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Optimization of Growth medium for Efficient Cultivation of *Lactobacillus salivarius* i 24 using Response Surface Method

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ABSTRACT

Production of *Lactobacillus salivarius* i 24, a probiotic strain for chicken, was studied in batch fermentation using 500 mL Erlenmeyer flask. Response surface method (RSM) was used to optimize the medium for efficient cultivation of the bacterium. The factors investigated were yeast extract, glucose and initial culture pH. A polynomial regression model with cubic and quartic terms was used for the analysis of the experimental data. Estimated optimal conditions of the factors for growth of *L. salivarius* i 24 were; 3.32 % (w/v) glucose, 4.31 % (w/v) yeast extract and initial culture pH of 6.10.

Keywords: Lactobacillus salivarius, optimization, response surface method, probiotic for chicken.

INTRODUCTION

The fermentation conditions, such as temperature, pH, medium composition, dissolved oxygen tension (DOT) and types of neutralizer greatly influence the growth of lactobacilli (Gilliland, 1985). The factors to be considered in the formulation of growth medium are costs, ability to produce a large number of cells and must be able to ease the harvesting method.

The "change one factor at a time" method is widely used as a conventional technique for multifactor experimental design. This method, which involves changing one independent variable while maintaining all others at a fixed level, is extremely time consuming and expensive when a large number of variables are evaluated. This method may also lead to unreliable results and wrong conclusions. It is inferior to the factorial design method (Logothetis and Wynn, 1989). Response surface methodology (RSM), which includes factorial design and regression analysis, is more suitable to be used with multifactor experiments. Hence, it can be employed to overcome the above difficulty. RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effect of factors, and searching optimum conditions of factors for desirable responses (Montgomery, 1991). The relationships between a response and several related factors are quantitative which cover the tested experimental range and include the interactions. The models obtained can be used to calculate any or all combinations of variables, and their effects within the test range.

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Optimization through factorial design and response surface analysis is a common practice in fermentation technology. This technique has been applied for the optimization of culture conditions (Rao *et al*., 1993; Cordenunsi *et al*., 1985) and medium composition (Sen, 1997) for various fermentation processes. From our previous work, growth medium for efficient cultivation of *Lactobacillus salivarius* i 24, a potential probiotic for chicken, has been formulated (Lim, 2006). Among the difference carbon and nitrogen sources tested, growth of this bacterium was enhanced by glucose and yeast extract. Growth was also greatly influence by the culture pH. Optimization of the medium and culture conditions shall be carried out to further enhance the cultivation performance of *L. salivarius* prior to large scale production for commercialization.

The main objective of this study was to find the optimum conditions of important factors that affected the cultivation performance of *L. salivarius* i 24 using RSM. The response investigated was the growth of *L. salivarius* i 24 represented by $\log_{10}$ CFU/mL and the factors were glucose, yeast extract and pH.

MATERIALS AND METHODS

Microorganism

The bacterium, *Lactobacillus salivarius* i 24, isolated from chicken intestine (Jin *et al*., 1996), was used throughout the study. It was kindly provided by the Digestive Microbiology Unit, Laboratory of Industrial Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, Serdang. This bacterium has very good probiotic effect to

chicken, such as improved resistance to infectious disease, increased growth rate and improved feed digestion (Jin *et al*., 1996; Jin *et al*., 1997). The strain was inoculated into De-Man, Rogosa and Sharpe (MRS) broth and incubated at 37 °C for 24 h. The bacterial cells were then harvested by centrifugation at 12,857 x *g* for 5 min at 4 °C. The bacterial pellets were resuspended in 15% (v/v) glycerol and stored at –80 °C until used in inoculum preparation.

