JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Optimization of Growth of *Lactobacillus acidophilus* FTCC 0291 and Evaluation of Growth Characteristics in Soy Whey Medium: A Response Surface Methodology Approach

WAI-YEE FUNG, YUH-PING WOO, AND MIN-TZE LIONG*

School of Industrial Technology, Food Technology Division, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

Four strains of probiotics were evaluated for their α-galactosidase activity. *Lactobacillus acidophilus* FTCC 0291 displayed the highest specific α-galactosidase activity and was thus selected to be optimized in soy whey medium supplemented with seven nitrogen sources. The first-order model showed that meat extract, vegetable extract, and peptone significantly ($P < 0.05$) influenced the growth of *L. acidophilus*. The second-order polynomial regression estimated that maximum growth was obtained from the combination of 7.25% (w/v) meat extract, 4.7% (w/v) vegetable extract, and 6.85% (w/v) peptone. The validation experiment showed that response surface methodology was reliable with a variation of only 1.14% from the actual experimental data. Increased utilization of oligosaccharides and reducing sugars contributed to increased growth of *L. acidophilus* in the soy whey medium. This was accompanied by increased production of short-chain fatty acids and a decrease in pH.

KEYWORDS: ***Lactobacillus*; growth; soy whey; RSM; optimization**

INTRODUCTION

Probiotics are defined as viable microorganisms that when administered in adequate amounts promote or support a beneficial balance of the autochthonous microbial population of the gastrointestinal tract (*1*). *Lactobacillus* and *Bifidobacterium* are the most common probiotic bacteria used as food adjuncts. A number of health benefits have been reported upon consumption of probiotic organisms including antimicrobial activity, alleviation of lactose intolerance, antidiarrheal properties, anticarcinogenic properties, and modulation of immune system and hypocholesterolemia (*2*). However, commercially available probiotic supplements are often costly, mainly due to their expensive production processes and media.

Soy is rich in protein and has been associated with hypocholesterolemia, reduced risks of atherosclerosis, cancer, hypertension, osteoporosis, and alleviation of postmenopausal symptoms. Strains of probiotics have been shown to grow well in soy milk, being able to utilize soy oligosaccharides and producing lower amounts of short-chain fatty acids (SCFA).

Approximately 225.6 million metric tons of soybeans is produced annually worldwide (*3*). Disposal of soybean wastes is often costly and conjures environmental issues. Soy whey is the liquid waste from pressing of coagulated soy milk, produced abundantly during the manufacture of tofu. Soy whey has a rich nutritive content comprising protein, fat, starch, and sugars. Most of the sugars comprise stachyose (6.4 g/L), raffinose (1.6 g/L), sucrose (11.3 g/L), fructose (1.1 g/L), and glucose (1.2 g/L) (*5*). Much of the oligosaccharides are carried in the whey, whereas the protein content is lower due to partial precipitation during the coagulation of soy milk. The total nitrogen was reported to be 0.82 g/L, suggesting a protein content of 5.1 g/L (*4*). Thus, with proper evaluation and development, soy whey could be utilized as a high-quality microbial fermentation medium to harvest various nutrients, enzymes, and microorganisms.

Response surface methodology (RSM) has become increasingly favorable in optimizing compositions of microbiological media, parameters for food processes, and enzyme hydrolysis. RSM is robust and effective in analyzing responses that are affected by many factors and their interactions. In optimization processes, RSM is less time-consuming and tedious compared to the conventional one-factor-at-a-time method (*5*).

Thus, the aim of this study was to optimize the growth of a probiotic in soy whey medium supplemented with nitrogen sources using RSM. In addition, the growth properties of the probiotic in soy whey medium such as pH, production of acids, and utilization of sugars within the optimized region for maximum growth were also evaluated.

MATERIALS AND METHODS

Bacteria and Medium Preparation. Strains of *Lactobacillus acidophilus* FTCC 0291, *Lactobacillus casei* FTCC 0442, *Lactobacillus fermentum* FTD 13, and *Bifidobacterium bifidum* BB12 were obtained

* Corresponding author (telephone +604 653 2114; fax +604 657 3678; e-mail mintze.liong@usm.my).

10.1021/jf801567j CCC: $40.75
Published on Web 08/08/2008

from the Culture Collection Centre of Universiti Sains Malaysia. *L. acidophilus* ATCC 4962 was purchased from the Australian Starter Culture Research Center (ASCRC), Werribee, Australia. The cultures were grown in sterile de Mann, Rogosa, Sharpe (MRS) broth supplemented with filter-sterilized (0.45 μm) L-cysteine hydrochloride 0.05% (w/v) (Bioshop, Burlington, Canada) at 37 °C for 24 h. The organisms were activated three successive times prior to use.

Soy whey supplemented with 0.1% (v/v) Tween 80 was used as the base medium. Seven types of nitrogen sources were used in the screening stage including peptone (Sigma-Aldrich, Steinheim, Germany), tryptone (Fluka Biochemika, Neu-ulm, Switzerland), meat extract (Merck, Darmstadt, Germany), vegetable extract (Fluka Biochemika), yeast extract (Merck), urea (Fluka Biochemika), and ammonia (Merck).

α-Galactosidase Activity. Soy is rich in α-galactoside sugars, and strains of probiotic with the ability to produce α-galactosidase would proliferate better in soy-based growth medium (*6*). Strains were thus screened for their α-galactosidase activities. Grown cells were harvested by centrifugation (10000*g*), and the cell pellet was sonicated (30 min). Cell debris was removed from the sonicated suspension, and the resulting supernatant was used for analysis as crude enzyme extract. The crude enzyme extracts were assayed for α-galactosidase activity according to the methods of Scalabrini et al. (*7*). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from pNPG per milliliter per minute under assay conditions. The protein concentration of the crude enzyme extract was determined using the Bradford method (*9*). The specific activity of each strain was expressed in units as activity of α-galactosidase per milligram of protein. The strain with the highest specific activity was selected for subsequent optimization experiments.

Measurement of Growth. Growth of probiotics was determined by measuring the optical density at 600 nm. The supernatant was reserved for analyses of reducing sugar, pH, and SCFA.

Determination of pH. The pH of samples was measured using the DELTA 320 pH-meter (Mettler Toledo, Shanghai, China).

Determination of Titratable Acidity. The concentrations of lactic and acetic acids produced in the soy whey were determined individually according to the titratable acidity method (*9*) with some modifications. Briefly, the supernatant was neutralized using 0.01 N sodium hydroxide (NaOH). The end point of titration (pH 7.0) was determined using a pH-meter. The volume of 0.01 N NaOH used was recorded, and the amount of acids produced was determined using the following equation:

$$\text{acid (mg mL}^{-1}) = \frac{\text{base normality (mequiv/mL)} \times \text{base vol (mL)} \times \text{equiv wt of acid (mg/equiv)} \times 10}{\text{sample wt (mg)}}$$

Assay for Reducing Sugars and Oligosaccharides. The concentration of reducing sugars was determined according to the dinitrosalicyclic acid (DNS) assay (*10*). The concentration of oligosaccharides was determined by comparing the content of hydrolyzed reducing sugars with that of nonhydrolyzed reducing sugars. To hydrolyze the oligosaccharides, 2 mL of the supernatant was added to 1% (v/v) enzyme solution containing α-amylase amyloglucosidase (Stargen; Genencor, Rochester, NY). The treated samples were assayed to determine their reducing sugar content.

