Korean J. Food Sci. An. Vol. 36, No. 3, pp. 427~434(2016)
© 2016 Korean Society for Food Science of Animal Resources
DOI http://dx.doi.org/10.5851/kosfa.2016.36.3.427
ISSN 1225-8563 eISSN 2234-246X

ARTICLE

Benzoic Acid Production with Respect to Starter Culture and Incubation Temperature during Yogurt Fermentation using Response Surface Methodology

Hyung-Seok Yu, Na-Kyoung Lee, Hye-Lin Jeon, Su Jin Eom, Mi-Young Yoo[1], Sang-Dong Lim[1], and Hyun-Dong Paik*

Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 05029, Korea
[1]Korea Food Research Institute, Seongnam 13539, Korea

Abstract

Benzoic acid is occasionally used as a raw material supplement in food products and is sometimes generated during the fermentation process. In this study, the production of naturally occurring yogurt preservatives was investigated for various starter cultures and incubation temperatures, and considered food regulations. *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Bifidobacterium longum*, *Bifidobacterium lactis*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, and *Bifidobacterium breve* were used as yogurt starter cultures in commercial starters. Among these strains, *L. rhamnosus* and *L. paracasei* showed the highest production of benzoic acid. Therefore, the use of *L. rhamnosus*, *L. paracasei*, *S. thermophilus*, and different incubation temperatures were examined to optimize benzoic acid production. Response surface methodology (RSM) based on a central composite design was performed for various incubation temperatures (35-44°C) and starter culture inoculum ratios (0-0.04%) in a commercial range of dairy fermentation processes. The optimum conditions were 0.04% *L. rhamnosus*, 0.01% *L. paracasei*, 0.02% *S. thermophilus*, and 38.12°C, and the predicted and estimated concentrations of benzoic acid were 13.31 and 13.94 mg/kg, respectively. These conditions maximized naturally occurring benzoic acid production during the yogurt fermentation process, and the observed production levels satisfied regulatory guidelines for benzoic acid in dairy products.

Keywords: benzoic acid, yogurt starter, natural preservative, response surface method, food regulation

Received May 19, 2016; Revised June 10, 2016; Accepted June 12, 2016

Introduction

Benzoic acid is used as a food preservative in various products owing to its antioxidant and antimicrobial activity to prevent spoilage by enzymes, oxidation, or microorganisms (Dong and Wang, 2006; Pan *et al*., 2005). Benzoic acid is generally recognized as safe; however, adverse effects related to allergic reactions, such as asthma, urticarial, metabolic acidosis, and convulsions, have been reported in sensitive individuals (Qi *et al*., 2009; Safford *et al*., 1990). Therefore, it is important to determine the levels of benzoic acid in food for both quality assurance and food safety purposes (Shan *et al*., 2008). These preservatives are allowed by legislation that establishes the maximum permitted concentrations in each type of food. The excessive addition of these preservatives to food products could be harmful to human health (Dong and Wang, 2006; Javanmardi *et al*., 2015). The addition of benzoic acid to yogurt is not allowed, and the amount of benzoic acid in yogurt should not exceed 50 mg/kg (Cakir and Cagri-Mehmetoglu, 2013; Choi *et al*., 2008; Hejtmankova *et al*., 2000). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) (WHO, 2000) has established acceptable daily intakes of 0-5 mg/kg body weight for benzoic acid.

The concentrations of benzoic acid in milk and yogurt are typically 2-5 mg/kg and up to 50 mg/kg, respectively (Urbiene and Leskauskaite, 2006). As lactic acid bacteria grows, lactic, acetic, and propionic acid increase and hippuric, orotic, and citric acid decrease (Urbiene and Leskauskaite, 2006). Benzoic acid is produced via lactic acid bacterium-mediated enzymatic conversion of hippuric acid, which is naturally present in milk; degradation of pheny-

*Corresponding author: Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 05029, Korea. Tel. +82-2-2049-6011, Fax: +82-2-450-0381, E-mail: hdpaik@konkuk.ac.kr

lalanine; and auto-oxidation of benzaldehyde (Sieber *et al*., 1995; Urbiene and Leskauskaite, 2006). Additionally, benzoic acid can be produced by β-oxidation in the catabolism of fatty acids in bacterial cells (Hertweck *et al*., 2001). These processes and results are depending on the fermentation starter and milk type (Horníèková *et al*., 2014). Strains that are known to produce benzoic acid in milk include *Lactococcus lactis*, *Lactobacillus casei*, *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Escherichia coli*, and *Pseudomonas fluorescens* (Garmiene *et al*., 2010; Sajko *et al*., 1984). The benzoic acid in fermented milk is detected immediately after the inoculation of the starter culture in the fermentation stage at 2.28-10.48 mg/kg (Lim *et al*., 2013).