Medium Composition

The basal fermentation medium for good growth of *L. salivarius* as reported in our previous work (Lim, 2007) was used in this study. This medium consisted of (g/L): K_2HPO_4, 2; $MgSO_4$, 0.02; Tween 80, 1mL/L. The amounts of yeast extract and glucose added were varied according to the requirement of each experiment. The initial pH of each medium was adjusted to the required value either with 5M NaOH or 5M HCl. The respective fermentation media of yeast extract, glucose and pH are shown in Table 1.

Cultivation Experiments

For inoculum preparation, stock culture of *L. salivarius* I 24 was sub-cultured in a 250 mL Erlenmeyer flask containing 50 mL of MRS broth. The flask was incubated at 37 °C for 16-18 h to obtain the final cell concentration of approximately 10^7 CFU/mL. For rapid estimation of cell concentration, correlation between CFU/mL with cell turbidity measured using a spectrophotometer (Spectronic 20 Genesys) at 620 nm was used (data not shown).

The cultivation experiments were performed using 100-mL volumes of culture medium in 500 mL Erlenmeyer flasks. The culture medium was inoculated with 4% (v/v) of inoculum, which was prepared as described above. The flasks were incubated at 37 °C in an orbital shaker at 100 rpm, to provide good mixing, for 14 to 18 h. Lactobacilli are not obligate anaerobes and could survive under minimal exposure to oxygen. Flask agitated at low speeds provided better growth conditions to *Lactobacillus* spp as compared to static flask, due to improvement in mixing (Liew, 2005). During the cultivation, pH was not controlled but the initial pH was set at different values ranging from 5 to 7 (Table 1). During cultivation, samples were withdrawn at 2 h intervals for analysis.

Analytical Techniques

The number of viable cells was determined as colony forming units (CFU) viability counts, serial decimal dilutions of each sample (10^{-5} to 10^{-9}) were prepared and plated in triplicates onto MRS agar plates. The plates were incubated at 37 °C for 48 h, after which the colonies on the plate were counted. Each colony was derived from a single viable cell or a colony forming unit (CFU).

Table 1: Actual factor level corresponding to coded factor levels

Factor	Symbol	Actual factor levels at coded factor level of				
		-1.682	**-1**	**0**	**1**	**1.682**
Glucose (%)	X_1	1.5	2.3	3.5	4.7	5.5
Yeast Extract (%)	X_2	1	2	3.5	5	6
pH	X_3	5	5.4	6	6.6	7

Experimental Design using RSM

A central composite design in two blocks was used to allocate treatment combinations in this experiment (Table 2). The blocks were used to determine the effect of uncontrolled environment to the result of the experiments, in this case the effect of the difference days in conducting the experiment (Liew *et al*., 2005). The experiment was conducted for two days. The first block, representing the first day of the experiment, contains the factorial runs and 3 centre runs. The second block, representing the second day of the experiment, contains 1 factorial run, 6 axial runs and 3 centre runs.

In this experiment, the growth of *L. salivarius* i 24 as measured by $\log_{10}$CFU/mL, was studied under the influence of 3 main factors; glucose, yeast extract and pH. This design was based on the results of the preliminary study on the formulation of medium suitable to support good growth of *L. salivarius* i 24 (Lim, 2006). Growth of *L. salivarius* i 24 was enhanced when glucose and yeast extract was used as carbon and nitrogen source, respectively. Growth of this bacterium was also increased with increasing glucose and yeast extract up to 5% and 4.5%, respectively. It is well known that growth of *Lactobacillus* spp is greatly influence by the culture pH (Schepers *et al*., 2002; Liew, 2004). The central composite design was aimed at finding the optimum combination of these three factors (glucose, yeast extract and pH) on final cell concentration could be attained and also the productivity, which related to the fermentation time.

To set up a statistical model, let Y denotes $\log_{10}$CFU/mL and determined code factor levels as follows: X_1 = (glucose-3.5)/1.2, X_2 = (yeast extract-3.5)/1.5 and X_3 = (pH-6)/0.6. Table 1 contains actual factor levels corresponding to coded factor levels. For each factor, a centre point level was set to zero as coded level. Using this design, we can fit a second or higher order polynomial regression model to the data. Treatment combinations and observed responses are presented in Table 2.