Experimental Design and Statistical Analyses. Screening experiments were performed to select the significant nitrogen sources on probiotic growth. Seven independent factors were used, namely, peptone (X_1), tryptone (X_2), yeast extract (X_3), meat extract (X_4), vegetable extract (X_5), ammonia (X_6), and urea (X_7). All nitrogen sources were purchased from Sigma-Aldrich. Screening was performed using a two-level partial factorial design (2^7-2^2) resulting in 64 experimental runs (including duplicates) and 5 middle-point runs. A first-order empirical equation was used to exclude insignificant factors and to generate the steepest ascent. Optimization was performed using a rotatable central composite design (CCD). The modeling and statistical analyses were performed using Design Expert 5 software version 5.07 (Stat-Ease Corp., Minneapolis, MN). One-way ANOVA was employed to analyze statistical difference between samples of the α-galactosidase analysis (SPSS Inc., version 11.5, Chicago, IL). A statistical level of significance was preset at $\alpha = 0.05$. Multiple comparisons of means were performed using Tukey's test. All data presented were mean values of duplicates, obtained from two separate runs.

A validation experiment was conducted to confirm the validity and reproducibility of the model. The growth was assessed using the optimum-point, center-point, high-point, and low-point media that were produced from the prediction. Growth was also compared with the commercial MRS medium.

RESULTS AND DISCUSSION

α-Galactosidase Activity. The main sugars of soy whey are stachyose, raffinose, sucrose, fructose, and glucose (*4*). The sucrose moiety in raffinose and stachyose is linked by α-1,6 bonds to one (raffinose) and two (stachyose) units of galactose. α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) catalyzes the cleavage of terminal α-1,6-linked galactosyl residues from a wide range of substrates, including raffinose, stachyose, and synthetic substrates such as *p*-nitrophenyl-α-D-galactopyranoside (*13*). Raffinose and stachyose are broken down to glucose, galactose, and fructose by α-galactosidase prior to use as microbial substrates. Therefore, a high α-galactosidase activity exhibited by probiotic strains would indicate better proliferation in soy-based medium (*6*). The α-galactosidase screening of probiotics thus was conducted on the basis of this prerequisite for growth.

All strains of probiotic studied exhibited various degrees of the enzyme activities (**Table 1**). The highest activity of *L. casei* FTCC 0442 was achieved at 12 h, and those of *L. fermentum* FTD 13 and *B. bifidum* BB 12 at were achieved at 16 h, whereas the highest activity of *L. acidophilus* ATCC 4962 was achieved at 24 h. *L. acidophilus* FTCC 0291 recorded the highest activity ($P < 0.05$) compared to the other strains studied, and this was achieved at 4 h. The total intracellular protein showed a constant trend for all of the strains studied, indicating a relatively similar enzyme production rate except for *B. bifidum* BB 12, which showed an increase at 12 h. The total intracellular protein also did not differ within strains over the 24 h period.

The specific intracellular α-galactosidase activity of *L. fermentum* FTD 13 and *B. bifidum* BB 12 increased after 16 h, as did that of *L. casei* FTCC 0442 after 20 h, whereas *L. acidophilus* ATCC 4962 showed an increase at the end of the 24 h period. The specific intracellular α-galactosidase activity of *L. acidophilus* FTCC 0291 showed a consistent increase after 4 h before decreasing at 24 h and was highest ($P < 0.05$) compared to the other strains studied. Thus, *L. acidophilus* FTCC 0291 was selected for subsequent optimization experiments.

Optimization of Growth. *Screening of Nitrogen Sources.* The total nitrogen content of soy whey was reported to be low (0.82 g/L) (*5*) compared to that of the commercial MRS medium (1.94 g/L of nitrogen). Thus, supplementation of soy whey with nitrogen sources is needed to promote probiotic growth. Screening was conducted using a two-level partial factorial design (2^7-2^2). A complete replication of the 2^7 design would involve 128 experimental runs. However, only 7 degrees of freedom are needed to estimate the main effects and 21 degrees of freedom to estimate the 2-factor interaction effects, whereas the remaining 99 degrees of freedom would estimate the error and/or 3- or higher-factor interaction effects (*14*). Assuming that certain high-order interactions were negligible, a partial factorial design could be used to reduce the number of experimental runs, identify the important factors, and determine interaction between factors without loss of information on the main effects and their interactions (*15*).

Table 1. α-Galactosidase Activity of Selected Probiotic Microorganisms Studied in Supplemented Media at 37 °C for 24 h

strain	time[a,b] 0 h	4 h	8 h	12 h	16 h	20 h	24 h
				Total Activity (Units per Milliliter)			
L. acidophilus FTCC 0291	0.03 ± 0.00^{Ca}	0.38 ± 0.00^{Aa}	0.36 ± 0.18^{Aa}	0.15 ± 0.01^{Ba}	0.36 ± 0.11^{Aa}	0.27 ± 0.02^{ABa}	0.03 ± 0.00^{Cb}
L. casei FTCC 0442	0.01 ± 0.00^{Ba}	0.03 ± 0.01^{Bb}	0.07 ± 0.02^{Bb}	0.10 ± 0.03^{ABa}	0.22 ± 0.07^{Ab}	0.27 ± 0.14^{Aa}	0.19 ± 0.09^{Aab}
L. fermentum FTD 13	0.02 ± 0.00^{Ba}	0.03 ± 0.00^{Bb}	0.05 ± 0.01^{Bb}	0.05 ± 0.02^{Bab}	0.21 ± 0.01^{Ab}	0.31 ± 0.10^{Aa}	0.04 ± 0.01^{Bb}
B. bifidum BB 12	0.01 ± 0.00^{Ca}	0.02 ± 0.01^{Cb}	0.14 ± 0.02^{BCb}	0.13 ± 0.14^{BCa}	0.28 ± 0.01^{ABab}	0.38 ± 0.01^{Aa}	0.05 ± 0.00^{Cb}
L. acidophilus ATCC 4962	0.01 ± 0.00^{Ba}	0.03 ± 0.04^{Bb}	0.03 ± 0.00^{Bb}	0.02 ± 0.02^{Bb}	0.01 ± 0.00^{Bc}	0.02 ± 0.02^{Bb}	0.26 ± 0.00^{Aa}
				Total Protein (Milligrams per Milliliter)			
L. acidophilus FTCC 0291	0.02 ± 0.00^{Aa}	0.02 ± 0.00^{Aa}	0.03 ± 0.00^{Aa}	0.05 ± 0.00^{Aa}	0.05 ± 0.04^{Aa}	0.03 ± 0.00^{Aa}	0.03 ± 0.02^{Aa}
L. casei FTCC 0442	0.01 ± 0.00^{Aa}	0.04 ± 0.00^{Aa}	0.04 ± 0.00^{Aa}	0.05 ± 0.01^{Aa}	0.06 ± 0.01^{Aa}	0.03 ± 0.00^{Aa}	0.03 ± 0.02^{Aa}
L. fermentum FTD 13	0.01 ± 0.00^{Aa}	0.04 ± 0.00^{Aa}	0.04 ± 0.00^{Aa}	0.05 ± 0.00^{Aa}	0.07 ± 0.02^{Aa}	0.05 ± 0.00^{Aa}	0.03 ± 0.00^{Aa}
B. bifidum BB 12	0.01 ± 0.00^{Ba}	0.05 ± 0.00^{ABa}	0.09 ± 0.01^{ABa}	0.13 ± 0.06^{Aa}	0.04 ± 0.00^{ABa}	0.03 ± 0.01^{ABa}	0.03 ± 0.01^{ABa}
L. acidophilus ATCC 4962	0.01 ± 0.00^{Aa}	0.04 ± 0.00^{Aa}	0.07 ± 0.00^{Aa}	0.05 ± 0.00^{Aa}	0.07 ± 0.02^{Aa}	0.06 ± 0.00^{Aa}	0.04 ± 0.00^{Aa}
				Specific Activity (Units per Milligram of Protein)			
L. acidophilus FTCC 0291	2.40 ± 0.20^{Ba}	16.90 ± 1.86^{Aa}	10.96 ± 4.85^{Aa}	3.19 ± 0.21^{ABa}	8.75 ± 7.17^{Aa}	10.24 ± 0.08^{Aa}	1.68 ± 1.37^{Bb}
L. casei FTCC 0442	0.93 ± 0.00^{Bb}	0.76 ± 0.22^{Bb}	1.69 ± 0.65^{Bb}	2.10 ± 1.09^{Bab}	3.90 ± 0.17^{Bb}	10.54 ± 3.85^{Aa}	7.28 ± 1.38^{ABa}
L. fermentum FTD 13	1.60 ± 0.32^{Bab}	0.86 ± 0.01^{Bb}	1.08 ± 0.32^{Bb}	0.90 ± 0.42^{Bb}	3.08 ± 0.92^{Ab}	6.75 ± 2.59^{Ab}	1.41 ± 0.57^{Bb}
B. bifidum BB 12	1.05 ± 0.26^{Bb}	0.45 ± 0.13^{Bb}	1.49 ± 0.17^{Bb}	1.44 ± 1.74^{Bb}	7.81 ± 0.59^{Aa}	9.57 ± 0.21^{Aa}	1.52 ± 0.36^{Bb}
L. acidophilus ATCC 4962	1.01 ± 0.09^{Bb}	0.59 ± 0.80^{Bb}	0.45 ± 0.00^{Bb}	0.24 ± 0.23^{Bb}	0.14 ± 0.00^{Bc}	0.36 ± 0.29^{Bc}	7.21 ± 0.26^{Aa}