Benzoic acid production has been studied for various starter cultures and incubation temperatures (Lim *et al*., 2013; Urbiene and Leskauskaite, 2006). However, the optimization of benzoic acid production has not been reported. Therefore, the objective of this study was determining the optimized benzoic acid production with respect to starter culture and incubation temperature using response surface methodology (RSM) in a range of regulation.

Materials and Methods

Materials and reagents

Benzoic acid, tetrabutyl ammonium hydroxide solution, cetyltrimethyl ammonium chloride, hydrogen chloride, and sodium hydroxide were purchased from Sigma-Aldrich (USA). Commercially available milk (Maeil Dairy Co., Korea) was used for yogurt preparation. All solvents were analyzed as high-performance liquid chromatography (HPLC) grade (J. T. Baker, USA).

Yogurt preparation

Yogurt was prepared as described by Donkor *et al*. (2007), with a few modifications. The reconstituted milk was standardized with milk and skim milk powder (SNF value, 11%). The yogurt base was prepared by heating at 85°C for 30 min, and followed by cooling to 45°C. The starter strains were *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *L. delbrueckii* subsp. *bulgaricus, L. rhamnosus*, *L. casei*, *L. paracasei*, *L. reuteri*, *L. plantarum*, *Bifidobacterium longum*, *B. lactis*, *B. bifidum*, *B. infantis*, and *B. breve*. These strains were purchased from Chr. Hansen (Denmark). The starter was aseptically inoculated (inoculation ratio, 0.02% w/v), and the inoculated milk was incubated at 37°C, 42°C, and 45°C until pH of 4.5±0.1 was reached. Manufactured yogurt was used for analysis directly.

Determination of pH and bacterial cell count

The pH was determined using a pH meter (VTW, Germany). The bacterial cell count was determined using the plate counting method described by Dave and Shah (1996) and Korean Food Standards Codex (KFDA, 2015). Sampled yogurt was diluted using 0.1% peptone water, and spread on BCP (Bromocresol purple) plate count agar (Eiken chemical, Japan). The plates were incubated at 42°C for 48 h, and the cell number was counted.

Benzoic acid analysis

The benzoic acid analysis was performed according to the methods of Korean Food Standards Codex (KFDA, 2015). Methanol (10 mL) and 0.005 M CTA solution (10 mL) were loaded on a Sep-Pak C18® cartridge sequentially at a 2 mL/min flow rate. Yogurt samples (5 g) were diluted in 25 mL of water, and 5 mL of diluent was obtained and mixed with 1 N HCl (0.5 mL) and 0.005 M CTA solution (0.5 mL). The diluent yogurt sample was injected, and H_2O (10 mL) and methanol (10 mL) were added to the activated Sep-Pak C18® cartridge. The obtained sample was filtered by using a 0.45-μm cellulose acetate filter and used for the benzoic acid analysis.

Benzoic acid was quantified with HPLC analysis (Agilent 1100 apparatus; Agilent Technologies, USA) with a C8 column (Shiseido, 5 μm, 150 mm × 4.6 mm) and a UV detector (217 nm). The flow rate was 1 mL/min, and the injection volume was 10 μL; a mixture of 0.1% TBA (tetrabutylammonium hydroxide)-OH solution (A) and acetonitrile (B) was used as the mobile phase. Gradient conditions were 0-2.5 min, 75% A and 25% B; 7 min, 65% A and 35% B; 12 min, 60% A and 40% B; 15 min, 70% A and 30% B.

To evaluate validation of the proposed Sep-Pak-HPLC method, the linearity, limit of detection, and limit of quantification were studied. We analyzed the rate of linearity recovery by using CRM (certificated reference material) to confirm the specificity and appropriacy of benzoic acid in this method.

Experimental design

The optimization of benzoic acid production was performed using RSM with a central composite design. The following were evaluated with respect to benzoic acid production during yogurt fermentation: incubation temperature (a), *L. rhamnosus* (b), *L. paracasei* (c), and *S. thermophilus* (d). The incubation temperatures ranged from 35°C

Table 1. Variables, codes, and experimental values used in the central composite design

Variables	Coded levels				
	-2	-1	0	1	2
Temperature (a, °C)	35	37	40	42	44
L. rhamnosus (b, %)	0	0.01	0.02	0.03	0.04
L. paracasei (c, %)	0	0.01	0.02	0.03	0.04
S. thermophilus (d, %)	0	0.01	0.02	0.03	0.04

to 44°C and starter cultures ranged from 0% to 0.04% (Table 1). For each factor, 5 levels were defined, and were designed by the following codes: -2, -1, 0, +1, +2 (see Table 2 for definitions). End point of fermentation was controlled by final pH (pH 4.5±0.1). A single type of yogurt was used for the optimization process, and the production of benzoic acid was assessed using HPLC.

