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Journal of Biotechnology 93 (2002) 27–34

JOURNAL OF Biotechnology

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Optimization of a cultural medium for bacteriocin production by *Lactococcus lactis* using response surface methodology

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Received 31 January 2001; received in revised form 11 July 2001; accepted 19 July 2001

Abstract

The medium composition for bacteriocin production by *Lactococcus lactis* ATCC 11454 was optimized using response surface methodology. The selected six factors based on CM medium were sucrose, soybean peptone, yeast extract, KH_2PO_4, NaCl, and $MgSO_4 \cdot 7H_2O$. Fractional factorial designs (FFD) and the path of steepest ascent were effective in searching for the main factors and approaching the optimum region of the response. By a 2^{6-2} FFD, sucrose, soybean peptone, yeast extract, KH_2PO_4 were found to be significant factors and had positive effects on cell growth, however, only soybean peptone and KH_2PO_4 were shown to be the two significant factors for bacteriocin production and had negative and positive effects, respectively. The effects of the two main factors on bacteriocin production were further investigated by a central composite design and the optimum composition was found to be 1% sucrose, 0.45% soybean peptone, 1% yeast extract, 2.84% KH_2PO_4, 0.2% NaCl, and 0.02% $MgSO_4 \cdot 7H_2O$. The optimal medium allowed bacteriocin yield to be doubled compared to CM medium.

Keywords: Bacteriocin; *Lactococcus lactis*; Response surface methodology; Optimization

1. Introduction

Cell growth and the accumulation of metabolic products are strongly influenced by medium compositions such as carbon sources, nitrogen sources, growth factors, and inorganic salts. It is difficult to search for the major factors and to optimize them for biotechnological processes including multivariables. The traditional 'one-factor at a time' technique used for optimizing a multivariable system not only is time-consuming, but also may result in wrong conclusions (Oh et al., 1995). Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the ef-

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0168-1656/02/$ - see front matter
PII: S0168-1656(01)00377-7

fects of factors, and searching optimum conditions of factors for desirable responses. With the development of computing software and the wide application of personal computers, RSM has been successfully applied in many areas of biotechnology such as optimization of a cultural medium (Ooijkaas et al., 1999), enzyme synthesis (Ismail et al., 1998), gibberellic acid production (Escamilla et al., 2000), lactic acid esterification (Kiran et al., 1999), and aqueous two-phase extraction of bacteriocin (Li et al., 2001), but it has not yet been reported as a means to study and optimize conditions of a medium for bacteriocin production.

Bacteriocin-producers are lactic acid bacteria which need complex nutritions to grow, and this not only increases the production cost, but also gives rise to the difficulties for their purification. Various media are used to cultivate the bacteriocin-producer such as CM (De Vuyst and Vandamme, 1992), SM8 (De Vuyst, 1995), M17 (Terzaghi and Sandine, 1975), M17S (Li et al., 2000) and MRS (De Man et al., 1960) media. All of these media are good for neutralizing lactic acid and improving cell growth, but do not consider the accumulation of bacteriocin and high content of nitrogen sources, especially proteins and peptides, that may bring about the difficulties of bacteriocin purification (Carolissen-Mackay et al., 1997).

The objectives of this work were to evaluate the effects of the medium components on nisin production and cell growth, and to search for the optimal medium composition for attaining a higher nisin yield.

2. Materials and methods

2.1. Materials

Yeast extract and peptone were obtained from Oxoid, and nisin from Sigma.

2.2. Bacterial strains and media

In this research work, CM medium was used as a starting medium for optimization and as a cultural medium for *Lactococcus lactis* ATCC 11454, a nisin-producing strain. The initial pH of the medium was adjusted to 6.8. *Micrococcus flavus* NCIB 8166, which was used as the indicator organism in nisin assay, was grown on medium SI (Tramer and Fowler, 1964). CM medium and SI medium were autoclaved at 121 °C for 20 min and 115 °C for 30 min, respectively.

2.3. Cultivation

The cells of *L. lactis* were pregrown in 10 ml CM medium without peptone overnight at 30 °C, and then 0.5 ml of the culture was added aseptically to 50 ml CM medium or other media for the optimization in a 100 ml serum bottle with a rubber plug. The culture was shaken at 180 rpm and 30 °C anaerobically. Samples were taken from the bottle with a 1 ml disposable syringe at a set time of incubation.

2.4. Cell biomass and nisin activity determination

Cell biomass was measured by optical density of the culture at 600 nm. For detecting nisin activity, a 1 ml sample was diluted with 9 ml 0.02 mol l^{-1} HCl containing 0.1% Tween 80. The tubes were then placed in a boiling-water bath for 5 min. The nisin activities of the samples were determined by an agar diffusion method (Tramer and Fowler, 1964).

