Growth Optimization of a Probiotic Candidate, *Bifidobacterium pseudocatenulatum* G4, in Milk Medium Using Response Surface Methodology

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Abstract *Bifidobacterium pseudocatenulatum* G4, a wild strain isolated from infant stools that has previously exhibited probiotic characteristics, was used in this study. The aim of this research was to improve the growth potential of this strain in milk-based medium. An initial screening study using a 2^3 full factorial design was carried out to identify the impact on biomass production of the various components of the medium which were skim milk, yeast extract, and glucose. Statistical analysis suggested that yeast extract had a significant positive effect on viable cell count whereas glucose had a negative effect. Response surface methodology (RSM) was then applied to optimize the use of skim milk and yeast extract. A quadratic model was derived using a 3^2 face-centered central composite design to represent cell mass as a function of the two variables. The optimized medium composition was found to be 2.8% skim milk and 2.2% yeast extract, w/v. The optimized medium allowed a maximum biomass of 9.129 $\log_{10}$ cfu/mL, 3.329 log units higher than that achieved with 10% skim milk, which is the amount commonly used. The application of RSM resulted in an improvement in the biomass production of this strain in a more cost-effective milk medium, in which skim milk use was reduced by 71.8%. © KSBB

Keywords: bifidobacteria, medium, milk, optimization, probiotic, RSM

INTRODUCTION

The complex ecosystem of gut microflora plays a significant role in the gastrointestinal health of humans and animals. This has attracted worldwide interest and intensive research has been conducted on issues pertaining to gut health. It is desirable to have a predominance of 'good' bacteria over harmful ones, however many factors including aging, stress, diet, and ingestion of antibiotics may disturb this equilibrium [1]. This has paved the way to the use of probiotics which are live beneficial microorganisms orally administered in order to encourage them to proliferate in the intestine.

For a long time, probiotics have been associated with dairy products to transform yogurts, cultured milk drinks or cheese into functional foods. Bifidobacteria, a commonly used genus in probiotic applications, is gaining importance in the industry due to its health benefits. Although bifidobacteria generally grow well on commercial media, these media are inappropriate for large-scale production due to the possibility of generating off-flavors in the food products [2]. Thus, most companies opt for milk-based media for probiotic bacterial cell mass production [3]. Milk, which contains carbohydrates, fat, casein protein, vitamins, and minerals, is a very nutritious growth medium for many microorganisms [4].

The ability of organisms to grow well in milk depends on their ability to metabolize milk protein and lactose and this ability varies considerably among strains [5]. Generally, probiotics grow relatively slowly in milk due to a lack of proteolytic activity and therefore they require supplements of peptides and amino acids [6]. In an effort to enhance the

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growth potential of probiotic strains in milk, researchers have added various concentrations of glucose as a carbon source and growth factors, such as yeast extract. Various concentration ranges have been used: 10.0~12.0% (w/v) reconstituted skim milk; 0.2~1.0% (w/v) yeast extract [6-10]; and 0.5~2.0% (w/v) glucose [6,8,9]. Avonts *et al*. [7] reported that addition of yeast extract (0.3~1.0%, w/v) to 10.0% (w/v) reconstituted skim milk powder enhanced both growth and bacteriocin production of lactobacillus strains. In a different study, a yeast extract concentration as high as 6.0% was used to optimize non milk-based media for the growth of *Lactobacillus rhamnosus* [11].

So far, published reports on the supplementation of milk media with sugar and growth factors have been limited to lab-scale studies that were mainly for subculturing or propagation of inoculum. There were no justifications given for the concentrations used. Moreover, industrial media formulations are rarely revealed to maintain the company's competitive advantage.

One of the major constraints involved in designing new fermentation media is the high number of experiments involved. Response surface methodology (RSM), a combination of good experimental design, regression modeling techniques and optimization, is a useful tool for process improvement. This methodology has been applied to the optimization of the growth of probiotic organisms in non-milk media [11,12]. A central composite design is one of the most commonly used designs for response surface optimization. While rotatability is a preferred characteristic in most central composite experiments, Neter *et al*. [13] commented that when it is physically inconvenient to extend axial points beyond the upper and lower limits of the experimental region, a face-centered central composite design (with axial distance $\propto = 1$) provides a good alternative. In face-centered central composite design, the region of interest and the region of operability are the same. In a study to optimize microbial growth in olive juice, a face-centered design was also used due to difficulty in experimenting outside the upper and lower limit of the factors [14].