[a] Results are expressed as means ± standard deviation; values are means of duplicates from two separate runs; $n = 2$. Means in the same row followed by different upper case letters are significantly different ($P < 0.05$). Means in the same column followed by different lower case letters are significantly different ($P < 0.05$) [b] α-Galactosidase activity from cell-free extracts of selected probiotic strains grown on sterile MRS broth supplemented with 5% (w/v) L-cysteine·HCl.

Table 2. Nitrogen Sources and Their Respective Nitrogen Contents

nitrogen source	nitrogen content (% w/w)
peptone	5.4
tryptone	12.0
meat extract	12.0
vegetable extract	10.0
yeast extract	10.0
urea	45.0
ammonia[a]	25.0

[a] Ammonia: nitrogen content % v/v.

The nitrogen sources used were varied over two levels, namely, the high concentration (+1) and the low concentration (−1), with five replicates of center points (0). Each of the nitrogen sources varied in nitrogen content (**Table 2**) and, thus, all seven nitrogen sources were standardized to provide similar total nitrogen contents prior to use. Therefore, growth would be influenced only by the type of nitrogen source, each containing equal amounts of nitrogen. The high level (+1) was set at 0.03% (w/v) nitrogen, whereas the low level was set at 0% (w/v) nitrogen for each factor. These levels were selected on the basis of the nitrogen content of the commercial MRS medium, which contained approximately 0.2% (w/w) nitrogen. When all seven factors were at their high concentration (+1), the total nitrogen content added would be approximately 0.21% (w/v). The coded values of factors and the response for growth (OD_{600}) from the screening experiment are shown in **Table 3**.

The analysis of variance (ANOVA) for the first model indicated that the model used was significant ($P < 0.0001$) with 25.86% variation not explained by the model. The insignificant lack of fit ($P = 0.9998$) and the acceptable coefficient of regression showed that the model represented the data adequately. Of the seven factors studied, only meat extract (X_1; $P < 0.0001$), vegetable extract (X_2; $P = 0.0001$), and peptone (X_3; $P = 0.0093$) significantly influenced the growth of *L. acidophilus* FTCC 0291 in the soy whey medium. Other factors were insignificant and hence were excluded from further optimization procedures. The following first-order equation (coded term) was generated for the response of growth (Y), with the significant factors now redefined as meat extract (X_1), vegetable extract (X_2), and peptone (X_3):

$$Y = 0.021 + 5.859 \times 10^{-4} X_1 + 3.234 \times 10^{-4} X_2 + 2.109 \times 10^{-4} X_3 \quad (1)$$

Steepest Ascent. Considering that meat extract produced the most influential effect with the highest coefficient estimate, it was subsequently used as the fundamental scale for the steepest ascent experiment. This determined the path of steepest ascent, and movement was generated along that path until no improvement occurred. The steepest ascent design was based on an increase of 0.03% (w/v) nitrogen concentration for X_1. On the basis of eq 1, the movement for X_2 was 0.55 design unit (3.234/5.859 = 0.55) and that for X_3 was 0.36 design unit (2.109/5.859) = 0.36). The steepest ascent coordinates and growth response obtained are presented in **Table 4**. The growth in terms optical density (OD_{600}) increased with each step of steepest ascent until maximum growth was achieved at step 7, and a decrease was observed beyond that. The highest OD_{600} was achieved at 5.15, from the combination of meat extract (6.38% w/v), vegetable extract (4.28% w/v), and peptone (5.28% w/v). This combination was used as the middle point for the subsequent optimization experiment.

Optimization of Growth. Optimization was conducted using a standard central composite design (CCD) with fixed middle points of meat extract (6.38% w/v), vegetable extract (4.28% w/v), and peptone (5.28% w/v). An α value of ±1.682 was used to produce design rotatability. Rotatability implies that the variation in the response predicted by the model will be constant at any given point from the center of the design. Such stability of prediction variance provides insurance that the quality of predicted values was approximately the same throughout the region of interest. The rotatable CCD is a near spherical design with points approximately a distance of $\sqrt{k}$ ($k = 3$) from the center of the design (*16*). The design matrix and response for growth are shown in **Table 5**.

The regression analyses (**Table 6**) indicated that the quadratic model was significant ($P < 0.0001$) and the model accurately represented the data in the experimental region. The coefficient of regression supported the sufficiency of the second-order terms

Table 3. Treatment Combinations and Response for the Screening Experiment

	coded factor level[a,b]							
standard order	X_1: peptone	X_2: tryptone	X_3: yeast extract	X_4: meat extract	X_5: vegetable extract	X_6: ammonia	X_7: urea	growth (OD_{600})[c]
1	−1	−1	−1	−1	−1	1	1	2.11
2	1	−1	−1	−1	−1	−1	−1	2.10
3	−1	1	−1	−1	−1	−1	−1	1.94
4	1	1	−1	−1	−1	1	1	1.99
5	−1	−1	1	−1	−1	−1	1	1.96
6	1	−1	1	−1	−1	1	−1	2.01
7	−1	1	1	−1	−1	1	−1	2.10
8	1	1	1	−1	−1	−1	1	2.14
9	−1	−1	−1	1	−1	−1	−1	2.01
10	1	−1	−1	1	−1	1	1	2.09
11	−1	1	−1	1	−1	1	1	2.09
12	1	1	−1	1	−1	−1	−1	2.01
13	−1	−1	1	1	−1	1	−1	2.10
14	1	−1	1	1	−1	−1	1	2.12
15	−1	1	1	1	−1	−1	1	2.01
16	1	1	1	1	−1	1	−1	2.01
17	−1	−1	−1	−1	1	1	−1	2.11
18	1	−1	−1	−1	1	−1	1	2.20
19	−1	1	−1	−1	1	−1	1	1.91
20	1	1	−1	−1	1	1	−1	1.93
21	−1	−1	1	−1	1	−1	−1	1.95
22	1	−1	1	−1	1	1	1	2.04
23	−1	1	1	−1	1	1	1	1.96
24	1	1	1	−1	1	−1	−1	2.05
25	−1	−1	−1	1	1	−1	1	2.11
26	1	−1	−1	1	1	1	−1	2.20
27	−1	1	−1	1	1	1	−1	2.21
28	1	1	−1	1	1	−1	1	2.36
29	−1	−1	1	1	1	1	1	2.28
30	1	−1	1	1	1	−1	−1	2.19
31	−1	1	1	1	1	−1	−1	2.22
32	1	1	1	1	1	1	1	2.23
33	0	0	0	0	0	0	0	2.02

[a] X_1: peptone (0−0.56% w/v); X_2: tryptone (0−0.25% w/v); X_3: yeast extract (0−0.30% w/v); X_4: meat extract (0−0.25% w/v); X_5: vegetable extract (0−0.30% w/v); X_6: ammonia (0−0.12% v/v); X_7: urea (0−0.07% w/v). [b] Nitrogen concentration for all nitrogen sources ranged between 0 and 0.03% (w/v nitrogen). [c] Results are means of duplicate values ($n = 2$), except for the center point (mean of five replicates).

