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ARTICLE

Application of Response Surface Methodology (RSM) for Optimization of Anti-Obesity Effect in Fermented Milk by *Lactobacillus plantarum* Q180

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Abstract

Obesity, a condition in which an abnormally large amount of fat is stored in adipose tissue, causing an increase in body weight, has become a major public health concern worldwide. The purpose of this study was to optimize the process for fermented milk for the production of a functional product with an anti-obesity effect by using *Lactobacillus plantarum* Q180 isolated from human feces. We used a 3-factor, 3-level central composite design (CCD) combined with the response surface methodology (RSM). Concentration of skim milk powder (%, X_1), incubation temperature (°C, X_2), and incubation time (h, X_3) were used as the independent factors, whereas pH (pH, Y_1), anti-lipase activity (%, Y_2) and anti-adipogenetic activity (%, Y_3) were used as the dependent factors. The optimal conditions of fermented milk for the highest anti-lipase and anti-adipogenetic activity with pH 4.4 were the 9.5% of skim milk powder, 37°C of incubation temperature, 28 h of incubation time. In the fermentation condition, the predicted values of pH, anti-lipase activity and anti-adipogenetic activity were 4.47, 55.55, and 20.48%, respectively. However, the actual values of pH, anti-lipase activity and anti-adipogenetic activity were 4.50, 52.86, and 19.25%, respectively. These results demonstrate that 9.5% of skim milk powder and incubation at 37°C for 28 h were the optimum conditions for producing functional fermented milk with an anti-obesity effect.

Keywords: *Lactobacillus plantarum*, optimization, anti-lipase activity, anti-adipogenetic activity

Introduction

Obesity is becoming increasingly prevalent among adults, adolescents and children and has become a major public health concern worldwide (Yanovski and Yanovski, 2002). Indeed, obesity is closely related with several metabolic syndromes such as hypertension, diabetes, hyperlipidemia, and arteriosclerosis (Tanida *et al*., 2008). For these reasons, numerous people have an interest in this issue.

Lactic acid bacteria (LAB) possess special physiological activities and are generally regarded as safe (GRAS). LAB have been widely used in a number of fermented foods, particularly in the production of dairy and vegetable products with functional and probiotic properties (Karahan *et al*., 2010; Leroy and Vuyst, 2004). As regards their use as probiotics, LAB are reported to have various beneficial effects on the health of hosts once consumed in adequate amounts. These effects include the modulation of immune responses (Salminen *et al*., 2002), and anti-carcinogenic and anti-oxidative activities (Choi *et al*., 2006). In addition to these effects, certain LAB have been found to be effective in regulating adipose tissue in overweight adults (Kadooka *et al*., 2010) as well as in a diet-induced obese animal model (Takemura *et al*., 2010).

Individual LAB has a specific fermentation profile, such as the ability to form functional substances and to produce acid. Thus, taking the profiles of LAB into consideration is a significant factor when it is used as a starter in the production of fermented foods (Komatsuzaki *et al*., 2005).

The response surface methodology (RSM), which was first described by Box and Wilson (Box and Wilson, 1951), is a collection of statistical and mathematical techniques. It is based on the fit of a polynomial equation to experimental data (Bezerra *et al*., 2008). Because RSM is an efficient experimental strategy for seeking optimal conditions for a multivariable system, it has been successfully employed in optimizing the culture conditions (Box *et al*., 1978).

The aim of this study is to optimize the fermentative parameters in order to apply them to functional food products which have an anti-obesity effect.

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Materials and Methods

Bacterial strains

A LAB strain having an anti-obesity effect, namely, *L. plantarum* Q180, was isolated from feces of healthy adults. In our previous study, *L. plantarum* Q180 was found to have lipase inhibitory activity of 83.61±2.32% and to inhibit the adipocyte differentiation of 3T3-L1 cells (14.63 ±1.37%) at a concentration of 100 μg/mL (Park *et al*., 2014). The strain was incubated in Lactobacilli MRS broth (Difco, USA) as the growth medium at 37°C for 18 h.