This paper outlines the optimization of milk-based fermentation medium using RSM for the maximum biomass production of *Bifidobacterium pseudocatenulatum* G4, expressed as $\log_{10}$ cfu/mL. This strain was isolated from infant stools and in previous studies demonstrated the probiotic characteristics of bile tolerance and deconjugation of bile acids [15], antibacterial activity, antimicrobial susceptibility, and adherence properties [16].

MATERIALS AND METHODS

Microorganism and Preparation of Inoculum

The strain used in this study, *B. pseudocatenulatum* G4, was obtained from the Probiotic Laboratory, Faculty of Food Science and Technology, Universiti Putra Malaysia. It was originally isolated from infant stools [17,18]. The strain was stored at −20°C in a mixture of glycerol and trypticase phytone yeast extract (TPY) broth (Scharlau, Spain), in a ratio of 80:20. For propagation of inoculum, a colony of this strain was transferred from TPY agar to TPY broth and incubated anaerobically at 37°C for 48 h. The culture was further propagated twice in TPY broth by 10% inoculation to obtain an approximate initial biomass concentration of 10^7 cfu/mL. In order to minimize carryover of previous media during inoculation, cells were harvested by centrifugation at 3,000 rev/min for 15 min at 4°C. The cells were resuspended as inoculum (10%) in various media.

Preparation of Media and Fermentation Conditions

Fermentation was carried out in 500 mL screw cap glass bottles (Schott Duran, Mainz, Germany) containing 500 mL of fermentation broth. The media components were skim milk (NZMP medium heat skim milk powder, New Zealand), yeast extract (Bio Springer, Maisons-Alfort Cedex, France), and glucose (Merck, Darmstadt, Germany). All media were flushed with oxygen-free nitrogen gas for 30 min before sterilization at 121°C for 15 min. The media were cooled to room temperature prior to inoculation with the cell pellet. The bottles were incubated at 37°C for 24 h. The culture was incubated in an anaerobic jar containing Anaerocult A gas packs (Merck) in order to maintain anaerobic conditions. No pH control was used in the experiment. Approximately 5 mL of sample was withdrawn at every 2 h for analysis. New pieces of Anaerocult A gas packs were used after each sampling. Microbiological plating was not performed in an anaerobic chamber but rather in laminar air flow because this particular strain is less sensitive to oxygen. Although bifidobacteria are anaerobic organisms, some strains may tolerate oxygen in the presence of CO_2. The anaerobic requirements of bifidobacteria are known to be strain-dependent [19].

Microbiological Analysis

For the enumeration of viable cells, samples were serially diluted in 0.1% (w/v) sterile peptone water (Merck) and plated in duplicate onto TPY agar. Plates were incubated anaerobically at 37°C for 3 days. Viability was expressed as $\log_{10}$ cfu/mL.

Experimental Design and Statistical Analysis

Experimental design was carried out using Design-Expert software (Stat-Ease Inc., Minneapolis, MN, USA, version 6.0.4). An initial screening study using a 2^3 full factorial design was carried out to identify the impact of the medium components skim milk, yeast extract, and glucose on biomass production. Each factor was varied over 2 concentration levels (−1, +1), with 5 times replication of center points (0). The range and the levels of the variables investigated in this study are given in Table 1. A total of 13 sets of experiments were conducted to determine the significant factors affecting growth.

The design was further expanded to a face-centered central composite design with biomass achieved at 20 h ex-

Table 1. Experimental range and levels of the independent variables used in the 2^3 full factorial design

ndependent variable	Unit	Range and levels	
		−1[a]	+1[a]
Skim milk (x_1)	% (w/v)	4.0[b]	10.0[b]
Yeast extract (x_2)	% (w/v)	0	1.0
Glucose (x_3)	% (w/v)	0	1.0

[a]–1 and +1 are coded levels.
[b]Actual media concentration expressed in % (w/v).