Table 2: Treatment combinations and responses

Run	Block[a]	Coded variable level X1	X2	X3	Response[b] Y
1	-1	-1	-1	-1	9.115
2	-1	-1	-1	1	9.319
3	-1	-1	1	-1	9.469
4	-1	-1	1	1	9.264
5	-1	1	-1	-1	9.053
6	-1	1	-1	1	9.271
7	-1	1	1	-1	9.369
8	-1	0	0	0	9.402
9	-1	0	0	0	9.472
10	-1	0	0	0	9.415
11	1	1	1	1	9.447
12	1	1.682	0	0	9.301
13	1	-1.682	0	0	9.351
14	1	0	1.682	0	9.444
15	1	0	-1.682	0	8.738
16	1	0	0	1.682	9.344
17	1	0	0	-1.682	9.129
18	1	0	0	0	9.379
19	1	0	0	0	9.347
20	1	0	0	0	9.412

[a] -1, first day of the experiment; 1, second day of the experiment.
[b] $\log_{10}$CFU/mL.

Statistical Analysis

Statistical analysis was aimed at determining the fitness of the equations in predicting the number of viable cells in the terms of $\log_{10}$ as response of the independent variables. The data were analysed by the Statistical Analysis System (SAS). SAS/STAT procedures were used for regression analysis (SAS Institute Inc., 1990a). Our regression model permitted evaluation of the effect of linear, interaction, quadratic, cubic and quartic terms of the independent variables (glucose, yeast extract and pH) on the response. The α-level at which every term in the selected model should be significant was set as 0.05. Optimum conditions were found through SAS data-step programming. Response surface plots were generated by SAS/GRAPH (SAS Institute Inc., 1990b).

RESULTS AND DISCUSSION

Development of a Regression Model

Firstly, the second-order polynomial regression model containing three linear, three quadratic and three interaction terms plus one block term was employed by using the RSREG procedure of SAS/STAT. Table 3 shows that the second-order model was significant and that the R^2 = 0.9030. However, the lack of fit was significant (P = 0.0286< 0.05). This result suggests that the second-order model did not accurately represent data in the experimental region. Therefore, higher order might have to be included in the regression model. Cubic and quartic terms can be included in the model using a model selection procedure in order to eliminate the lack of fit (Oh *et al*., 1995; Schepers *et al*., 2002). Since each factor has five levels, up to quartic terms could be included into the model (Box and Draper, 1982).

Variable selection techniques were used to find a better model. Among the variable selection techniques available in the REG procedure of SAS, the smallest Mallows' C*p* selection and maximum R^2 improvement techniques were used. Besides that, the sparcity of effect principle was also taken into account when trying to select good predictors from the following candidates for model terms:

Block,
X_1, X_2, X_3
X_1X_2, X_1X_3, X_2X_3
X_1^2, X_2^2, X_3^2
X_1^3, X_2^3, X_3^3
X_1^4, X_2^4, X_3^4

Table 3: Analysis of variance for evaluation of the second-order model[a]

Source of variation	No of degrees of freedom	Sum of square	Mean square	F value	P value
Model	10	0.539235	0.053924	8.381	0.0019
Residual	9	0.057910	0.006434		
Lack of fit	5	0.053024	0.0010605	8.683	0.0286
Pure error	4	0.004885	0.001221		
Total	19	0.597145			

[a] R^2 = 0.9030, Coefficient of variance = 0.8623

The same 9-variables model was identified by application of all three of the variable selection methods mentioned above. The functional form of this model is as follows:

$$Y= b_0+ b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{222}X_2^3 + b_{2222}X_2^4 + b_{33}X_3^2 + b_{23}X_2X_3 \quad \text{[equation 1]}$$