Anti-adipogenetic activity

Cell line and cell culture

3T3-L1 cells were cultured as described by Hemati *et al.* (1997). The 3T3-L1 cells were obtained from the American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) containing a high glucose content supplemented with 10% bovine calf serum (BCS; GIBCO, USA) and 1% penicillin/streptomycin (Sigma, USA) at 37°C in a humidified 5% CO_2 atmosphere. To induce differentiation, 2-d post-confluent cells (0 d) were stimulated for 2 d with an adipocyte differentiation cocktail medium containing 5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma, USA), 1 mM dexamethasone (Dex; Sigma, USA), and 5 g/mL of insulin (Sigma, USA) in DMEM supplemented with 10% fetal bovine serum (FBS; GIBCO, USA) and 1% penicillin/streptomycin. On 2 d, the medium was replaced with DMEM containing 10% FBS, 1% penicillin/streptomycin, and 5 g/mL of insulin, and incubated for 2 d, followed by culturing with DMEM containing 10% FBS and 1% penicillin/streptomycin for an additional 4 d (8 d), at the end of which more than 90% of the cells were mature adipocytes with accumulated fat droplets.

Cell viability

Cell viability was assessed by the MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay. The MTT assay was performed according to the modified method of Mosmann (1983). The 3T3-L1 preadipocytes were placed in 96-well microliter plates at a density of 16×10^4 cells/well. After 24 h incubation, the culture medium was replaced by 100 μL of serial dilutions (10, 100, 1000 mg/mL) of the sample, and the cells were incubated for 24 h. After incubation, 20 μL of sterile filtered MTT solution (5 mg/mL) in PBS (PBS, 0.85% NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4 and 1.76 mM KH_2PO_4 were dissolved in distilled water, pH 7.4) was added to each well. Unreacted dye was removed after 4 h incubation. Insoluble formazan crystals were dissolved in 100 μL/well of dimethyl sulfoxide (DMSO) and measured spectrophotometrically in an ELISA reader (BioTek, USA) at 550 nm (sample A). The non-treated cell was also dissolved in 100 μL/well of DMSO and the absorbance was recorded at 550 nm (control A). The percent viability was expressed using the following formula:

$$\text{Cell viability (\%)} = 100-\left[\left(\frac{\text{control A}-\text{sample A}}{\text{control A}}\right)\times 100\right]$$

Sample preparation and treatment *L. plantarum* Q180

L. plantarum Q180 was incubated at 37°C for 18 h in MRS broth. All of the purified strains were kept at 70°C until use. After culturing the *L. plantarum* Q180, all of the strains were harvested in a refrigerated centrifuge (1,500 *g* for 15 min at 4°C) and washed three times with distilled water to remove any remaining MRS broth. The washed *L. plantarum* Q180 was freeze-dried and re-suspended in distilled water at a concentration of 10 mg/mL and homogenized for 50 sec followed by 1 min of rest (repeated 3 times) using a sonicator. The 3T3-L1 cells were treated with 100 g/mL of the sample. The concentration of the sample was determined according to the result of the MTT assay.

Oil red O staining of 3T3-L1 adipocyte

Intracellular lipid accumulation was measured using oil red O (Sigma, USA). Oil red O staining of 3T3-L1 cells was performed using a modified version of the method described by Ramirez-Zacarias *et al.* (1992). 3T3-L1 cells were washed with PBS twice, fixed with 10% formaldehyde/PBS at 4°C for 1 h, and stained with filtered oil red O solution (stock solution: 3.5 mg/mL in isopropanol; working solution: 60% oil red O stock solution and 40% distilled water) at room temperature for 30 min. The quantification of lipid accumulation was achieved by the oil red O obtained from stained cells with isopropyl alcohol and measured spectrophotometrically at 520 nm. The material stained with oil red O was expressed on a per cell basis using the number of cells determined from similar plates. The percentage of the material stained with oil red O relative to the control wells containing the cell culture medium without compounds was calculated as 520 nm (Q180)/520 nm (control) × 100.

Table 1. Independent variables and their levels in the 3-factor, 3-level central composite rotatable design optimizing the incubation condition of *L. plantarum* Q180

Independent variables	Symbol	Level		
		-1	0	1
Skim milk powder (%)	X_1	9	10	11
Incubation temp. (°C)	X_2	34	37	40
Incubation time (h)	X_3	20	30.5	41

Anti-lipase activity

The method of determining lipase activity proposed by Lee *et al.* (1993) was modified. Pancreatic lipase activity was measured using porcine pancreatic lipase (Sigma, USA). 0.1 mg/mL of a sample solution dissolved in water, 0.167 mM *p*-Nitrophenylpalmitate (PNP; Sigma, USA) solution and 0.061 M (pH 8.5) Tris-HCl buffer were mixed in the well of a plate, and 0.3 mg/mL of the lipase solution was then added to start the enzyme reaction. After incubation at 25°C for 10 min, its absorbance was measured at 405 nm.