Table 2. Screening of factors using a 2^3 full factorial design with maximum biomass as the response

Run	Skim milk (%, w/v)	Yeast extract (%, w/v)	Glucose (%, w/v)	Maximum biomass ($\log_{10}$ cfu/mL)
	x_1[b]	x_2[b]	x_3[b]	Y[c]
1	−1[a]	−1[a]	−1[a]	5.531
2	−1	−1	+1	5.250
3	−1	+1	+1	8.100
4	−1	+1	−1	8.140
5	0	0	0	7.310
6	0	0	0	6.902
7	0	0	0	6.841
8	0	0	0	7.092
9	0	0	0	7.060
10	+1	−1	−1	5.800
11	+1	−1	+1	4.855
12	+1	+1	+1	8.030
13	+1	+1	−1	8.210

[a]–1 and +1 are coded levels. The center point (0) was repeated 5 times.
[b]x_1, x_2, x_3 are skim milk, yeast extract, and glucose concentration, respectively, expressed in % (w/v).
[c]Maximum biomass count achieved at 20 h, expressed in $\log_{10}$ cfu/mL.

pressed as $\log_{10}$ cfu/mL as the response. In this design, the axial distance $\propto$ was chosen to be 1 to make the design cuboidal. The axial points were at the centers of the faces in a cube, rather than outside the faces as in the case of a conventional central composite design. A face-centered design was used because it was not feasible to explore the effect of skim milk concentration lower than 1.0% (w/v), as there would be to insufficient nutrients for growth. Earlier studies showed that growth in skim milk concentration < 1.0% resulted in drastic drop in viability. There were three coded factor levels: −1, 0, and +1, where −1 corresponded to the low level of each factor, 1 to the high level and 0 to the middle level. The coded value of each factor can be calculated using Eq. (1) as follows [13].

$$\text{Coded value} = \frac{\text{Actual level} - \dfrac{\text{High level} + \text{Low level}}{2}}{\dfrac{\text{High level} - \text{Low level}}{2}} \quad (1)$$

The center point was repeated five times, for a total of 13 experiments. Replications of center points enable the estimation of pure error in order to predict whether the models give a significant lack-of-fit.

Table 3. Regression analysis of the 2^3 full factorial design with maximum biomass ($\log_{10}$ cfu/mL) as the response

Variable	*F*-value	*P*-value
x_1[a]	0.059	0.820
x_2	452.930	< 0.001
x_3	7.760	0.049
$x_1 x_2$	0.059	0.820
$x_1 x_3$	2.400	0.196
$x_2 x_3$	3.760	0.125
$x_1 x_2 x_3$	1.020	0.370
Model	66.860	0.001
Curvature	8.310	0.045
C.V. = 2.68%	R^2 = 0.99	Adjusted R^2 = 0.98

[a]x_1, x_2, x_3 are skim milk, yeast extract, and glucose concentration, respectively, expressed in % (w/v).

RESULTS AND DISCUSSION

Initial Screening of Significant Medium Components

An initial screening of medium components was performed. The effects of skim milk (x_1), yeast extract (x_2), and glucose (x_3) on biomass production were examined using a 2^3 full factorial design (Table 2). The response, which was the maximum biomass count achieved at 20 h, varied from 4.855 to 8.210 $\log_{10}$ cfu/mL. Regression analysis of the variables, as shown in Table 3, revealed that yeast extract (P < 0.001) and glucose (P = 0.049) were the only significant factors at a 95% confidence interval. The P-values for skim milk variable and interaction terms were higher than 0.05, and were therefore considered not significant. Taking into consideration only the significant factors, Eq. (2) was obtained as shown below.

$$Y = 5.540 + 2.761\, x_2 - 0.362\, x_3 \quad (2)$$

The regression model can be applied in screening crucial medium components as it indicates how each factor affects the response. For every unit increase in x_2, an increase of 2.761 units in Y would be expected. In contrast, for every unit increase in x_3, Y will decrease by 0.362 units. Based on the statistical output, biomass count was the highest when the maximum level of yeast extract (1.0%, w/v) and the minimum level of glucose content (0%, w/v) were used as shown in Fig. 1. Thus, improvement could be achieved by using a higher concentration of yeast extract (> 1.0%, w/v) and by not adding glucose. Since the levels of skim milk (4.0~10.0%, w/v) did not affect the response, this component which was crucial as a basal medium was further reduced in