Tables 4 and 5 show how the above model was fitted to the data. The fourth-order subset model in Table 5, which was to be used as the response surface model for subsequent analysis, was superior to the second-order-full model in Table 3; it had a larger R^2 (0.9393 > 0.9030) and smaller coefficient of variation (0.64746 < 0.8623) and smaller number of variables (9 < 10) and all the regression coefficients were lower than 5%, indicating

that they were significant. The larger the R^2, the more accurately the values of the response can be predicted by the model. Furthermore, the lack of fit was insignificant (P = 0.0903). However, X_1, X_2 and X_2^2 with p > 0.05 were forced into the final equation due to the fact that its corresponding higher-order terms were chosen for inclusion. The intercept b_0 is the estimated response at the center point (X_1, X_2, X_3) = (0, 0, 0).

Table 4: Analysis of variance in the regression model selected through variable selection[a]

Source of variation	No of degrees of freedom	Sum of square	Mean square	F value	P value
Model	9	0.56087	0.06232	17.181	0.0001
Residual	10	0.03627	0.00363		
				4.284	0.0903
Lack of fit	6	0.031385	0.005231		
Pure error	4	0.004885	0.001221		
Total	19	0.597145			

[a] R^2 = 0.9393, Coefficient of variance = 0.64746

Table 5: Coefficient estimates in the regression model selected through variable selection

Variable	Coefficient estimate	Standard error	t value	P value
Intercept	9.404500	0.02458758	382.490	0.0001
X_1	-0.008134	0.01629649	-0.499	0.6285
X_2	0.038193	0.03572549	1.069	0.3102
X_3	0.048076	0.01629649	2.950	0.0145
X_1^2	-0.027747	0.01738176	-1.596	0.1415
X_2^2	0.015734	0.04772295	0.330	0.7484
X_2^3	0.060682	0.01808672	3.355	0.0073
X_2^4	-0.044730	0.01842177	-2.428	0.0356
X_3^2	-0.059382	0.01738176	-3.416	0.0066
X_2X_3	-0.068625	0.0219347	-3.223	0.0091

Note: The variables which gave P values higher than 0.5, which are not significant, are not presented in this table.

Determining the Optimum Point of the Factors

This response surface model can be written as:

$$Y = b_0 + f_1(X_1) + f_{23}(X_2, X_3)$$

[equation 2]

Where

$$f_1(X_1) = b_1X_1 + b_{11}X_1^2$$

$$f_{23}(X_2, X_3) = b_2X_2 + b_{22}X_2^2 + b_{222}X_2^3 + b_{2222}X_2^4 + b_3X_3 + b_{33}X_3^2 \; b_{23}X_2X_3$$

The optimum value of X_1 that maximized f_1 (X_1) was found through differentiation. X_2 and X_3 that maximized f_{23} (X_2, X_3) were maximized through calculation and sorting of f_{23} (X_2, X_3) values on a grid of points for X_2 and X_3. The search was done with computer programs written in SAS (data not shown).

The optimum point obtained through this study was (X_1, X_2, X_3) = (-0.147, 0.54, 0.15). By encoding the coded levels back to the original levels, the following results were obtained: glucose = 33.24 g/L, yeast extract = 43.1g/L and pH = 6.1. The estimated maximum response corresponding to the optimum factor levels was 9.440 $\log_{10}$CFU/mL, which was slightly higher than the center factor levels, 9.404 $\log_{10}$ CFU/mL. This was a slight improvement claimed by the regression model. A validation experiment would ascertain whether there was a real improvement.