Experimental design

To optimize the fermentative condition of *L. plantarum* Q180, concentration of skim milk powder (%, X_1), incubation temperature (°C, X_2), and incubation time (h, X_3) were used as the independent factors. In this design there are three experimental levels: -1, 0, 1. The range and center point values of the three independent factors were chosen after a series of preliminary single factor experiments (Table 1). pH (pH, Y_1), anti-lipase activity (%, Y_2), and anti- adipogenetic activity (pH, Y_3) were selected as the dependent factors.

Response surface methodology

The central composite design (CCD) described by Box and Wilson (1951) was adopted for the optimization of the anti-obesity activity of *L. plantarum* Q180. The CCD in the experimental design consisted of 2^3 factorial points, six axial points (α=2), and three replicates of the central point (Table 2). Experimental runs were randomized in order to minimize the effects of unexpected variabilities in the observed responses.

Analysis of the data

The statistical analysis of the data and the multiple response optimizations were calculated by the desirability function of MINITAB statistical software (Version 13, Minitab Inc., USA). The statistical analysis was performed to fit the following quadratic polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j$$

Where Y represents the dependent variables (pH, anti-lipase activity and anti-adipogenetic activity), β_0 is constant, β_i, β_{ii}, β_{ij} are regression coefficients, and *Xi*, *Xj* are levels of the independent variables. Multiple response optimizations were performed to search for the condition

Table 2. Central composite design and responses of dependent variables for fermented milk with *Lactobacillus plantarum* Q180 to independent variables

Run No.	Coded levels of variables			Responsee		
	X_1	X_2	X_3	Y_1	Y_2	Y_3
1	-1	-1	-1	5.56	22.92	19.17
2	1	-1	-1	5.87	11.94	-2.39
3	-1	1	-1	4.70	46.49	13.73
4	1	1	-1	4.72	5.41	5.94
5	-1	-1	1	5.38	55.40	10.29
6	1	-1	1	5.44	51.05	-4.02
7	-1	1	1	4.14	67.44	12.28
8	1	1	1	4.29	33.47	5.58
9	-1.68179	0	0	4.4.	48.65	26.78
10	1.68179	0	0	4.63	56.54	-2.57
11	0	-1.68179	0	6.02	5.58	13.91
12	0	1.68179	0	4.72	47.20	5.76
13	0	0	-1.68179	5.40	21.75	30.04
14	0	0	1.68179	4.23	60.71	10.11
15	0	0	0	4.55	56.60	18.44
16	0	0	0	4.43	53.75	16.45
17	0	0	0	4.40	56.54	15.00

X_1: skim milk powder (%), X_2: incubation temp. (°C), X_3: incubation time (h); Y_1: pH, Y_2: anti-lipase activity (%), Y_3: anti-adipogenetic activity (%).