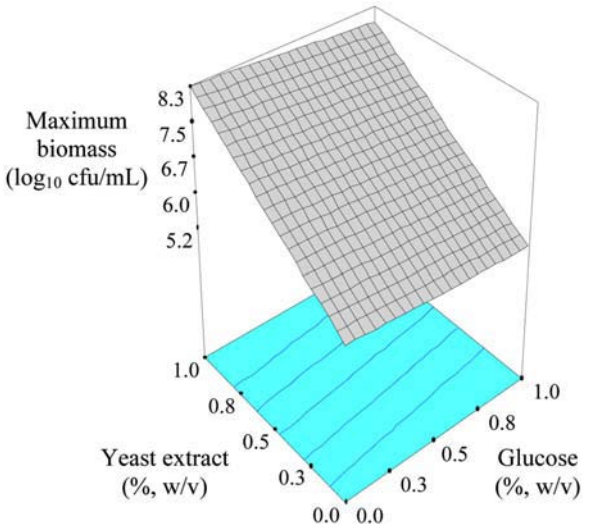

Fig. 1. Response surface plot of maximum biomass as a function of yeast extract and glucose concentrations.

order to reduce cost and minimize wastage of the carbon source. Confirmation that skim milk levels were unnecessarily high came from high performance liquid chromatography (HPLC) analysis which indicated that high concentrations of milk lactose and its hydrolyzed monosaccharides remained at the end of fermentation time (results not shown).

Lactose, the primary carbon source in milk, comprised 52.5% of the skim milk powder used in this study. One of the criteria for microorganisms to flourish in milk is the ability to hydrolyze lactose into its monosaccharides, glucose, and galactose, which requires the activity of β-galactosidase. Thus, researchers commonly add glucose into the fermentation media to facilitate growth. However in this study, results demonstrated that *B. pseudocatenulatum* G4 grows well in skim milk-yeast extract media without the addition of glucose. This also provided evidence of the ability of this strain to assimilate milk lactose.

In a study to examine the effect of various carbon sources (lactose, glucose, and galactose) and nitrogen sources (yeast extract, peptone, and $(NH_4)_2SO_4$) on β-galactosidase production of *B. longum*, Hsu *et al*. [20] discovered that the highest level of the enzyme was produced with lactose and yeast extract as carbon and nitrogen sources respectively. They noted significantly repressed β-galactosidase activity in lactose-containing broth when glucose or galactose was added to the medium. The highest *B. longum* cell count was achieved in cultures containing either lactose or glucose as the sole carbon source, but with glucose resulting in the lowest enzyme activity. Since lactose was the major substrate in milk in this study, β-galactosidase activity of this strain may be exceptionally high. When glucose was added to milk media, the enzyme activity was suppressed, which led to incomplete utilization of the lactose in the milk. This presumably contributed to the lower biomass count when glucose was present in the media in this research.

The free monosaccharides which accumulated in the media also served as available substrates for cell metabolism. Thus, we do not rule out the possibility of a glucose inhibition effect when glucose was added to the fermentation media. Excessive carbon source in the culture broth reduces biomass yield and energetic growth efficiency due to metabolism overflow [21]. For all of these reasons, glucose was eliminated from the study. Senthuran *et al*. [22] revealed that in a free cell cultivation of *Lactobacillus casei*, this organism preferred lactose to glucose as a carbon source for its growth. This improved performance with lactose was linked to the presence of different transport mechanisms for lactose and glucose. Although the actual mechanism of lactose utilization is unclear, Ostlie *et al*. [5] suggested that bifidobacteria metabolize lactose by the bifidus pathway. More in-depth study is needed to confirm the metabolic pathway of *B. pseudocatenulatum* G4 in milk.