Table 4. Steepest Ascent Coordination Path for All Chosen Factors

		coded factors[a]			natural factors[b] (% w/v)			growth
step		ε_1	ε_2	ε_3	X_1	X_2	X_3	(OD_{600})[c]
1	base	0	0	0	0.13	0.15	0.28	2.42
2	base + Δ	1.00	0.55	0.36	0.25	0.23	0.38	2.90
3	base + 10Δ	10.00	5.50	3.60	1.38	0.98	1.28	3.70
4	base + 20Δ	20.00	11.00	7.20	2.63	1.81	2.28	4.58
5	base + 30Δ	30.00	16.50	10.80	3.88	2.64	3.28	4.68
6	base + 40Δ	40.00	22.00	14.40	5.13	3.47	4.28	4.95
7	base + 50Δ	50.00	27.50	18.00	6.38	4.28	5.28	5.15
8	base + 60Δ	60.00	33.00	21.60	7.63	5.13	6.28	4.13
9	base + 70Δ	70.00	38.50	25.20	8.88	5.96	7.28	3.79
10	base + 80Δ	80.00	44.00	28.80	10.13	6.79	8.28	3.32
11	base + 90Δ	90.00	49.50	32.40	11.38	7.62	9.28	2.27
12	base + 100Δ	100.00	55.00	36.00	12.63	8.45	10.28	1.32

[a] ε_1: meat extract; ε_2: vegetable extract; ε_3: peptone. [b] X_1: meat extract; X_2: vegetable extract; X_3: peptone. [c] All results are means of duplicates from two separate runs; $n = 2$; all data were obtained upon regulation of dilution factors.

and showed that only 15.5% of total variation was unexplained by the model.

Meat extract (X_1) and peptone (X_2) showed significant linear effects ($P < 0.01$) on the growth of *L. acidophilus* FTCC 0291. Quadratic effects of all factors were significant, whereas only

Table 5. Combination Matrix of the CCD Using Coded Levels for the Factors and Seven Responses

					responses[a,b]					
std run	block[c]	X_1[d]	X_2[d]	X_3[d]	Y_0	Y_1	Y_2	Y_3	Y_4	Y_5
1	1	−1	−1	−1	4.27	4.28	7.18	4.79	1.94	9.17
2	1	1	−1	−1	4.79	4.57	7.90	5.27	0.24	2.52
3	1	−1	1	−1	5.09	4.44	7.27	4.85	1.70	8.15
4	1	1	1	−1	5.39	4.71	7.40	4.93	0.65	4.56
5	1	−1	−1	1	5.57	4.25	10.34	6.89	0.50	3.45
6	1	1	−1	1	5.68	4.45	11.07	7.38	0.64	4.41
7	1	−1	1	1	5.30	4.39	9.76	6.51	1.19	6.20
8	1	1	1	1	5.93	4.57	10.74	7.16	0.18	1.80
9	1	0	0	0	5.88	4.50	9.37	6.25	0.50	2.82
10	1	0	0	0	5.92	4.38	10.09	6.73	0.50	2.82
11	2	−α	0	0	5.36	4.18	5.73	3.82	1.42	6.41
12	2	α	0	0	5.87	4.60	9.62	6.42	0.74	4.62
13	2	0	−α	0	5.86	4.26	9.61	6.41	1.17	6.05
14	2	0	α	0	5.67	4.53	9.35	6.23	0.57	4.23
15	2	0	0	−α	4.60	4.62	6.49	4.32	0.82	4.89
16	2	0	0	α	6.30	4.46	9.84	6.56	0.89	5.25
17	2	0	0	0	5.89	4.38	10.06	6.71	0.83	4.89

[a] Y_0: growth (OD_{600}), Y_1 = pH, Y_2 = lactic acid (mg mL^{-1}), Y_3 = acetic acid (mg mL^{-1}), Y_4 = oligosaccharides (mg mL^{-1}), Y_5 = reducing sugars (mg mL^{-1}). [b] All results are means of duplicates from two separate runs; $n = 2$. [c] 1, first day of experiment; 2, second day of experiment. [d] X_1: meat extract [3.88−8.88% (w/v); $\pm\alpha = 2.18-10.58\%$ (w/v)]; X_2: vegetable extract [2.63−5.93% (w/v); $\pm\alpha = 1.5-7.05\%$ (w/v)]; X_3: peptone [3.28−7.28% (w/v); $\pm\alpha = 1.91-8.65\%$ (w/v)].

Table 6. Regression Coefficients of the Second-Order Equation for the Five Responses[a]

	values for indicated responses					
coefficient[b]	Y_0	Y_1	Y_2	Y_3	Y_4	Y_5
c	5.946	4.410	0.976	0.650	0.623	3.590
c_1	0.177[c]	0.122[c]	0.067[c]	0.044[c]	−0.350[c]	−1.225[c]
c_2	0.077	0.074[c]	−0.013	−0.009	−0.046	−0.140
c_3	0.424[c]	−0.044[c]	0.130[c]	0.087[c]	−0.139[c]	−0.581[c]
c_{11}	−0.176[c]	−0.007	−0.063[c]	−0.042[c]	0.150[c]	0.638[c]
c_{22}	−0.122[c]	−0.005	0.001	0.000	0.077	0.505
c_{33}	−0.235[c]	0.046[c]	−0.046[c]	−0.030[c]	0.069	0.479
c_{12}	0.037	−0.007	−0.004	−0.003	−0.063	−0.289
c_{13}	−0.010	−0.022	0.011	0.007	0.235[c]	0.851[c]
c_{23}	−0.181[c]	−0.006	−0.006	−0.004	0.008	−0.109
R^2	0.8450	0.9404	0.8564	0.8564	0.7178	0.6353
P value	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	0.0018

[a] Y_0 = growth (OD_{600}), Y_1 = pH, Y_2 = lactic acid (mg mL^{-1}), Y_3 = acetic acid (mg mL^{-1}), Y_4 = oligosaccharides (mg mL^{-1}), Y_5 = reducing sugars (mg mL^{-1}). [b] c = intercept (estimated response at the center point with coded values of X_1, X_2, and X_3 set at 0; c_1, c_2, and c_3 = regression coefficients for meat extract, vegetable extract, and peptone, respectively. [c] Significant at an α level of 0.05.

the interaction of vegetable extract−peptone (X_2X_3) was significant ($P < 0.01$) on the growth of *L. acidophilus* FTCC 0291. Also, the coefficient estimates of all the quadratic terms (c_{11}, c_{22}, c_{33}) had negative signs, implying that the parabola would open downward, leading to a maximum point of the model. It must also be noted that the coefficient estimate of peptone (X_3) was highest, indicating the strongest effect.