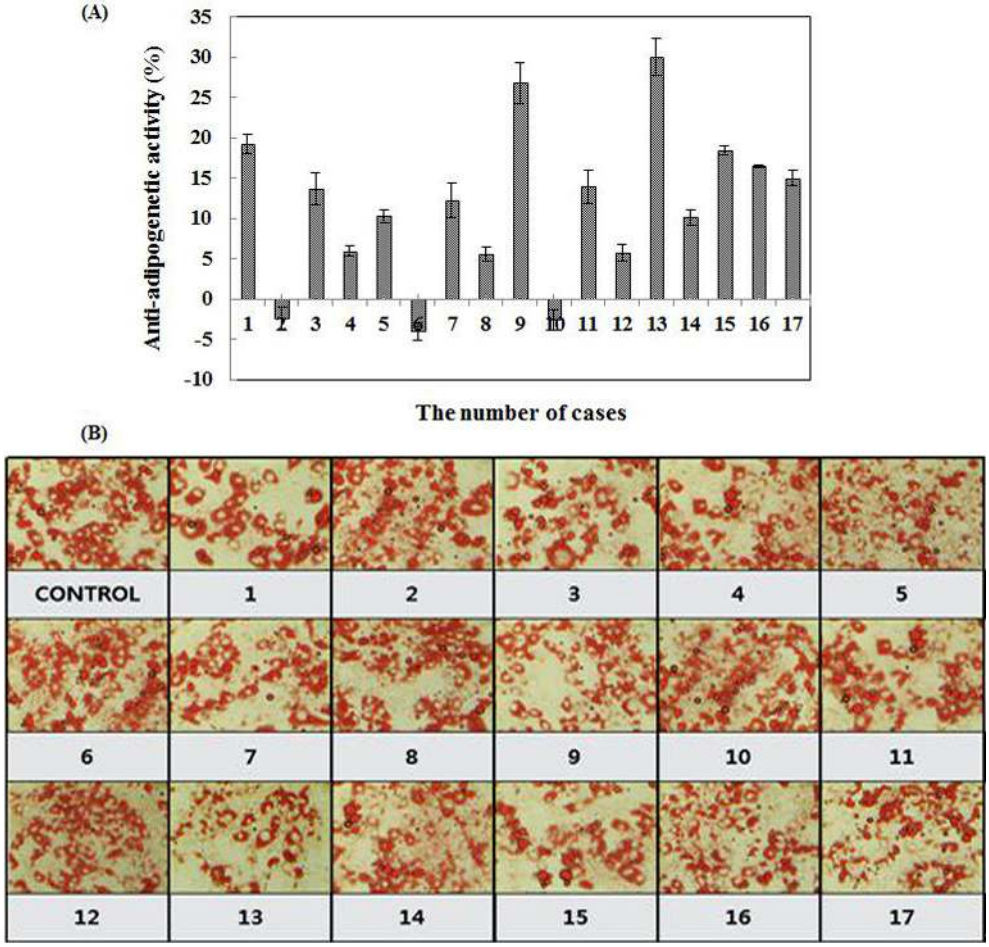

Fig. 1. The effects of *L. plantarum* Q180 on oil red O stained in 3T3-L1 adipocyte. (A) Anti-adipogenetic activity. All values are the mean±standard deviation of three replicates. (B) Photograph of oil red O staining. Cells were stained with oil red O observed by using a microscope (original magnification × 200).

that could simultaneously satisfy the three dependent variables (Y_1, Y_2 and Y_3). The response surface plots were developed using Maple software (Maple 7, Waterloo Maple Inc., Canada), and represented a function of two independent variables, while keeping the another independent variables at the optimal values.

Results and Discussion

Diagnostic checking of the fitted models

The pH, anti-lipase activity, and anti-adipogenetic activity were measured by the seventeen fermentation conditions (Table 2 and Fig. 1). MINITAB statistical software was employed to fit the quadratic polynomial equation to the experimental data. All the coefficients of linear (X_1, X_2, X_3), square (X_{11}, X_{22}, X_{33}) and interaction (X_{12}, X_{13}, X_{23}) were calculated with a *t*-statistic to determine their significance. The estimated coefficients of each model are presented in Table 3. As a result of the significance test, in the case of Y_1 (pH), X_2 and X_3 were found to be lower than the significance level (*p*-value) of 0.05 in the first-order term, and are thus statistically significant, and exercised great influence on the dependent variables. The cross-terms were not statistically significant, except for X_2X_2, and X_3X_3. In the case of Y_2 (anti-lipase activity, %), X_3 was significant and exercised a great influence on the dependent variables. It was shown that the cross-terms were not statistically significant, except for X_2X_2. In the case of Y_3 (anti-adipogenetic activity, %), X_1 showed significance and had a great influence on the dependent variables, and none of the cross-terms was statistically significant. The reaction equation obtained on the basis of the above results is shown in Table 4. A proper second-order polynomial expression model was obtained on the basis of the results of the response surface analysis. The coefficients of determination (R^2) for Y_1, Y_2 and

Table 3. Estimated effects and coefficients for pH, anti-lipase activity and anti-adipogenetic activity (coded units) about *Lactobacillus plantarum* Q180

