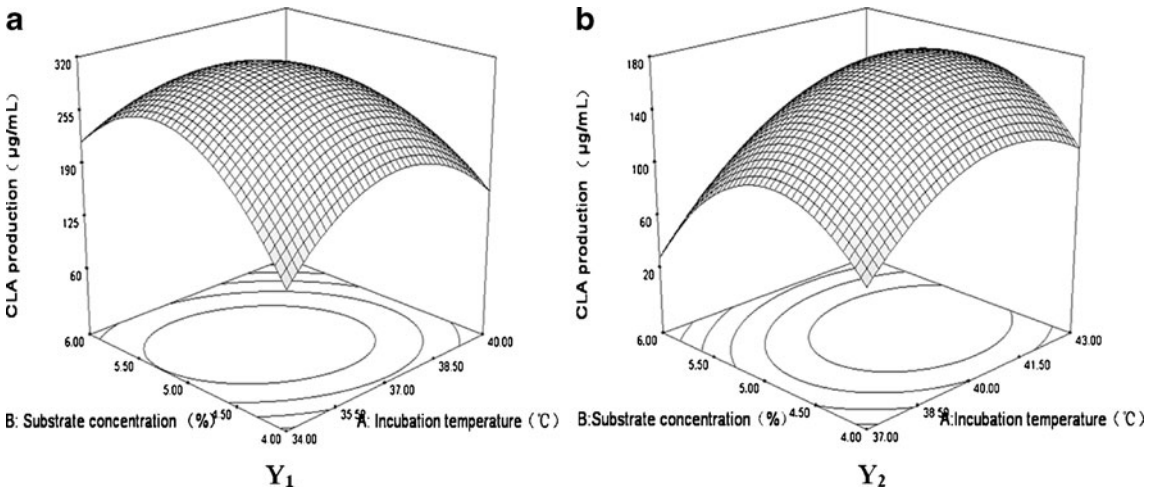

Fig. 4 Response surfaces for the effect on CLA production of **a** incubation temperature, and **b** substrate concentration for Y_1 and Y_2

processes in microbial cells. CLA is an intermediate metabolite of lactic acid bacteria for converting polyunsaturated fatty acid to saturated fatty acid in order to reduce the toxicity of free fatty acids (Jun et al. 2005). Linoleic acid isomerase plays an important role in this process. These microorganisms were reported to be able to form CLA by the action of LA isomerase at an optimal pH ranging from 4.5 to 7.5 (Cao et al. 2004; Miao et al. 2005; Zhang et al. 2006; Rosson et al. 2004). In our study, the co-cultures of La–Lp and La–St showed different abilities to produce CLA within this pH range, with the La–Lp co-culture reaching a peak at pH 6.5 and La–St at pH 5.5. Further studies will be needed to investigate the mechanism of co-culture of these strains to produce CLA.

Lactic acid bacteria are capable of isomerizing linoleic acid (LA) to CLA in vitro through LA isomerase (Lin 2000, 2006; Van-Nieuwenhove et al. 2007). LA isomerase is a membrane-bound enzyme. Enzyme activity is quite sensitive to temperature. The main effect of temperature is on the migration of hydrogen (Allen 1981). If the temperature is too high, the double bonds in LA migrate and break the structure of the enzyme, or the migration process cannot occur in the original configuration changes (Young et al. 2000). The optimal temperature could promote the formation of linoleic acid isomerase, thereby increasing production of CLA. From our study, the conversion of LA to CLA by both co-cultures seemed to be favored by 37– 40 °C.

In our study, incubation time was closely related to CLA production. The formation of LA isomerase was induced by LA, following a sequential process: the longer the incubation time, the greater the enzyme activity and the higher the production rate of CLA. However, prolonged incubation time did not yield a further increase in CLA synthesis. LA isomerase did not recycle like a normal enzyme to catalyze more substrate, and CLA production was highly cell-density-dependent. CLA production increased when more LA was added, but only at low LA concentration. Because CLA was as toxic as LA, there was no advantage in releasing large amounts of free CLA. At the same time, polyunsaturated fatty acids would ultimately restore the saturated or monounsaturated fatty acids by bio-hydrogenation (Young et al. 2000).

The growth of lactic acid bacteria and the production of LA isomerase were both limited by smaller inoculum sizes. Therefore, appropriate initial loads of inoculum accelerate the fermentation process. However, if the initial inoculation is overloaded, nutrients are absorbed rapidly in the early stage of fermentation, and the pH of the system would decrease in consequence, greatly inhibiting the growth and metabolism of cells, leading to retarded LA isomerase activity. Therefore, too high an initial inoculum was also not favor CLA production.

LA has inhibitory effects on bacterial growth (Jiang et al. 1998), and different strains have various tolerances. One of the known underlying mechanisms is related to the capability of these strains in converting LA to CLA through intrinsic detoxification to eliminate free fatty acids, such as oleic and linoleic acids. CLA production when LA was added into the culture medium was more than expected. So another mechanism underlying the observed detoxification was isomerization (Rainio et al. 2001). In the present work, the tolerance to LA of the co-culture strains was tested by adding hydrolyzed safflower oil to SKM at various concentrations. Cell growth would be certainly restricted under over-loading concentrations. In contrast, the bacteria would produce less LA isomerase when the substrate was insufficient at the initial LA levels.

The experiment was carried out at the optimum conditions defined by this study. The production of CLA by La–Lp and La–St at the optimum levels were 316.52 μg/mL and 210.51 μg/mL, respectively. Amino acids from hydrolyzed protein of La were important for the growth of St and Lp, while the acid production rate was slow in La. Growth promoting substances of St and Lp could promote the growth of La. Metabolic products of St were lactic acid, formic acid and CO_2, and metabolic products of Lp included lactic acid (Xiao et al. 2005). Lp produced more lactic acid than St. The conclusion that La–Lp produced more CLA than La–St due to lactic acid was maybe the most important factor among all the factors considered. And the difference in symbiotic mechanism between La–St and La–Lp probably underlies the above hypothesis.

The experimental optimal value was lower than the value computed by the regression model. This phenomenon may be due to the fact that the calculated amount of CLA production was the theoretical value under the optimal conditions. Earlier studies on protein extraction also demonstrated such a pattern (Wani et al. 2006).

Conclusion

The present work optimized operation parameters for the co-culture of La–St and La–Lp using hydrolyzed safflower oil as the substrate in a skim-milk-based medium. The promising results form a foundation for future scale-up and field studies targeting the dairy product industry. The optimal ratios for co-cultures of La–St and La–Lp were 1:1 and 1:4, respectively. Other operation parameters related to co-culture conditions, such as initial medium pH, incubation temperature, substrate concentration, inoculum size and incubation time were studied and the optimal combinations were established by RSM. Different factors showed different effects on CLA production; comparing the two co-cultures, La–Lp showed a higher ability to produce CLA than La–St.

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