Statistical analysis

All experiments were repeated at least three times. The results are expressed as mean±standard deviation of the treatments in triplicate, and were analyzed by a one-way analysis of variance, Duncan's multiple range test, and RSM. The threshold level for statistical significance was set at $p<0.05$. SPSS version 18 (USA) and SAS 9.4 (SAS Institute Inc., USA) were used for analyses.

Results and Discussion

Method validation

The correlation coefficient (R^2) was 0.999 for benzoic acid, indicating a linear response in the range of 0.5-100 mg/kg. The limit of detection and limit of quantification for benzoic acid were 0.11 and 0.32 mg/kg, respectively. In case of CRM, correlation coefficient of linearity and rate of recovery were 0.998 and 99.96±4.18%, respectively in the range of 0.5-8.00 mg/kg.

Benzoic acid production with respect to the starter culture

Changes in benzoic acid during yogurt fermentation

Table 2. Experimental design and benzoic acid production results

Run no.	a (°C)	b (%)	c (%)	d (%)	Max. benzoic acid (mg/kg)	Cell no. (Log CFU/mL)	pH	Incubation time (h)
1	0	-2	0	0	12.12 ± 0.02	9.37 ± 0.00	5.03	3
2	+2	0	0	0	10.95 ± 0.03	9.41 ± 0.03	4.59	6
3	0	0	-2	0	11.39 ± 0.01	9.35 ± 0.02	4.53	5
4	+1	-1	+1	-1	11.55 ± 0.03	9.31 ± 0.01	4.50	6
5	0	0	0	-2	11.23 ± 0.02	9.53 ± 0.08	5.07	13
6	+1	-1	-1	+1	11.23 ± 0.04	9.36 ± 0.02	5.12	3
7	+1	+1	-1	-1	11.86 ± 0.03	9.17 ± 0.05	4.86	4
8	-1	+1	+1	-1	11.64 ± 0.04	9.55 ± 0.01	5.05	5
9	-1	-1	+1	-1	10.82 ± 0.03	9.45 ± 0.01	4.70	6
10	-1	+1	+1	+1	12.26 ± 0.02	9.57 ± 0.02	4.74	5
11	0	0	0	0	10.51 ± 0.01	9.34 ± 0.03	4.73	4
12	0	0	0	0	10.72 ± 0.03	9.38 ± 0.02	4.77	4
13	0	0	0	0	10.71 ± 0.02	9.45 ± 0.02	4.74	4
14	-1	+1	-1	+1	12.73 ± 0.06	9.39 ± 0.01	4.70	5
15	+1	+1	-1	+1	11.94 ± 0.04	9.17 ± 0.03	5.01	3
16	0	0	0	0	10.59 ± 0.04	9.11 ± 0.03	5.02	3
17	+1	+1	+1	+1	11.99 ± 0.04	9.45 ± 0.02	4.95	3
18	-2	0	0	0	11.73 ± 0.06	9.60 ± 0.02	4.58	7
19	0	0	+2	0	11.38 ± 0.03	9.55 ± 0.02	4.88	4
20	0	+2	0	0	12.86 ± 0.01	9.47 ± 0.02	4.56	4
21	-1	+1	-1	-1	12.71 ± 0.01	9.44 ± 0.01	4.72	6
22	+1	-1	-1	-1	12.70 ± 0.03	9.40 ± 0.03	4.98	3
23	0	0	0	+2	11.88 ± 0.03	9.21 ± 0.02	4.73	4
24	+1	-1	-1	-1	11.07 ± 0.02	9.31 ± 0.01	5.13	3
25	+1	+1	+1	-1	11.29 ± 0.02	9.38 ± 0.01	5.13	3
26	-1	-1	-1	+1	12.33 ± 0.05	9.37 ± 0.01	4.94	4
27	-1	-1	+1	+1	12.19 ± 0.01	9.52 ± 0.05	4.55	6
28	-1	-1	-1	-1	11.28 ± 0.03	9.53 ± 0.03	4.62	6

a, temperature; b, *L. rhamnosus*; c, *L. paracasei*; d, *S. thermophilus*.