Variable and interaction	Y_1		Y_2		Y_3	
	Coeffficient	*p* value	Coeffficient	*p* value	Coeffficient	*p* value
Intercept	4.45481	0.000	7.807	0.000	4.684	0.002
X_1	0.06417	0.216	-1.682	0.136	-4.276	0.004
X_2	-0.48227	0.000	1.778	0.119	0.034	0.974
X_3	-0.26124	0.001	4.061	0.005	-1.965	0.090
X_1X_1	0.04262	0.439	-0.433	0.678	-1.593	0.155
X_2X_2	0.33961	0.000	-2.941	0.022	-2.019	0.083
X_3X_3	0.14338	0.028	-1.521	0.172	-0.093	0.928
X_1X_2	-0.02500	0.679	-1.703	0.132	1.198	0.270
X_1X_3	-0.01500	0.815	0.392	0.707	0.467	0.655
X_2X_3	-0.04750	0.466	-0.644	0.540	0.487	0.641

X_1: skim milk powder (%), X_2: incubation temp. (°C), X_3: incubation time (h); Y_1: pH, Y_2: anti-lipase activity (%), Y_3: anti-adipogenetic activity (%).

Table 4. Response surface model for making condition

Responses	Quadratic polynomial model	R^2	*p*-value
Y_1	$Y_1=4.45481+0.06417X_1-0.48227X_2-0.26124X_3+0.04262X_1X_1+0.33961X_2X_2+0.14338X_3X_3-0.02500X_1X_2-0.01500X_1X_3-0.04750X_2X_3$	0.963	0.000
Y_2	$Y_2=55.784-5.646X_1+5.967X_2+13.628X_3-1.599X_1X_1-10.864X_2X_2-5.617X_3X_3-7.466X_1X_2+1.717X_1X_3-2.822X_2X_3$	0.835	0.000
Y_3	$Y_3=17.0408-7.3044X_1+0.0573X_2-3.3572X_3-2.9947X_1X_1-3.7956X_2X_2-0.1755X_3X_3+2.6731X_1X_2+1.0420X_1X_3+1.0874X_2X_3$	0.810	0.002

X_1: skim milk powder (%), X_2: incubation temp. (°C), X_3: incubation time (h); Y_1: pH, Y_2: anti-lipase activity (%), Y_3: anti-adipogenetic activity (%).

Table 5. Analysis of varience for pH, GABA concentrations (coded units) about *Lactobacillus plantarum* K154

	Source of variation	DF	SS	MS	F-value	*p*-value
Y_1	Main effects	9	5.57201	0.61911	20.39	0.000
	Linear	3	4.16466	1.38822	45.72	0.000
	Square	3	1.38249	0.46083	15.18	0.002
	2-way interactions	3	0.02485	0.00828	0.27	0.843
	Residual error	7	0.21254	0.03036		
	Lack of fit	5	0.19994	0.03999	6.35	0.042
	Total	16	5.78455			
Y_2	Main effects	9	5454.00	606.00	3.94	0.042
	Linear	3	3457.83	1152.62	7.50	0.014
	Square	3	1462.97	487.66	3.17	0.094
	2-way interactions	3	533.17	177.72	1.16	0.392
	Residual error	7	1076.48	153.78		
	Lack of fit	5	1071.19	214.24	280.98	0.012
	Total	16	6530.48			
Y_3	Main effects	9	1188.26	132.029	3.31	0.064
	Linear	3	882.63	294.210	7.38	0.014
	Square	3	230.32	76.774	1.93	0.214
	2-way interactions	3	75.31	25.103	0.63	0.619
	Residual error	7	279.01	39.859		
	Lack of fit	5	273.03	54.607	18.27	0.023
	Total	16	1467.27			

DF, Degrees of freedom; SS, sum of squares; MS, Mean square (MS=SS/DF).
Y_1: pH, Y_2: anti-lipase activity (%), Y_3: anti-adipogenetic activity (%).

Y_3 were 0.963, 0.935 and 0.810, respectively, which indicates that the model is suitable to represent the real relationships among the selected reaction parameters. The values of R_2 for all models were extremely high for the response surface and significant at p=0.00. The reason why the values obtained for R^2 are quite high is that the

experimental design was based on an adequately performed preliminary test.