Table 4. Face-centered central composite design, representing the maximum biomass achieved as influenced by skim milk and yeast extract concentration

Run	Skim milk, x_1		Yeast extract, x_2		Maximum biomass, Y (log_{10} cfu/mL)[c]
	Coded value	Actual value (%, w/v)	Coded value	Actual value (%, w/v)	
1	–1[a]	1.0[b]	–1[a]	2.0[b]	8.282
2	–1	1.0	0	4.0	8.371
3	–1	1.0	+1	6.0	5.310
4	0	2.0	–1	2.0	8.908
5	0	2.0	0	4.0	8.720
6	0	2.0	0	4.0	8.551
7	0	2.0	0	4.0	8.290
8	0	2.0	0	4.0	8.529
9	0	2.0	0	4.0	8.543
10	0	2.0	+1	6.0	4.771
11	+1	3.0	–1	2.0	8.934
12	+1	3.0	0	4.0	8.468
13	+1	3.0	+1	6.0	5.353

[a]–1, 0, and +1 are coded values.
[b]Actual concentration expressed in % (w/v).
[c]Maximum biomass count achieved at 20 h, expressed in log_{10} cfu/mL.

Medium Optimization Using Response Surface Methodology

A second experiment was conducted as a result of the aforementioned findings, in an attempt to optimize the levels of skim milk and yeast extract using a face-centered central composite design. Skim milk levels were reduced to 1.0, 2.0, and 3.0% (w/v) whereas the amount of yeast extract was further increased to 2.0, 4.0, and 6.0% (w/v). The experimental design and results are shown in Table 4. The biomass count obtained varied from 4.771 to 8.934 log_{10} cfu/mL. Center points with a coded value (0,0) were repeated five times in order to estimate pure error for the lack of fit test. The lack of fit test shows how well the model fits the data. Moreover, replications of center points also help in estimat-

Table 5. Regression analysis of face-centered central composite design with maximum biomass (log_{10} cfu/mL) as the response

Variable	Degree of freedom	*F*-value	*P*-value
x_1[a]	1	1.820	0.219
x_2[b]	1	333.320	< 0.001
x_1^2	1	0.003	0.961
x_2^2	1	119.170	< 0.001
x_1 x_2	1	1.620	0.244

[a]x_1 represents skim milk, expressed in % (w/v).
[b]x_2 represents yeast extract, expressed in % (w/v).

ing curvature in the response system.

Regression analysis was performed to fit the response function with the experimental data. The data obtained were fitted to a quadratic polynomial model. A general second-order model is shown in Eq. (3) and the actual obtained model in Eq. (4).

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad (3)$$

$$Y = 4.923 + 0.408\, x_1 + 2.407\, x_2 + 0.007\, x_1^2 - 0.393\, x_2^2 - 0.076\, x_1 x_2 \quad (4)$$

Here, x_1 and x_2 represent skim milk and yeast extract respectively and β_0, β_1, β_2, β_{11}, β_{22}, and β_{12} are constant coefficients. The statistical significance of the second-order model was analyzed using analysis of variance (ANOVA) as shown in Table 5. This table shows that only yeast extract, x_2 and x_2^2 were highly significant terms with $P < 0.001$. Thus, the quadratic model was further reduced, eliminating non-significant terms. However, it was decided that skim milk (x_1) with $P > 0.05$ should be included in the final equation because this study was aimed at improving the growth potential of this strain in milk-based medium. Moreover, skim milk was a very important basal medium in this case. The coefficient estimate changed after dropping insignificant terms as they were added to the error term. The final adjusted model that describes the experimental data of this system is as below Eq. (5).

$$Y = 5.516 + 0.132\, x_1 + 2.249\, x_2 - 0.392\, x_2^2 \quad (5)$$

The ANOVA results for the initial model and the adjusted model are shown in Table 6. From the low *P* value (< 0.001), it can be inferred that the equation obtained was appropriate after model reduction. The quality of fit of the equation was expressed by the determination coefficient, R^2. A value of 0.98 indicated that the model could explain ~98% of the variability in the response. In fact, a lower coefficient of variation (*C.V.*) of 3.02 was obtained compared to the first model. The adjusted model had no significant lack of fit at the 5% level ($P = 0.130$). Therefore, the final model represents the data adequately.