Table 3. Benzoic acid production with respect to yogurt starter

Strains	Benzoic acid (mg/kg)	
	Single strain[A]	Mixed culture with *S. thermophilus*[B]
S. thermophilus	1.30 ± 0.24[c]	3.68 ± 0.48[b]
L. acidophilus	0.79 ± 0.00[b]	0.85 ± 0.11[a]
L. bulgaricus	2.45 ± 0.18[cd]	2.15 ± 0.18[b]
L. rhamnosus	13.89 ± 0.63[f]	10.12 ± 0.49[e]
L. casei	4.04 ± 0.87[d]	0.76 ± 0.04[a]
L. paracasei	17.46 ± 0.54[g]	11.36 ± 1.09[e]
L. reuteri	1.36 ± 0.16[c]	0.82 ± 0.17[a]
L. plantarum	13.99 ± 0.24[f]	8.90 ± 0.16[c]
B. longum	0.77 ± 0.22[b]	0.78 ± 0.13[a]
B. lactis	4.97 ± 0.25[e]	0.84 ± 0.14[a]
B. bifidum	1.43 ± 0.23[c]	0.86 ± 0.15[a]
B. infantis	N.D.	0.82 ± 0.10[a]
B. breve	4.87 ± 0.13[e]	0.83 ± 0.03[a]

[A]Yogurt incubated for 24 h. [B]Yogurt incubated for 6 h.
These values represented as mean±SD.
[a-f]The letters are different significantly.
N.D. means not detected.

were measured using commercial starters (Table 3). To estimate benzoic acid production, 0.02% inoculums of *S. thermophilus*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus L. rhamnosus*, *L. casei*, *L. paracasei*, *L. reuteri*, *L. plantarum*, *B. longum*, *B. lactis*, *B. bifidum*, *B. infantis*, and *B. breve* were incubated at 42°C for 24 h. The single strains, excluding *S. thermophilus*, reached to stationary phase at 24 h. The benzoic acid contents during yogurt fermentation were 0-17.46 mg/kg. The maximum benzoic acid contents for *L. rhamnosus* and *L. paracasei* were 13.89±0.63 mg/kg and 17.46±0.54 mg/kg, respectively.

S. thermophilus is the most widely used species in yogurt manufacturing because it has characteristics of β-galactosidase-producing and homofermentative strains. We examined mixed cultures to determine benzoic acid production in commercial conditions. The mixed cultures of 0.01% each strain with 0.01% *S. thermophilus* were incubated at 42°C for 6 h to reach the stationary phase. The benzoic acid contents of mixed cultures ranged from 0.76 to 11.36 mg/kg. Those of the mixed cultures of *L. rhamnosus* with *S. thermophilus* and *L. paracasei* with *S. thermophilus* were 10.12±0.49 mg/kg and 11.36±1.09 mg/kg, respectively (Fig. 1). Bacterial cell number increased rapidly at the initial fermentation and maintained cell growth when the stationary phase was reached. The benzoic acid content also increased and then decreased with cell growth because benzoic acid is primary metabolite.

Benzoic acid production was reported for the commercial starter YC-180, which is composed of *Lactobacillus delbrueckii* subsp. *lactis*, *S. thermophilus*, and *L. delbrueckii* subsp. *bulgaricus*, ABT-2 composed of *L. acidophilus*, *Bifidobacteria*, and *S. thermophilus*, and La-5, which is composed of *L. acidophilus* (Urbiene and Leskauskaite, 2006). Benzoic acid production was highest values in the yogurt fermented with La-5, at 24 mg/kg, and these levels were detected at the initial stationary phase (Urbiene and Leskauskaite, 2006). The commercial starters MYE 95, MY900, CH1, and LYOFAST Y 4.80F have been investigated in Lebanon (Mroueh *et al*., 2008). The benzoic