Analysis of variance

The statistical significance of the quadratic polynomial model equation was evaluated by conducting an analysis of variance (ANOVA). Table 5 shows the ANOVA for the models that explain the response of the three dependent variables, Y_1 (pH), Y_2 (anti-lipase activity) and Y_3 (anti-adipogenetic activity). The square terms and 2-way interaction terms for the dependent variables (Y_1, Y_2 and Y_3) were not significant, except for the square terms of Y_1 (Y_1: p=0.002 and p=0.843, Y_2: p=0.094 and p=0.392, Y_3: p=0.214 and p=0.619, respectively) at the 95% probability level (p<0.05), whereas the linear term and total regression model were significant at the 95% probability level, except for the total regression model of Y_3. In this design, the data were highly influenced by the linear term. As regards the results of the lack-of-fit test, which indicates the fitness of the model, all the dependent variables were significant at the 95% probability level.

Conditions for optimum responses

In order to find the condition for optimum responses, the desirability function of the MINITAB statistical software was used. Optimal conditions included the coded and un-coded values of each dependent variable (Y_1, Y_2 and Y_3), which are shown in Table 6. The results of performing the optimization of fermented milk each under conditions showing the highest anti-lipase activity and anti-adipogenetic activity and targeting the pH 4.4 showed that the critical values were different in all data. However, the result of the optimization satisfying both conditions at the same time showed that the coded values of the independent variables were the concentration of skim milk powder, X_1=-0.4572; incubation temperature, X_2=0.0548; and incubation time, X_3=-0.2134; respectively. The actual values of the independent variables against the coded values were X_1=9.5%, X_2=37°C and X_3=28 h. The predicted values of multiple response optimal conditions were Y_1= pH 4.47, Y_2=55.55% and Y_3=20.48%.

Response surface plots and the effect of factors

Fig. 2 shows the estimated response function and the effect of the independent variables (X_1, X_2 and X_3) and dependent variables (Y_1, Y_2 and Y_3). The response surface plot presents the interrelationship between two independent variables and one dependent variable, while keeping another independent variable at the optimal values. It is considered that two factors, i.e. incubation temperature and incubation time, affect anti-lipase activity (Y_2) among the fermentation conditions for fermented milk with an excellent anti-obesity effect, and that all three independent variables affect the pH (Y_1) and anti-adipogenetic activity (Y_3).

Verification of predicted values

Verification experiments were conducted under optimal conditions (concentration of skim milk powder = 9.5%, incubation temperature = 37°C, and incubation time = 28 h) to compare the predicted values and the actual values of the dependent variables (Table 7). The actual values, which were repeated three times, were pH = 4.50, anti-lipase activity = 52.86%, and anti-adipogenetic activity = 19.25% against the predicted values of pH = 4.47, anti-lipase activity = 55.55%, and anti-adipogenetic activity =

Table 6. Optimal conditions of pH and GABA concentrations

Dependent	Independent variables	Critical value		Predicted value	Stationary point
		Coded	Uncoded		
Y_1	X_1	0	10	4.4	Target
	X_2	0	37		
	X_3	0.2419	33.0399		
Y_2	X_1	-1.6818	8.3182	67.42	Maximum
	X_2	1.4017	41.2051		
	X_3	0.6063	36.8661		
Y_3	X_1	-1.4677	8.4677	31	Maximum
	X_2	-0.5395	65.3815		
	X_3	-1.6818	12.8412		
Multiple response optimization	X_1	-0.4572	9.5428		
	X_2	0.0548	37.1644		
	X_3	-0.2134	28.2593		

X_1: skim milk powder (%), X_2: incubation temp. (°C), X_3: incubation time (h); Y_1: pH, Y_2: anti-lipase activity (%), Y_3: anti-adipogenetic activity (%).