Assessment of Factor Effects with the Partial-effects Plot

The partial-effect functions and plots were used to determine the effect of each factor graphically. The partial-effect function of a certain factor is a function that describes how the response moves as the level of that factor changes when the other factors are fixed at their optimum levels (Liew, 2005). Let Y = f (X_1, X_2, X_3) denote the response surface model described in Tables 5.4 and 5.5 and (X_1*, X_2*, X_3*) denote the optimum points of the factors which are, (-0.147, 0.54, 0.15), in this study. Hence, the partial-effect function of X_1 is defined as

$$Y(X_1) = f(X_1, X_2^*, X_3^*)$$

[equation 3]

Similarly, the partial-effect functions of X_2 and X_3 are defined as:

$$Y(X_2) = f(X_1^*, X_2, X_3^*)$$

[equation 4]

$$Y(X_3) = f(X_1^*, X_2^*, X_3)$$

[equation 5]

The partial-effect curve was drawn with the vertical axis representing $Y(X_j)$ and the horizontal axis representing X_j. By overlaying all partial-effect curves, we would get the partial-effects plot. In the partial-effects plot, since all X_j have common coded-factor levels, we let the horizontal axis represent the common coded-factor level. Figure 1 is the partial-effects plot of the factors investigated. The partial-effect curve for pH (X_3) was the most prominent, as indicated by the changes within the -1 and +1 region of the coded level. This observation is also supported by the data of P values presented in Table 5, which shows that the quadratic effect of glucose (X1) and yeast extract (X_2) are higher than 0.05, indicating that the effect was insignificant. However, the P value of pH effect (X_3) is very much lower than 0.05, suggesting that the effect was very significant. In addition the coefficient estimate for X_3^2 (0.06) was higher than for X_1^2 (0.03) and for X_2^2 (0.02). As shown in Figure 1, the partial effect of yeast extract was higher than the partial effect of glucose, suggesting that yeast extract has more influence on growth of *L. salivarius* i 24 than glucose. Aeschlimann (1989) and Schepers *et al.* (2002) claimed that the growth of *L. helveticus* was limited by nitrogen substrate in whey permeate, due to low content of total nitrogen as compared to lactose. The partial-response curves of glucose and pH also showed a pronounced change; the estimated response increased rapidly until the coded levels of glucose and pH reached 0 and then declined gradually after the glucose and pH percentages became higher than their coded level of 0. This happened while the other factors were fixed at their optimum levels.

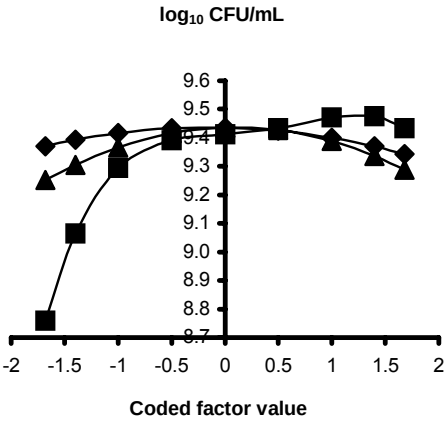

Figure 1: Partial-effects plot of (◆) glucose, (■) yeast extract and (▲) pH

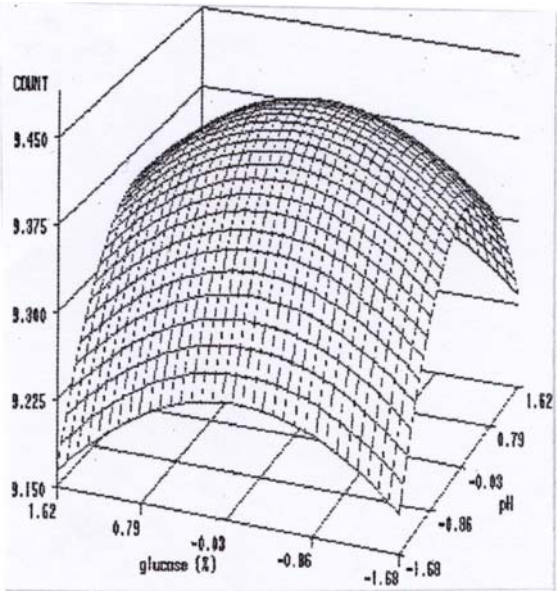

Figure 2: Response surface for the effects of glucose and yeast extract on the growth of *L salivarius* i 24 at pH = 6.10