The best explanatory second-order model equation to produce the response surface is expressed as

$$Y_0 = c + c_1X_1 + c_2X_2 + c_3X_3 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{12}X_1X_2 + c_{13}X_1X_3 + c_{23}X_2X_3 \quad (2)$$

where c_i, c_{ii}, and c_{ij} are regression coefficients and X_1, X_2, and X_3 are the coded independent factors. Intercept c is the estimated response at the center point with coded values of X_1, X_2, and X_3 set at 0.

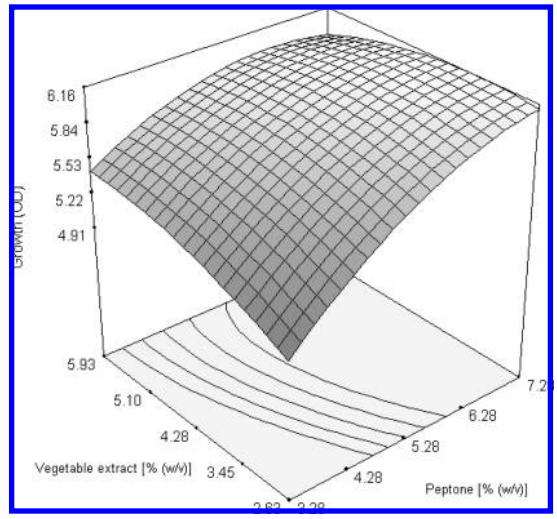

Figure 1. Response surfaces for growth (OD) of *L. acidophilus* FTCC 0219 in soy whey media from the effects of vegetable extract and peptone at 6.38% (w/v) meat extract.

Table 7. Treatment Combinations and Response for Validation Experiment

	natural factors[a] (% w/v)			nitrogen content	growth	variation
medium	X_1	X_2	X_3	(% w/v)	(OD_{600})[b]	(%)[c]
optimum point	7.25	4.7	6.85	1.710	6.08 ± 0.04^{A}	1.14
high point	8.88	5.93	7.28	2.051	4.44 ± 0.01^{E}	25.25
center point	6.38	4.28	5.28	1.478	5.08 ± 0.00^{C}	14.56
low point	3.88	2.63	3.28	0.905	5.46 ± 0.01^{B}	15.43
MRS	0.80	NA[d]	0.74	1.940[e]	4.66 ± 0.10^{D}	NA

[a] X_1, meat extract; X_2, vegetable extract; X_3, peptone. [b] Highest growth obtained over 24 h. All results are means of duplicates: $n = 2$; readings were adjusted to account for dilution factors. Means in the same column followed by different upper case letters were significantly different ($P < 0.05$). [c] Variation of experimental values from the prediction by the model. [d] MRS did not contain vegetable extract; thus, a prediction could not be established. [e] Nitrogen content was obtained from information provided by the supplier.

The second-order regression model involved three factors, thus producing three linear, three quadratic, and three interaction terms. The optimum response surface for growth (**Figure 1**) was generated on the basis of the following second-order equation:

$$Y_0 = 5.95 + 0.18X_1 + 0.078X_2 + 0.42X_3 - 0.18{X_1}^2 - 0.12{X_2}^2 - 0.24{X_3}^2 + 0.037X_1X_2 - 9.906 \times 10^{-3}X_1X_3 - 0.18X_2X_3 \quad (3)$$

The response surface was produced by plotting two factors at a time while the unrepresented factor was fixed at its actual optimum level. The vertical axis represented the response (growth OD_{600}), whereas the two horizontal axes represented the actual levels of two explanatory factors. An optimum point was produced with maximum growth (OD_{600}) obtained at 6.15 at a combination of 7.25% (w/v) meat extract, 4.7% (w/v) vegetable extract, and 6.85% (w/v) peptone.

The response surfaces of growth (**Figure 1**) indicated that growth was high at high levels of peptone and meat and vegetable extracts. The coefficient estimates (**Table 6**) indicated that the only significant interaction term was obtained from the combination of vegetable extract−peptone. This interaction produced a negative coefficient ($X_2X_3 = -0.18$). This implies that for an increase in the response of growth, the level of one factor must be increased, whereas the other must be decreased. The response surface showed that growth increased at the regions of increasing level of peptone and low level of vegetable extract (**Figure 1**).

Validation Experiments. The reliability of the model used was evaluated via validation. The growth patterns were compared over a 24 h period using five different media, namely, the high-point medium (all factors were held at their respective highest concentrations), low-point medium (all factors were held at their respective lowest concentrations), center-point medium (all factors were held at their respective middle concentrations), optimum medium (all factors were held at their respective optimum concentrations), and MRS medium (**Table 7**). The highest growth obtained from each medium over the 24 h period showed that the highest growth ($P < 0.05$) was obtained from the optimum-point medium (6.08) followed by low-point (5.46), center-point (5.08), MRS medium (4.66), and high-point (4.435) media.

Both the optimum- and center-point media produced high growths, significantly ($P < 0.05$) exceeding that obtained from the MRS medium. The growth performance of *L. acidophilus* FTCC 0291 in the low-point medium was significantly ($P < 0.05$) higher than those in center- and high-point media. Despite the differences, the performance of the optimum medium, with 1.14% variation from the prediction, indicated that the predictions generated from the model were reliable in optimizing the growth of *L. acidophilus* FTCC 0291 in soy whey medium.

Analyses of pH, utilization of sugars, and production of acids were conducted to evaluate the growth performance of *L. acidophilus* FTCC 0291 within the regions of optimized growth. The results of the analyses are as presented in **Table 5**, whereas the coefficient estimates are shown in **Table 6**.

Evaluation of Growth Characteristics. *pH and Titratable Acidity.* Anaerobic fermentation of lactic acid bacteria produces SCFA, which reduces the pH of the medium. A rapid decline in the pH of the medium could indicate an active metabolism. Thus, the pH of the medium in the region of optimal growth was analyzed as a growth characteristic of *L. acidophilus* in soy whey.

The response surfaces of pH (Y_1) (**Figure 2**) were generated on the basis of the following equation:

$$Y_1 = 4.41 + 0.122X_1 + 0.074X_2 - 0.044X_3 - 0.007{X_1}^2 - 0.005{X_2}^2 + 0.046{X_3}^2 - 0.007X_1X_2 - 0.022X_1X_3 - 0.006X_2X_3 \quad (4)$$

All factors showed significant ($P < 0.05$) linear effects as shown by the P values of the coefficient estimates (**Table 6**).