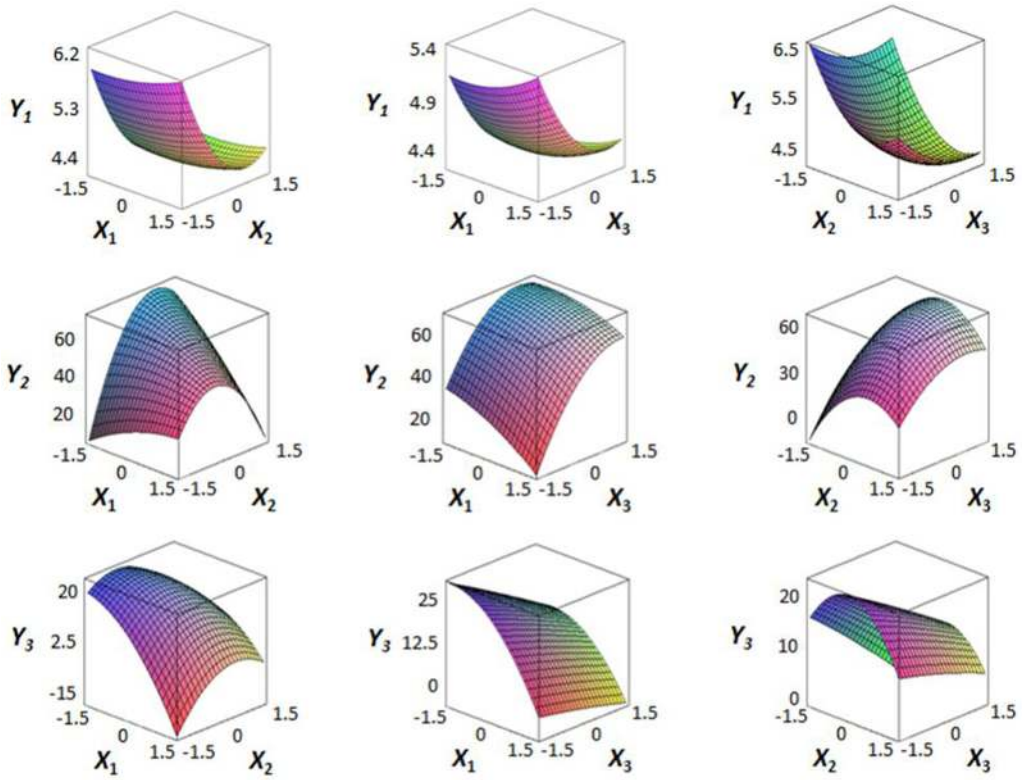

Fig. 2. Response surface plots for the effect of independent variables on dependent (pH, anti-lipase activity, and anti-adipogenetic activity). X_1: skim milk powder (%), X_2: incubation temp. (°C), X_3: incubation time (h); Y_1: pH, Y_2: anti-lipase activity (%), Y_3: anti-adipogenetic activity (%).

Table 7. Predicted results of verification under optimized conditions

Dependent	Predicted value	Experimental value
Y_1 (pH)	4.47	4.50±0.03
Y_2 (lipase, %)	55.55	52.86±0.86
Y_3 (adiposite, %)	20.48	19.25±0.53

All values are mean±standard deviation of three replicates.

20.48%. Both the actual values and the predicted values almost coincided with each other. According to Park *et al*. (2011), the lipid content in differentiated cells decreased by 11±3.6% when treated with 0.01% of *L. plantarum* KY1032, a strain isolated from kimchi. Therefore, the estimated response surface model has an excellent anti-obesity effect and can be adapted to optimize the production of functional fermented milk with an anti-obesity effect obtained from *L. plantarum* Q180.

Conclusion

We investigated the optimum condition for producing functional fermented milk with an anti-obesity effect by using *L. plantarum* Q180. We used a 3-factor, 3-level CCD combined with RSM. Concentrations of skim milk powder (%, X_1), incubation temperature (°C, X_2), and incubation time (h, X_3) were used as the independent factors, while pH (pH, Y_1), anti-lipase activity (%, Y_2) and anti-adipogenetic activity (%, Y_3) were used as the dependent factors. The optimal conditions of fermented milk for the highest anti-lipase and anti-adipogenetic activity with pH 4.4 were the 9.5% of skim milk powder, 37°C of incubation temperature, 28 h of incubation time. In the fermentation condition, the predicted values of pH, anti-lipase activity and anti-adipogenetic activity were 4.47, 55.55, and 20.48%, respectively. However, the actual values of pH, anti-lipase activity and anti-adipogenetic activity were 4.50, 52.86, and 19.25%, respectively. These results demonstrate that 9.5% of skim milk powder and incubation at 37°C for 28 h were the optimum conditions for producing functional fermented milk with an anti-obesity effect.

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