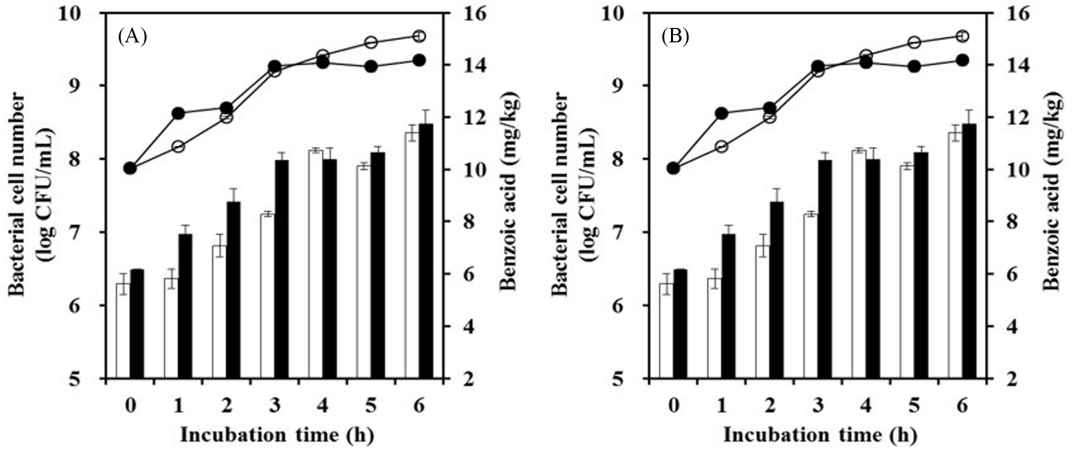

Fig. 1. Effect of benzoic acid production with respect to incubation temperature. (a) Yogurt produced by a mixed culture of *Lactobacillus rhamnosus* with *Streptococcus thermophilus*, (b) yogurt produced by a mixed culture of *Lactobacillus paracasei* with *Streptococcus thermophiles.* ○ , bacterial cell number of incubated yogurt at 37°C (Log CFU/mL); ● , bacterial cell number of incubated yogurt at 42°C (Log CFU/mL); □ , benzoic acid (mg/kg) of incubated yogurt at 37°C; ■ , benzoic acid (mg/kg) of incubated yogurt at 42°C.

acid contents using these strains were 4.7, not detected, 14.7, and 8.5 mg/kg, respectively. Based on these results, the production of benzoic acids was influenced by a starter type.

Effect of incubation temperature

S. thermophilus, *L. rhamnosus*, and *L. paracasei* were incubated at 37°C, 42°C, and 45°C (data not shown). The cell counts and benzoic acid contents for *S. thermophilus* at 37°C and 42°C were 9.26±0.01 Log CFU/mL and 0.89 ±0.01 mg/kg, and 9.41±0.11 Log CFU/mL and 3.53±0.25 mg/kg, respectively. The cell counts and benzoic acid contents of the mixed culture of *L. rhamnosus* with *S. thermophilus* at 37°C and 42°C were 9.21±0.03 Log CFU/mL and 10.48±0.17 mg/kg, and 9.29±0.05 Log CFU/mL and 10.62±0.03 mg/kg, respectively. The mixed culture of *L. paracasei* with *S. thermophilus* at 37°C and 42°C were 9.62±0.06 Log CFU/mL and 11.40±0.30 mg/kg, and 9.33 ±0.02 Log CFU/mL and 11.75±0.51 mg/kg, respectively. However, the tested strains did not grow at 45°C. Therefore, the benzoic acid contents for these strains was higher at 42°C.

Response surface modelling for benzoic acid production

Optimized conditions were evaluated in the range of 35-44°C for the incubation temperature and 0-0.04% for the inoculum ratio using *L. rhamnosus*, *L. paracasei*, and *S. thermophilus* (Table 2). A quadratic model was fitted to the obtained data (Table 4). This model was used to predict responses for any parameter value, and the RSM model in terms of coded values is as follows:

$$R = 0.04a^2 + 4816.85b^2 + 2050.19c^2 + 2471.02d^2 - 5.49ab + 8.94ac - 2.81ad - 2143.75bc - 1443.75bd + 1585.42cd - 3.04a + 116.26b - 428.25c + 36.49d + 74.41 \quad \text{(Eq. 1)}$$

Where R represents benzoic acid production as a function of temperature (a), *L. rhamnosus* (b), *L. paracasei* (c), and *S. thermophilus* (d). Based on the analysis of variance, the model was significant, with a *p*-value of less than 0.0001 and an R^2 value of 0.96 (Table 5). The explanatory variables had significant linear effects on the response variable. The interactive effects of ab, ac, ad, bc, bd, and cd were significant ($p<0.005$). The benzoic acid production was influenced by factor interactions of each variants (Fig. 2). The conditions for maximum benzoic acid production was 38.12°C, 0.04% a, 0.01% b, and 0.02% c, at which the maximum response for benzoic acid production is predicted (13.31 mg/kg).