Plotting Three Dimensional Response Surface Plots

For any two of the three significant factors, a three-dimensional response surface plot was drawn with the vertical axis representing $\log_{10}$ CFU/mL and two horizontal axes representing the coded levels of two explanatory factors. In each plot, the factors, which were not representing the two horizontal axes, were fixed at their optimum actual levels. All three plots were produced (Figures 2 to 4). In Figures 2 and 4, it can be seen that the effects of pairs of factors were additive since there were no interactions. Additivity of the two factor effects means that the effect of one factor on the response is independent of the level of the other factor (Ha *et al*., 2003; Liew, 2004).

Figure 3 shows non-additive effects of yeast extract and pH that were due to the significant interaction between them. The coefficient estimate of this interaction term had a negative sign (b_{23} = -0.068625). By considering this interaction only, the negative sign may imply that for an increase of the response, the coded levels of yeast extract and pH must have the opposite sign—one greater than zero or one smaller than zero (Oh *et al.,* 1995). However, the three-dimensional plot did not show this feature and at the optimum point, X_2 and X_3 = (0.54, 0.15). This is considered to be due to the other terms (linear, square, cubic and quartic terms) dominating the interaction term (Ha *et al*., 2003; Liew, 2004).

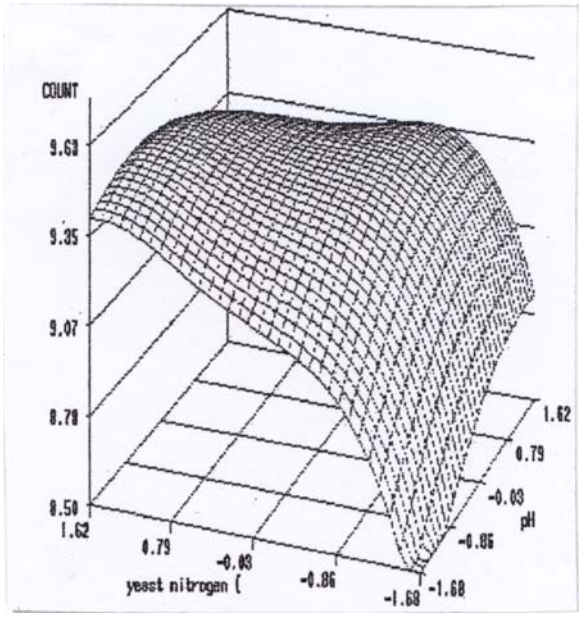

Figure 3: Response surface for the effects of yeast extract and pH on the growth of *L salivarius* i 24 at glucose = 3.324%

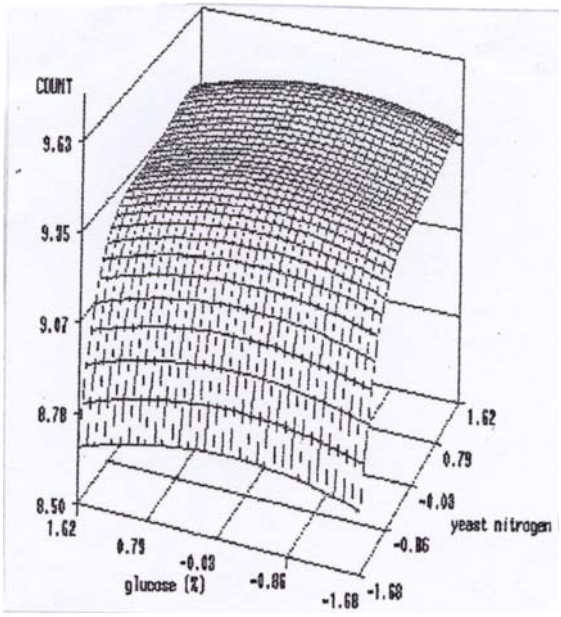

Figure 4: Response surface for the effects of glucose and pH on the growth of *L salivarius* i 24 at yeast extract = 4.31%