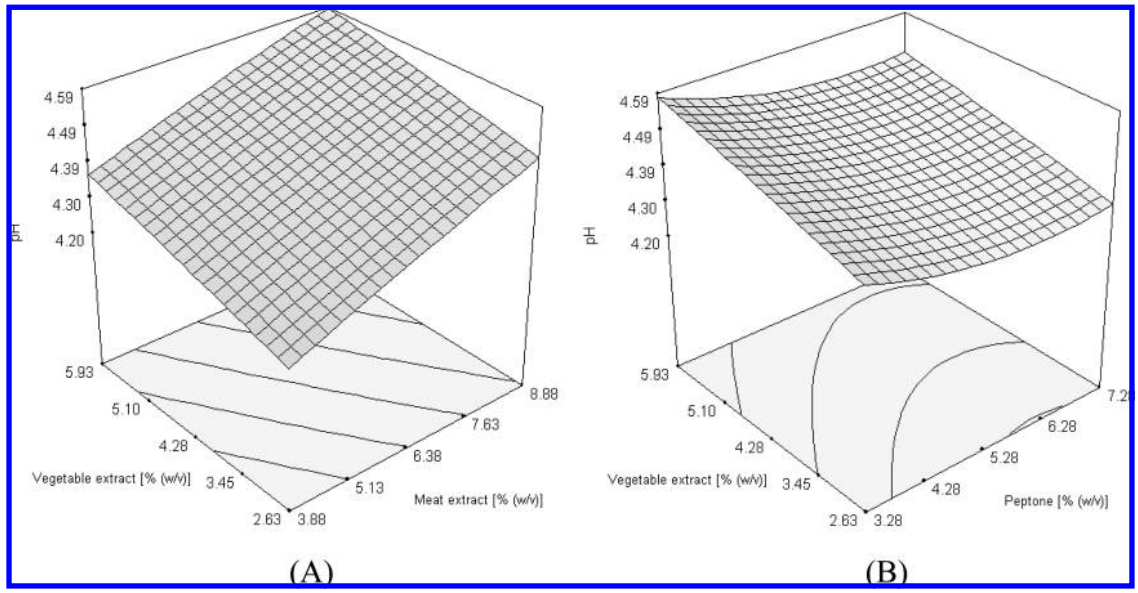

Figure 2. Response surfaces for pH of *L. acidophilus* FTCC 0219 fermented soy whey medium from the effects of (**A**) vegetable extract and meat extract at 5.28% (w/v) peptone and (**B**) vegetable extract and peptone at 6.38% (w/v) meat extract.

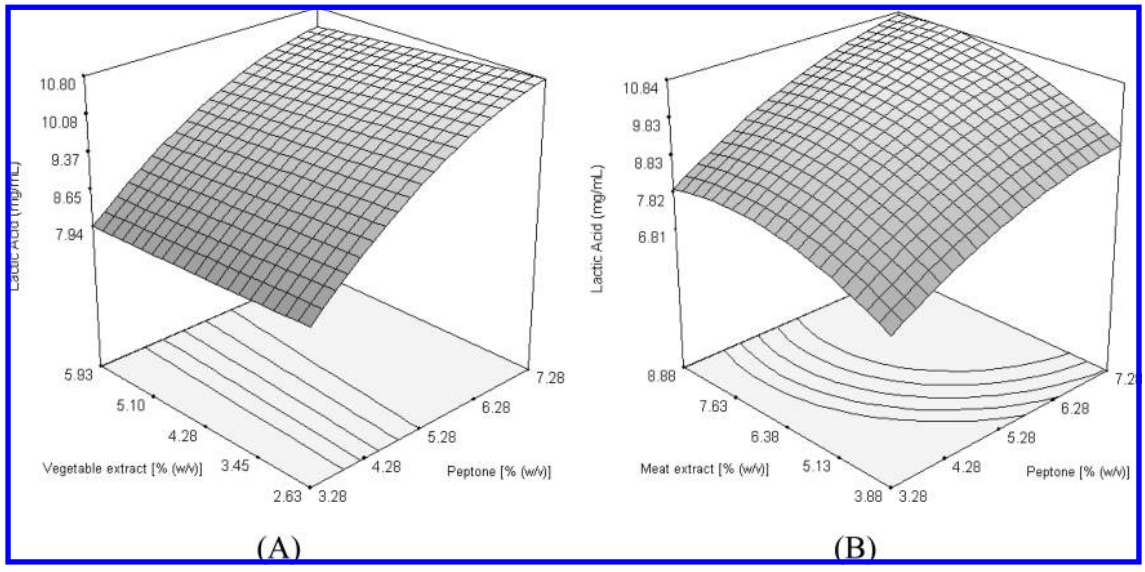

Figure 3. Response surfaces for concentration of lactic acid (mg mL^{-1}) in *L. acidophilus* FTCC 0219 fermented soy whey medium from the effects of (**A**) vegetable extract and peptone at 6.38% (w/v) meat extract and (**B**) meat extract and peptone at 4.28% (w/v) vegetable extract.

Only peptone showed a significant ($P < 0.05$) quadratic effect, whereas all interaction effects were insignificant ($P > 0.05$). The pH of the medium was decreasing with decreasing levels of vegetable and meat extracts (**Figure 2A**). However, pH decreased with increasing levels of peptone, although the change was minimal (**Figure 2B**). It must be noted that, in the presence of high levels of peptone, higher growth was achieved despite a lower value of pH (**Figures 1** and **2B**). A low pH has been associated with inhibitory effects on microbial growth, due to the inhibition of enzyme activities and cell functions. However, our results showed that growth was high in regions of low pH. Peptone has been found to contribute free amino acids such as L-glutamate, which could extrude protons from the cell as part of the pH homeostasis mechanism (*17*). We postulate that this subsequently increased the buffering capacity of the medium, thus minimizing pH fluctuation and enhancing bacterial survival.

Lactobacilli could produce >85% of the fermentation metabolites as lactic acid, with a small proportion as acetic acid (*18*). The growth of *L. acidophilus* FTCC 0291 resulted in the production of lactic and acetic acids, which were released into the medium. The rate of metabolite production was a better indicator of the growth rate of the probiotic in the medium compared to pH, because the latter can be influenced by the buffering capacity of the medium (*19*). Higher production of SCFA is an indication of higher growth rates and a shorter period to attain maximal growth.

The response surfaces for lactic acid content (Y_2) in the soy whey medium (**Figure 3**) were generated on the basis of the coded factor equation below:

$$Y_2 = 0.976 + 0.067X_1 - 0.013X_2 + 0.13X_3 - 0.063X_1^2 + 0.001X_2^2 - 0.046X_3^2 - 0.004X_1X_2 + 0.011X_1X_3 - 0.006X_2X_3 \quad (5)$$

The response surfaces for acetic acid content (Y_3) in the soy whey medium (**Figure 4**) were generated on the basis of the following coded factor equation:

$$Y_3 = 0.65 + 0.044X_1 - 0.009X_2 + 0.087X_3 - 0.042X_1^2 - 0.030X_3^2 - 0.003X_1X_2 + 0.007X_1X_3 - 0.004X_2X_3 \quad (6)$$

Meat extract and peptone produced significant ($P < 0.05$) linear and quadratic effects on the production of both lactic and acetic acids, whereas vegetable extract and the other interaction effects were insignificant ($P > 0.05$) (**Table 6**). Both acids showed similar trends (**Figures 3B** and **4B**), where the concentration of acids increased at high levels of meat extracts and peptone. However, the production of both acids was not affected by concentrations of vegetable extract.

This trend was reflective of the growth response, of which growth was high at increasing levels of meat extract and peptone, but less affected by concentrations of vegetable extract. In the presence of low levels of vegetable extract, high levels of peptone induced higher positive response in growth and, subsequently, higher production of acid (**Figures 3A** and **4A**) and greater decrease in pH (**Figure 2B**), compared to low levels of peptone. However, the influence of vegetable extract on these responses was not as prominent as those of meat extract and peptone, and this was supported by a low coefficient estimate (**Table 6**).

High growth and active metabolism led to a high concentration of acids (**Figures 1**, **3**, and **4**). pH was high despite high concentration of acids in the region of optimum growth (**Figures 2**−**4**). We postulated that this was due to the pH regulatory mechanisms of the probiotic, developed to endure the harsh gastrointestinal environment. The mechanisms include proton translocation, structural alterations in the cell membrane, alterations to metabolic pathways, and amino acid decarboxylation (*20*). *L. acidophilus* has been shown to induce an adaptive response at low pH that provided an elevated acid tolerance to the cells (*21*). We postulated that the maintainance of growth in high-acid conditions may be attributed to the pH homeostasis action of the probiotic, protecting it from reaching an intracellular pH that could inhibit cellular functions.