The response surface plot simulated by the adjusted model is shown in Fig. 2. A steep change in the response plot

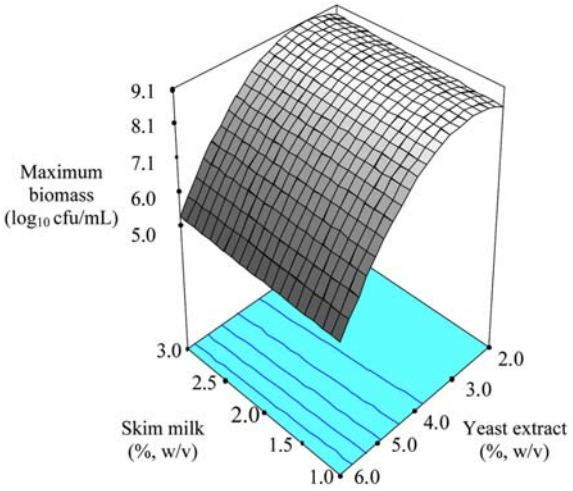

Fig. 2. Response surface plot of the maximum biomass count obtained from the adjusted quadratic mathematical model (experimental data).

shows that biomass count was particularly sensitive to yeast extract concentration in the medium. Yeast extract, which consists of nitrogenous compounds and growth factors, stimulates cell growth. The response surface obtained was a stationary ridge system. In a stationary ridge system there is a plane rather than a point where the response is at its maximum value. Therefore, there is flexibility in choosing the appropriate optimum points [23].

Results from the Design-Expert output have shown that optimal concentrations of skim milk and yeast extract for biomass production were calculated to be 2.8 and 2.2% (w/v), respectively. This software uses an optimization method that allows the criteria for all variables and responses to be set. There are five possibilities for a "goal" to construct desirability indices: maximum, minimum, target, in range, and equal to. This optimization method takes into consideration a combination of criteria in the calculation of the optimum points. Therefore, based on the criteria setting of skim milk (in range), yeast extract (in range), and response (maximize), the optimum point of 2.8% skim milk and 2.2% yeast extract was obtained. A lower yeast extract concentration was also desirable to minimize the cost of raw material [23].

The maximum response predicted from the model was 8.942 log_{10} cfu/mL. Repeated experiments were performed to verify the predicted optimum. A maximum biomass of 9.129 log_{10} cfu/mL was obtained from duplicate replications. Although the actual experimental response value at the optimum point was higher than the predicted value, statistically there was no difference. The maximum biomass obtained from the optimized medium was compared with growth performance in other media: TPY broth, skim milk 10.0% (w/v), and skim milk 10.0% (w/v) supplemented with yeast extract 1.0% (w/v) (Fig. 3). The predicted response based on the optimum points was also plotted for comparison. It was apparent that the optimized medium produced the highest biomass count at 20 h, followed by TPY broth and skim milk

Table 6. Comparison of ANOVA results between initial quadratic model and final adjusted model of face-centered central composite design

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-value
Initial model (Eq. 3)					
Model	27.20	5	5.440	94.930	< 0.001
Residual	0.40	7	0.057		
Total	27.60	12			
R^2 = 0.99	Adjusted R^2 = 0.98	Predicted R^2 = 0.89	*C.V.* = 3.08%		
Adjusted model (Eq. 4)					
Model	27.11	3	9.040	164.570	< 0.001
Residual	0.49	9	0.055		
Total	27.60	12			
R^2 = 0.98	Adjusted R^2 = 0.98	Predicted R^2 = 0.95	*C.V.* = 3.02%		

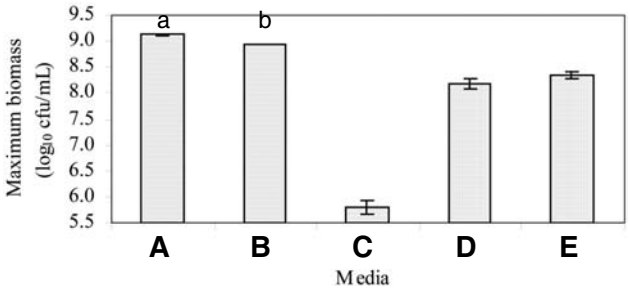

Fig. 3. Effect of different media compositions on the biomass production of *B. pseudocatenulatum* G4 after 20 h fermentation. (A) Optimized medium (experimental value); (B) optimized medium (predicted value); (C) skim milk 10.0%; (D) skim milk 10.0% + yeast extract 1.0%; (E) TPY broth. Error bars indicate the mean ± standard deviation of two experiments. [a]Actual experimental response obtained when grown in 2.8% skim milk and 2.2% yeast extract, w/v. [b]Predicted response based on 2.8% skim milk and 2.2% yeast extract.