Validation of the Optimum Points of the Factors

An experiment was conducted to validate the optimum point of the factors found in this study. Here, we compared three growth media; the MRS medium, the optimum-point medium and the center-point medium. The compositions of these media are given in Table 6. Figure 5 shows three growth curves with the vertical axis representing $\log_{10}$ CFU/mL and the horizontal axis representing the fermentation time in hours.

The MRS medium produced the highest number of viable cells at 16 h (Figure 5). As for the optimum-point medium, even though the final cell concentration was less than that of the MRS medium, it took a shorter time to reach the highest number of viable cells, which was 8 h. This indicated that its productivity was higher compared to the MRS medium. The productivity can be estimated as final number of viable cells divided by the fermentation time. Centre-point medium also produced the highest number of viable cells at 8 h, but the amount of viable cells was less than that of the optimum-point medium. Thus, the optimum-point medium seemed to be the most suitable among the three media in term of productivity of the cultivation process. It is also simpler in composition and might be cheaper than MRS medium.

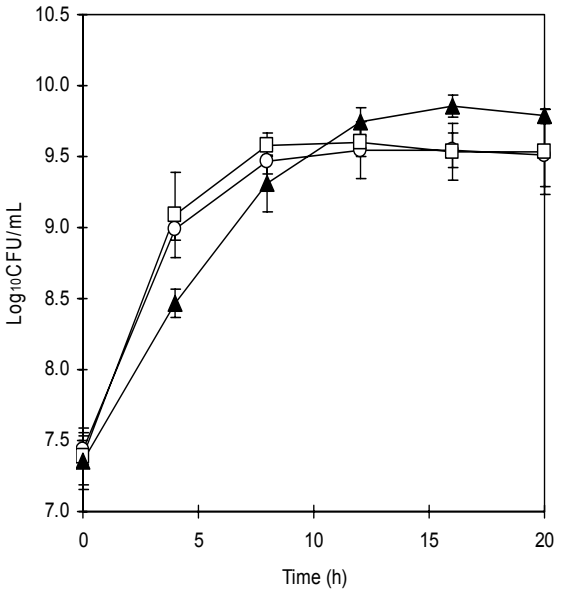

Figure 5: Growth curves of *L salivarius* i 24 in MRS broth (▲), optimum-point (□) and center point (o) media as obtained from the validation experiment

Table 6: Compositions of three media for the growth of *L salivarius* i 24

Composition	Amount of component (%) in		
	Optimum-point	Center-point	MRS
Glucose	3.324	3.5	2.0
Yeast extract	4.31	3.5	0.5
Tween 80	0.1	0.1	0.1
Proteose peptone	-	-	1.0
Beef extract	-	-	1.0
Ammonium citrate	-	-	0.2
Sodium citrate	-	-	0.5
Magnesium sulfate	0.002	0.002	0.01
Manganese sulfate	-	-	0.005
Dipotassium phosphate	0.2	0.2	0.2
pH	6.1	6	6.20

CONCLUSIONS

This study demonstrated that RSM was used successfully in designing, analyzing, finding the optimum point and assessing the effects of factors leading to a higher growth rate of *L. salivarius* i 24, which in turn, improve the overall productivity of the cultivation process. The optimum conditions of the factors for the growth of *L. salivarius* i 24 are as follows: glucose = 33.24 g/L, yeast extract = 43.1 g/L and pH = 6.1. Even though the optimum levels of glucose and yeast extract were higher than the centre point level, the composition of the medium was less complicated than the commercial MRS medium. Hence, it could imply a reduction in the cost of production, which would translate to an economic gain. Another advantage of using the optimized medium is significant increase in the productivity as compared to for the MRS medium.

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