Table 4. Results of the central composite design response surface regression analysis for benzoic acid production

Parameter	DF	Estimate	Standard error	T value	Significant level
Intercept	1	74.411029	5.621842	13.24	< 0.0001
a	1	-3.043319	0.278768	-10.92	< 0.0001
b	1	116.262240	36.399545	3.19	< 0.0001
c	1	-428.248725	36.399545	-11.77	< 0.0001
a × a	1	0.037506	0.003514	10.67	< 0.0001
b × a	1	-5.486842	0.884150	-6.21	< 0.0001
b × b	1	4816.854099	180.319220	26.71	< 0.0001
c × a	1	8.936404	0.884150	10.11	< 0.0001
c × b	1	-214.750000	222.506215	-9.63	< 0.0001
c × c	1	2050.187433	180.319220	11.37	< 0.0001
d × a	1	-2.807018	0.884150	-3.17	< 0.005
d × b	1	-1443.750000	222.506215	-6.49	< 0.0001
d × c	1	1585.416667	222.506215	7.13	< 0.0001
d × d	1	2471.020766	180.319220	13.70	< 0.0001

a, temperature; b, *L. rhamnosus*; c, *L. paracasei*; d, *S. thermophilus*.

Table 5. ANOVA results for the central composite design

Regression	DF	Type 1 sum of squares	R-square	F value	Significant level
Linear	4	10.440892	0.2680	109.84	< 0.0001
Quadratic	4	18.879068	0.4846	198.61	< 0.0001
Crossproduct	6	7.995400	0.2052	56.07	< 0.0001
Total model	14	37.315360	0.9579	112.16	< 0.0001