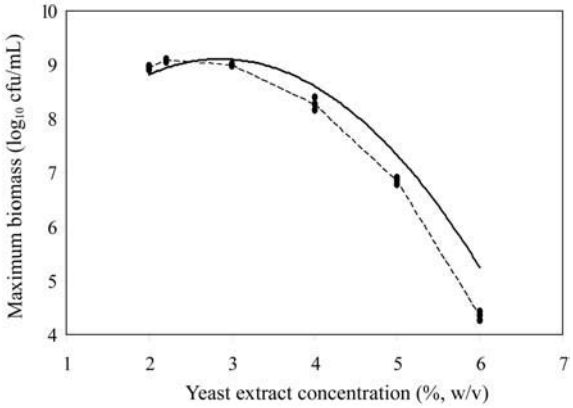

Fig. 4. Comparison between model-predicted plot and actual experimental data at various yeast extract concentrations (with a fixed skim milk concentration of 2.8%, w/v). (——) Predicted data, (–•–) experimental data. The experiment was repeated 3 times.

supplemented with yeast extract. Poor growth was observed when the strain was grown in 10% skim milk without the addition of growth factors.

This was a great improvement in the growth of *B. pseudocatenulatum* G4 in milk. The viable count was considered satisfactory because ideally the growth medium should produce minimum cell concentrations of 10^9 cfu/mL [24], which was not attainable using TPY media. In addition, the cost of raw material would be reduced as the skim milk concentration was decreased by 71.8%, from the commonly used 10.0 to 2.8% (w/v). In a similar study to optimize production of *L. rhamnosus*, a probiotic organism, a maximum biomass of 9.350 $\log_{10}$ cfu/mL was reported [11]. Different variables have been used by different researchers to represent biomass: viable cells, $\log_{10}$ cfu/mL [11], maximum specific growth rate, h^{-1} [25], and dry cell weight, g/L [12]. Therefore, comparison of results from this study with other published papers is limited. Moreover, since growth performance is strain-specific, medium optimization should be compared with individual strain performance on commonly used commercial media.

Validation of Mathematical Model

In order to assess the adequacy of the model (Eq. (5)), a validation experiment was performed by varying the level of yeast extract (2.0, 3.0, 4.0, 5.0, and 6.0%, w/v) at the optimum point of skim milk (2.8%, w/v) (Fig. 4). Cell count was the highest at 2.0~3.0% (w/v) yeast extract and diminished drastically with increased yeast extract concentration. Using Minitab 13, a quadratic equation representing the model-predicted plot and actual experimental plot was obtained (as illustrated in Eqs. 6 and 7, respectively).

$$Y_{\text{predicted}} = 5.889 + 2.249\, x_2 - 0.392\, x_2^2 \quad (6)$$
$$P = < 0.001$$

$$Y_{\text{experimental}} = 6.176 + 2.207\, x_2 - 0.414\, x_2^2 \quad (7)$$
$$P = < 0.001, \quad R^2 = 99.60\%$$

It was found that both sets of data have a high correlation (R = 0.99; P-value < 0.001) at the 95% confidence interval. Therefore, it was concluded that the predicted model fits well with the experimental data. In addition, these results suggest the possibility of applicability of the optimization.

CONCLUSION

The use of RSM as a statistical tool to improve the growth of B. pseudocatenulatum G4 in milk medium has been demonstrated in this study. This work determined the optimum amount of skim milk and yeast extract in the medium formulation for the cultivation of this particular strain. Following the development of a quadratic model, it was predicted that optimum points of 2.8% skim milk and 2.2% yeast extract resulted in the highest viable cell count. This growth optimization led to biomass increase in a more cost-effective milk medium, in which the skim milk requirement was reduced by 71.8%.

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NOMENCLATURE

α	Axial distance to the centerpoint
β	Constant coefficient
C.V.	Coefficient of variation
F	Fisher variance ratio
P	Probability
R	Correlation coefficient
R^2	Coefficient of determination
x_1	Skim milk variable (%, w/v)
x_2	Yeast extract variable (%, w/v)
x_3	Glucose variable (%, w/v)
Y	Response variable ($\log_{10}$ cfu/mL)

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