Glucose has also been shown to have protective effects on *L. acidophilus* under acidic conditions (*22*) because availability of metabolizable carbohydrates met the high energy demands of maintaining pH homeostasis (*23*). We suggested that the availability of metabolizable sugars in the medium encouraged the growth and viability of *L. acidophilus* FTCC 0291 during fermentation in soy whey, and high growth was maintained in regions of high acid concentrations (**Figures 1**, **3**, and **4**).

Utilization of Oligosaccharides and Reducing Sugars. The response surfaces for concentration of oligosaccharides (Y_4) in the soy whey medium (**Figure 5A**) were generated on the basis of the following coded factor equation:

$$Y_4 = 0.623 - 0.35X_1 - 0.046X_2 - 0.139X_3 + 0.15X_1^2 + 0.077X_2^2 + 0.069X_3^2 - 0.063X_1X_2 + 0.235X_1X_3 + 0.008X_2X_3 \quad (7)$$

In the region of optimum growth, the concentration of oligosaccharides (Y_5) decreased (**Figure 5A**) with increasing levels of meat extract and peptone. The hydrolysis of oligosaccharides by the probiotic as shown in the response surface was in tandem with the response surface of growth, where the regions with higher growth had lower concentrations of oligosaccharides. Meat extract and peptone showed significant ($P < 0.05$) linear effects and strong interaction effects, whereas only meat extract showed significant ($P < 0.05$) quadratic effect (**Table 6**).

The response surface for reducing sugar content (Y_5) in the soy whey medium (**Figure 5B**) was generated on the basis of the following coded factor equation:

$$Y_5 = 3.59 - 1.22X_1 - 0.14X_2 - 0.581X_3 + 0.638X_1^2 + 0.505X_2^2 + 0.479X_3^2 - 0.289X_1X_2 + 0.851X_1X_3 - 0.109X_2X_3 \quad (8)$$

The response surface of reducing sugar content (Y_6) (**Figure 5B**) also showed a correlation with the response surface of growth, where the region with a higher growth had lower concentrations of reducing sugars, indicating the utilization of reducing sugars for growth of probiotic in the soy waste medium. Similar to the response of oligosaccharides, meat extract and peptone showed significant ($P < 0.05$) linear and interaction effects on the growth of probiotic (**Table 6**).

However, the reducing sugar content was higher compared to oligosaccharides in the soy waste medium. *L. acidophilus* produces the enzyme α-galactosidase (EC 3.2.1.22), which hydrolyzes the α-D-galactosidic bonds present in raffinose and

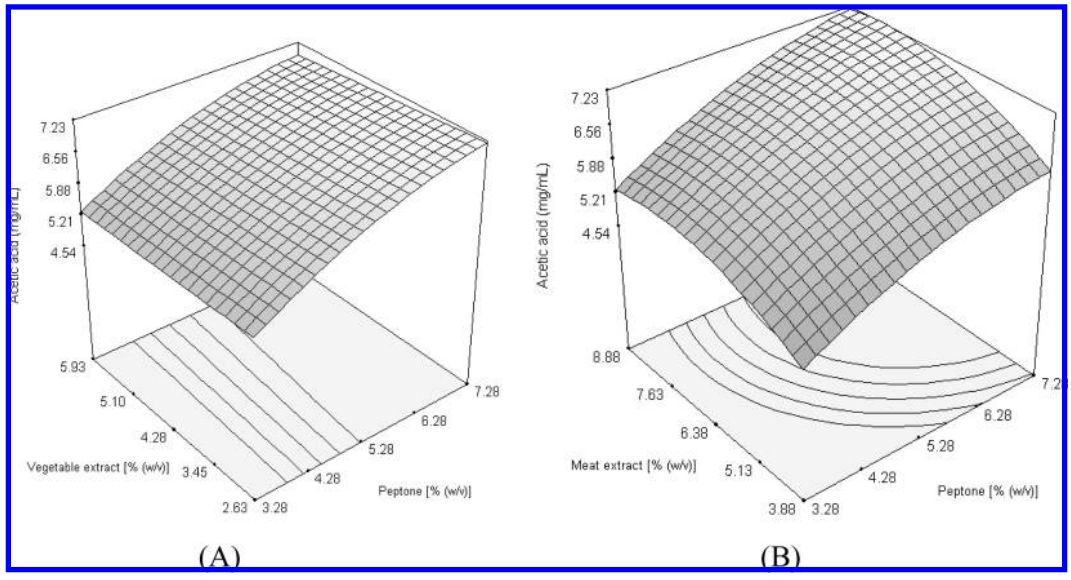

Figure 4. Response surfaces for concentration of acetic acid (mg mL^{-1}) in *L. acidophilus* FTCC 0219 fermented soy whey medium from the effects of (**A**) vegetable extract and peptone at 6.38% (w/v) meat extract and (**B**) meat extract and peptone at 4.28% (w/v) vegetable extract.

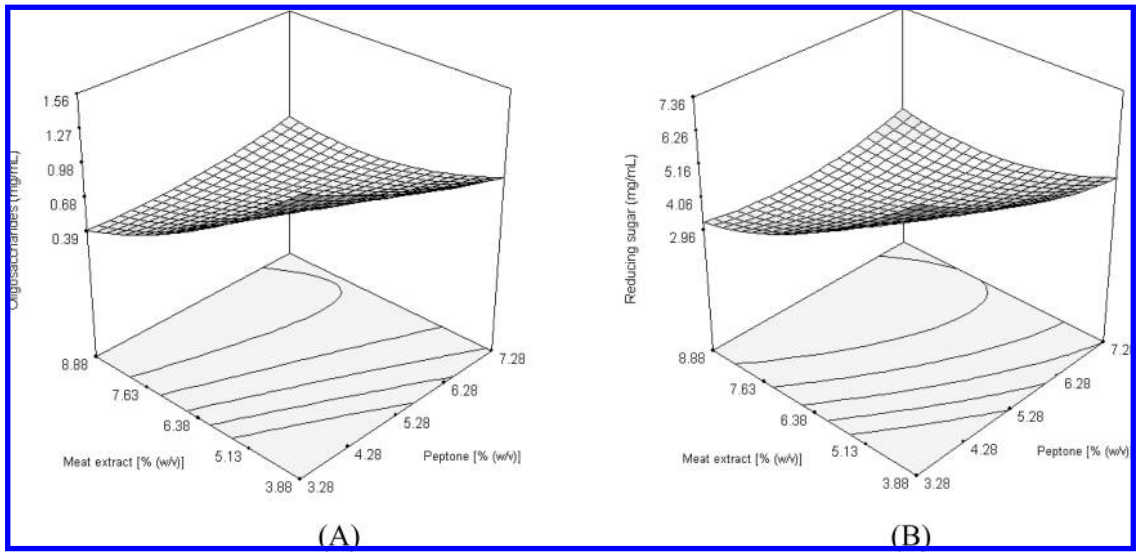

Figure 5. Response surfaces for the concentration of oligosaccharides (**A**) and reducing sugars (**B**) (mg mL^{-1}) in *L. acidophilus* FTCC 0219 fermented soy whey medium from the effects of meat extract and peptone at 4.28% (w/v) vegetable extract.

stachyose and breaks them down to glucose, galactose, and fructose. We suggested that the production of these sugars contributed to increased content of reducing sugars in the medium.

L. acidophilus uses three different carbohydrate transporters, namely, the phosphoenolpyruvate [sugar transferase system (PTS) transporters for uptake of glucose, fructose, and sucrose], the ATP-binding cassette (ABC) transporters for the uptake of raffinose, and a component of the galactosidepentose hexuronide (GPH) translocators for uptake of galactose (*24*). Sugars are translocated into the cell, where they are catabolized by hydrolases into readily fermentable sugars used as substrates in the Embden Meyerhof Parnas (EMP) pathway. Metabolites of glycolysis are further metabolized in a partial citrate cycle, characteristic of *L. acidophilus* (*25*).