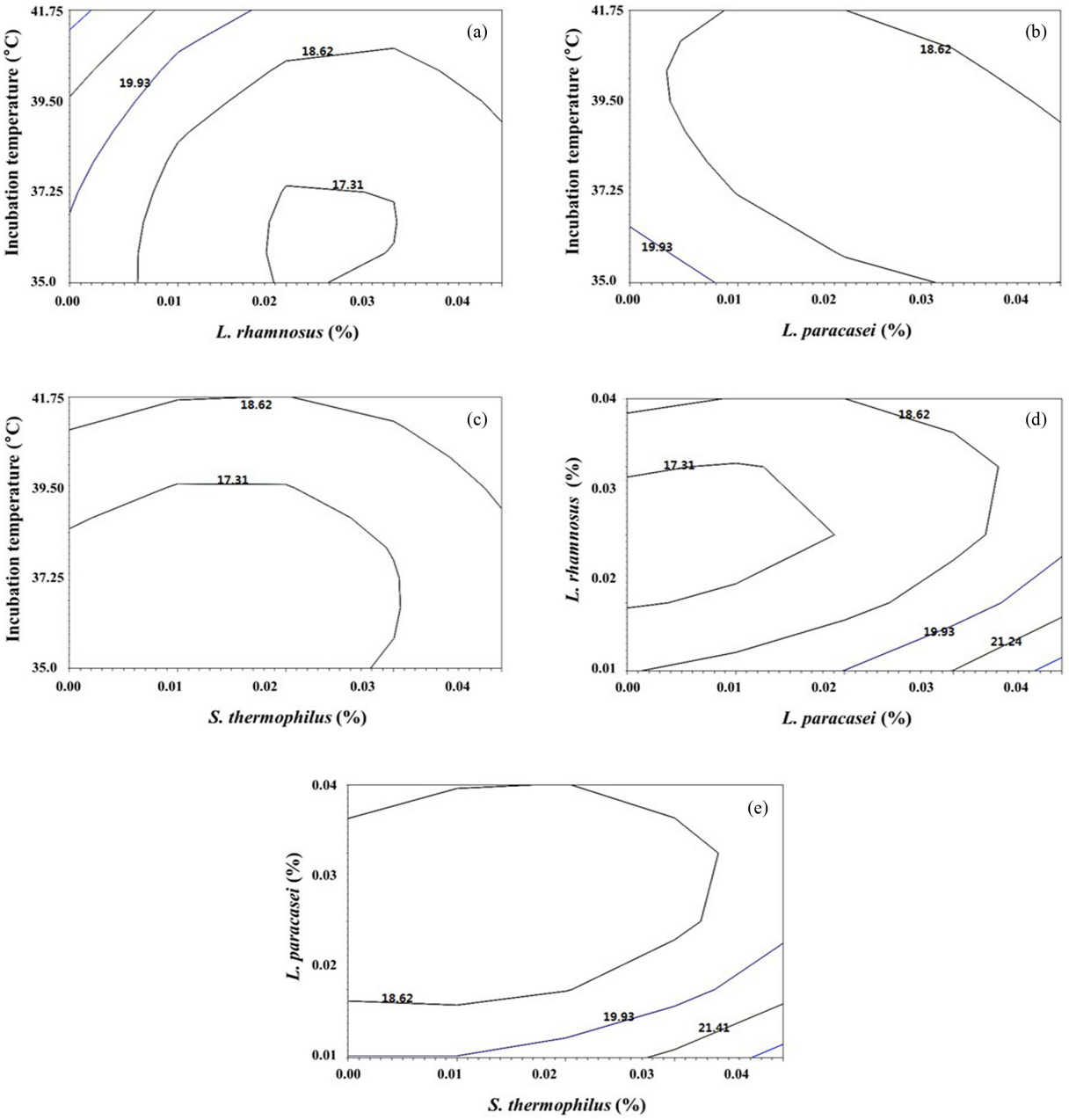

Fig. 2. Contour plots for benzoic acid production for the combination of (a) incubation temperature and *L. rhamnosus*, (b) incubation temperature and *L. paracasei*, (c) incubation temperature and *S. thermophilus*, (d) *L. rhamnosus* and *L. paracasei*, (e) *L. paracasei* and *S. thermophilus*.

Benzoic acid production in optimized conditions

The optimized conditions were applied to yogurt fermentation to empirically confirm the predicted values. The time course of benzoic acid production is shown for yogurt fermentation in Fig. 3. The maximum value of benzoic acid was reached at 4 h, the initial stationary phase (pH 4.8), and then decreased. Benzoic acid production was 5.90-13.96 mg/kg for the incubation period. The predicted and actual values were similar, i.e., 13.31 and 13.96 mg/kg, respectively. Maximal benzoic acid production has been reported for an incubation time of 3-6 h (≥ pH 4.5), which is consistent with our results (Lim *et al*., 2013; Urbiene and Leskauskaite, 2006). In case of *L. paracasei*, concentration of benzoic acid was higher than optimized condition after 24 h incubation as a single strain. However, considering the manufacturing process in commercial, fermentation would be complete around in 6 h. *L. paracasei* doesn't grow well as a single strain at initial

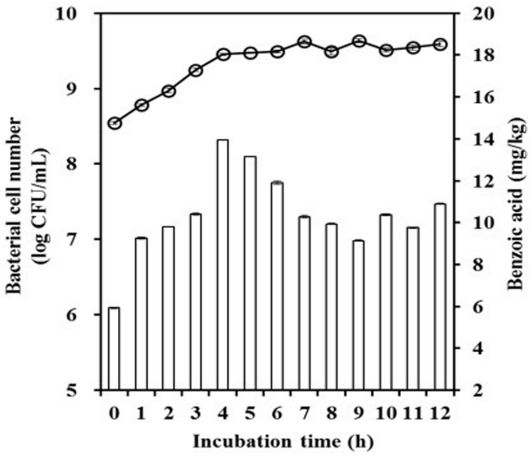

Fig. 3. Production of benzoic acid at optimized conditions (38.12°C, 0.04% *L. rhamnosus*, 0.01% *L. paracasei*, and 0.02% *S. thermophilus*) using response surface methodology. --, bacterial cell number (Log CFU/mL); ■, benzoic acid (mg/kg).

state, because of pH and temperature of milk. In mixed culture incubation, fermentation ends before *L. paracasei* reach to maximum growth. Thus, only obtained values during fermentation with *S. thermophilus* would be in consideration. In commercial yogurt, benzoic acid production has been reported as 12-47 mg/kg (Sieber *et al*., 1995) and 8.94-28.30 mg/kg (Yildiz *et al*., 2012). These naturally occurring levels of benzoic acid are within regulatory guidelines.

Conclusions

This study investigated the production of a naturally occurring yogurt preservative with respect to starter culture and incubation temperature. Among 12 commercial starter cultures, *L. rhamnosus* and *L. paracasei* showed the highest benzoic acid production. RSM of incubation temperature, *L. rhamnosus*, *L. paracasei*, and *S. thermophilus* was performed to optimize benzoic acid production. The optimum conditions for benzoic acid production were 38.12°C, 0.04% *L. rhamnosus*, 0.01% *L. paracasei*, and 0.02% *S. thermophilus*. The predicted production was similar to observed value. The results demonstrated maximal naturally occurring benzoic acid production during the yogurt fermentation process, and these values satisfy current regulations.

Acknowledgements

This research was supported by a grant (14162MFDS 087) from Ministry of Food and Drug Safety in 2014.

References

1. Cakir, R. and Cagri-Mehmetoglu, A. (2013) Sorbic and benzoic acid in non-preservative-added food products in Turkey. *Food Addit. Contam. B* **6,** 47-54.
2. Choi, S. H., Lee, J. Y., Park, E. Y., Won, J., Hong, K. K., Moon, G. I., Kim, M. S., and Hong, J. H. (2008) Assessment of estimated daily intakes of preservatives in the Korean population. *Korean J. Food Sci. Technol.* **40,** 503-509.
3. Dave, R. I. and Shah, N. P. (1996) Evaluation of media selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, and bifidobacteria. *J. Dairy Sci.* **79,** 1529-1536.
4. Dong, C. and Wang, W. (2006) Headspace solid-phase microextraction applied to the simultaneous determination of sorbic and benzoic acid in beverages. *Anal. Chim. Acta* **562,** 23-29.
5. Donkor, O. N., Henriksson, A., Vasiljevic, T., and Shah, N. P. (2007) Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of growth and in vitro angiotensin-converting enzyme inhibitory activity in fermented milk. *Lait* **87,** 21-38.
6. Garmiene, G., Salomskiene, J., Jasutiene, I., Macioniene, I., and Miliauskiene, I. (2010) Production of benzoic acid by lactic acid bacteria from *Lactobacillus*, *Lactococcus* and *Streptococcus* genera in milk. *Milchwissenschaft* **65,** 295-298.
7. Hejtmankova, A., Dolejskova, J., Horak, V., Dragounova, H., and Louda, F. (2000) Benzoic acid in some milk products from the market in the Czech Republic. *Czech J. Food Sci.* **18,** 99-102.
8. Hertweck, C., Jarvis, A. P., Xiang, L., Moore, B. S., and Oldham, N. J. (2001) A mechanism of benzoic acid biosynthesis in plants and bacteria that mirrors fatty acid β-oxidation. *Chembiochem* **2,** 784-786.
9. Horníèková, Š., Dragounová, H., Hejtmanková, K., Michlová, T., and Hejtmánkova, A. (2014) Production of benzoic acid in fermented goat's and sheep's milk. *Sci. Agic. Bohem.* **45,** 247-253.
10. Javanmardi, F., Nemati, M., Ansarin, M., and Arefhosseini, S. R. (2015) Benzoic and sorbic acid in soft drink, milk, ketchup sauce and bread by dispersive liquid-liquid microextraction coupled with HPLC. *Food Addit. Contam. B* **8,** 32-39.
11. KFDA (2015) Analysis method of food additives in food. Korean Food Standards Codex 9, 2.1.1.
12. Lim, S. D., Park, M. S., Kim, K. S., and Yoo, M. Y. (2013) Evaluation of Benzoic Acid Level of Fermented Dairy Products during Fermentation. *Korean J. Food Sci. An.* **33,** 640-645.
13. Mroueh, M., Issa, D., Khawand, J., Haraty, B., Malek, A., Kassaify, Z., and Toufeili, I. (2008) Levels of benzoic and sorbic acid preservatives in commercially produced yoghurt in Lebanon. *J. Food Agric. Environ.* **6,** 62-66.
14. Pan, Z., Wang, L., Mo, W., Wang, C., Hu, W., and Zhang, J. (2005) Determination of benzoic acid in soft drinks by gas chromatography with on-line pyrolytic methylation technique.

Anal. Chim. Acta **545,** 218-223.
15. Qi, P., Hong, H., Liang, X., and Liu, D. (2009) Assessment of benzoic acid levels in milk in China. *Food Control* **20,** 414-418.
16. Safford, R. J., Basketter, D. A., Allenby, C. F., and Goodwin, B. F. J. (1990) Immediate contact reactions to chemicals in the fragrance mix and a study of the quenching action of eugenol. *Br. J. Dermatol.* **123,** 595-606.
17. Sajko, W., Kisza, J., and Niedziecki, J. (1984) Benzoic acid in raw cow milk. *Zesz. Nauk. Akad. Roln. Tech. Olszt. Technol. Zywn.* **19,** 61-68.
18. Shan, D., Li, Q., Xue, H., and Cosnier, S. (2008) A highly reversible and sensitive tyrosinase inhibition-based amperometric biosensor for benzoic acid monitoring. *Sensor Actuat. B. Chem.* **134,** 1016-1021.
19. Sieber, R., Bütikofer, U., and Bosset, J. O. (1995) Benzoic acid as a natural compound in cultured dairy products and cheese. *Int. Dairy J.* **5,** 227-246.
20. Urbiene, S. and Leskauskaite, D. (2006) Formation of some organic acids during fermentation of milk. *Pol. J. Food Nutr. Sci.* **15,** 277-281.
21. WHO (2000) Benzoic acid and sodium benzoate: concise international chemical assessment document, 26, Geneva.
22. Yildiz, A., Erdogan, S., Saydut, A., and Hamamci, C. (2012) High-performance liquid chromatography analysis and assessment of benzoic acid in yogurt, ayran, and cheese in Turkey. *Food Anal. Method* **5,** 591-595.