In the optimized growth region, the response surfaces (**Figures 2** and **5**) showed that oligosaccharides and reducing sugars content decreased. This was indicative of a higher rate of metabolic breakdown of oligosaccharides and a higher uptake of reducing sugars for growth (**Figure 1**).

Our results also showed an unconventional finding. Although the concentrations of reducing sugars and oligosaccharides were low due to active metabolism, extracellular pH remained high (**Figures 2** and **5**). High concentrations of exogenous pyruvate from active metabolism had been found to favor the formation of acetoin over acidic end products, thus contributing to the maintenance of pH homeostasis (*26*). We suggest that not all metabolized sugars were converted to acidic metabolites, as the formation of acetoin could reduce the concentration of acids in the region of high growth. Daeschel (*27*) also showed that L-malate decarboxylation can, by the uptake of protons (H^+), neutralize the acidity from sugar fermentation as a means of resistance to pH change. This was a possible mechanism that led to the incidence of high extracellular pH despite high a sugar metabolism observed here.

In conclusion, optimum growth of *L. acidophilus* FTCC 0291 in soy waste medium was achieved at 6.15 OD_{600} in the presence of meat extract (7.25% w/v), vegetable extract (4.7% w/v), and peptone (6.85% w/v). The validation experiment showed that the prediction generated from the model using RSM was reliable. Analyses of growth, pH, and titratable acidity showed that increased growth in the medium was accompanied by increasing production of lactic and acetic acids. The decreased concentration of reducing sugars and oligosaccharides at regions of high growth indicated increased utilization of the sugars. Results from this study strongly indicated that soy whey could be a suitable growth medium for this organism and the fermented whey produced could be further developed into a functional beverage. The conversion of this waste to a valuable product could also reduce economical and environmental liabilities.

ACKNOWLEDGMENT

We thank Professor Abdul Karim Alias and Dr. Rosma Ahmad for their assistance with the project.

LITERATURE CITED

(1) Holzapfel, W. H.; Schillinger, U. Introduction to pre- and probiotics. *Food Res. Int.* **2001**, *35*, 109–116.
(2) Liong, M. T. Probiotics: a critical review of their potential role as antihypertensives, immune modulators, hypocholesterolemics, and perimenopausal treatments. *Nutr. Rev.* **2007**, *65*, 316–328.
(3) Dorff, E. The soybean, agriculture's jack-of-all-trades, is gaining ground across Canada. Component of Statistics Canada; http://www.statcan.ca/english/freepub/96-325-XIE/2007000/articles/10369-en.pdf, 2007 (accessed Jan 5, 2008).
(4) Thi, L. N.; Champagne, C. P.; Lee, B. H.; Goulet, J. Growth of *Lactobacillus paracasei* ssp. *paracasei* on tofu whey. *Int. J. Food Microbiol.* **2003**, *89*, 67–75.
(5) Liong, M. T.; Shah, N. P. Optimization of cholesterol removal by probiotics in the presence of prebiotics by using a response surface method. *Appl. Environ. Microbiol.* **2005**, *71*, 1745–1753.
(6) Donkor, O. N.; Henriksson, A.; Vasiljevic, T.; Shah, N. P. α-Galactosidase and proteolytic activity of selected probiotic and dairy cultures in fermented soymilk. *Food Chem.* **2006**, *104*, 10–20.
(7) Scalabrini, P.; Rossi, M.; Spettoli, P.; Matteuzzi, D. Characterization of *Bifidobacterium* strains for use in soymilk fermentation. *Int. J. Food Microbiol.* **1998**, *39*, 213–219.
(8) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(9) AOAC. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists: Washington, DC, 1984.
(10) Nielsen, S. S. *Food Analysis*, 2nd ed.; Aspen Publishers: Gaithersburg, MD, 1998.
(11) Church, F. C.; Swaisgood, H. E.; Porter, D. H.; Catignani, G. L. Spectrophotometric assay using *o*-phthaldialdehyde for determi-

nation of proteolysis in milk and isolated milk proteins. *J. Dairy Sci.* **1983**, *66*, 1219–1227.
(12) Cushman, D. W.; Cheung, H. S. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* **1971**, *20*, 1637–1648.
(13) Ademark, P.; Larsson, M.; Tjerneld, F.; Stålbrand, H. Multiple α-galactosidases from *Aspergillus niger*: purification, characterization and substrate specificities. *Enzyme Microb. Technol.* **2001**, *29*, 441–448.
(14) Liong, M. T.; Shah, N. P. Acid and bile tolerance and cholesterol removal ability of lactobacilli strains. *J. Dairy Sci.* **2005**, *88*, 55–66.
(15) Montgomery, D. C. *Design and Analysis of Experiments*; Wiley: New York, 1996.
(16) Myers, R. H.; Montgomery, D. C. *Response Surface Methodology*; Wiley: New York, 1995.
(17) Cotter, P. D.; Gahan, C. G.; Hill, C. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. *Mol. Microbiol.* **2001**, *40*, 465–475.
(18) Tannock, G. W. A special fondness for lactobacilli. *Appl. Environ. Microbiol.* **2004**, *70*, 3189–3194.
(19) Corcoran, B. M.; Stanton, C.; Fitzgerald, G. F.; Ross, R. P. Growth of probiotic lactobacilli in the presence of oleic acid enhances subsequent survival in gastric juice. *Microbiology* **2007**, *153*, 291–299.
(20) Sanchez, H. D. C.; Osella, A.; De La Torre, M. A. Use of response surface methodology to optimize gluten-free bread fortified with soy flour and dry milk. *Food Sci. Technol. Int.* **2004**, *10*, 5–9.
(21) Azcarate-Peril, M. A.; Altermann, E.; Hoover-Fitzula, R. L.; Cano, R. J.; Klaenhammer, T. R. Identification and inactivation of genetic loci involved with acid tolerance in *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.* **2004**, *70*, 5315–5322.
(22) Charalampopoulos, D.; Pandiella, S. S.; Webb, C. Evaluation of the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. *Int. J. Food Microbiol.* **2003**, *82*, 133–141.
(23) Shabala, L.; Budde, B.; Ross, T.; Siegumfeldt, H.; McMeekin, T. Responses of *Listeria monocytogenes* to acid stress and glucose availability monitored by measurements of intracellular pH and viable counts. *Int. J. Food Microbiol.* **2002**, *75*, 89–97.
(24) Barrangou, R.; Azcarate-Peril, M. A.; Duong, T.; Conners, S. B.; Kelly, R. M.; Klaenhammer, T. R. Global analysis of carbohydrate utilization by *Lactobacillus acidophilus* using cDNA microarrays. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 3816–3821.
(25) Altermann, E.; Russell, W. M.; Azcarate-Peril, M. A.; Barrangou, R.; Buck, B. L.; McAuliffe, O.; Souther, N.; Dobson, A.; Duong, T.; Callanan, M.; Lick, S.; Hamrick, A.; Cano, R.; Klaenhammer, T. R. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 1013–1022.
(26) Tsau, J. L.; Guffanti, A. A.; Montville, T. J. Conversion of pyruvate to acetoin helps to maintain pH homeostasis in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **1992**, *58*, 891–894.
(27) Daeschel, M. A. A pH control system based on malate decarboxylation for the cultivation of lactic acid bacteria. *Appl. Environ. Microbiol.* **1988**, *54*, 1627–1629.

Received for review May 20, 2008. Revised manuscript received July 4, 2008. Accepted July 9, 2008. This work was supported by Research University Grants 1001/PTEKIND/811020 through Universiti Sains Malaysia.

JF801567J