2.5. Experimental designs

Fractional factorial designs (*FFD*). The purpose of the first optimization step was to identify which ingredient(s) of the medium has a significant effect on nisin production. Factorial designs, one class of experimental designs, are very useful in identifying the important nutrients and interactions between two or more nutrients in relatively few experiments as compared to the one-factor-at a time technique. There are six ingredients in CM medium and each one was set as a factor in our optimization procedures. So, according to factorial designs there are 64 experiments, which are still a great number.

The number of experiments can be reduced by using only part of the factorial designs (fractional factorial design) without loss of information about the main effects. For a 2^{6-2} fractional factorial design with six factors at two levels, 16 experimental runs are required. In order to approach the vicinity of the optimum, a first-order model was fitted to the data obtained from the FFD experiments. For a region remote from the maximum, this first-order approximation is sufficient. The response surface is hence represented locally by a sloping plane. The next experiment was then carried out along the path of steepest ascent, that is, the direction at right angles to the contour lines representing equal yield, which shows the relative amounts by which the factors have to vary in order to attain a maximum increase of responses.

Central composite designs (*CCD*). In order to describe the nature of the response surface in the optimum region, a central composite design with five coded levels was performed. For the two factors, this design was made up of a full 2^2 factorial design with its four cube points, augmented with five replications of the center points (all factors at level 0) and the four star points, that is, points having for one factor an axial distance to the center of $\pm\alpha$, whereas the other factor is at level 0. The axial distance α was chosen to be 1.414 to make this design orthogonal. For predicting the optimal point, a second-order polynomial function was fitted to the experimental results. For two factors this equation is:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2, \quad (1)$$

where Y, predicted response, stands for nisin concentration or cell growth (OD_{600}).

Data analysis. SAS/Statistics, version 6.12 was used for the regression analysis of the experimental data obtained. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2, and its statistical significance was checked by an F-test. The significance of the regression coefficient was tested by a t-test. The level of significance was given as $***P < 0.01$, $**P < 0.05$, $*P < 0.1$. A differentiation calculation was then employed for predicting the optimum point.

3. Results and discussion

3.1. Effects of nitrogen sources on cell growth and nisin production

There are six components in CM medium, namely, 1.0% sucrose, 1.0% peptone, 1.0% yeast extract, 1.0% KH_2PO_4, 0.2% NaCl, and 0.02% $MgSO_4{\cdot}7H_2O$, and the high content of organic nitrogen sources in this medium may make bacteriocin purification difficult. Lowering the amount of the organic nitrogen sources in the medium while keeping the nisin yield constant is advantageous not only for bacteriocin purification, but also for decrease of the production cost. In order to investigate the effects of nitrogen sources on nisin production, four different media were designed as follows: CM medium (Treatment 1); CM medium without yeast extract (Treatment 2); CM medium without peptone (Treatment 3); and CM medium without yeast extract and peptone, but supplied with 2.0% corn syrup and 0.2% NH_4NO_3 (Treatment 4). The initial pH values for the four different media were adjusted to pH 6.8, and the media were autoclaved at 121 °C for 20 min and then inoculated with fresh inocula. The culture was shaken at 180 rpm and 30 °C anaerobically. Nisin production and OD_{600} were determined after 6 and 8 h of incubation. The results are presented in Fig. 1. CM medium was advantageous to cell growth, but not optimal for nisin accumulation. The biomass concentration for the cells grown in Treatment 3 was lower than that in CM medium (Treatment 1), but nisin concentration in Treatment 3 was higher than that in CM medium. Consequently, higher biomass concentration could not necessarily result in higher nisin concentration for *L. lactis* ATCC 11454. Kim et al. (1997) and Bogovic-Matijasic and Rogeli (1998) also reported that the maximization of cell growth might not result in maximization of bacteriocin production. Therefore, it was possible to obtain an optimal medium by optimizing the components of CM medium with nisin production as the desired response.

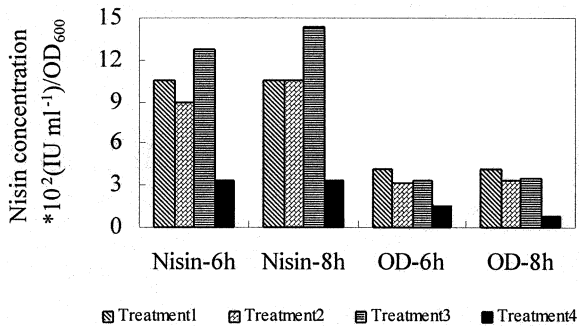

Fig. 1. Influence of nitrogen sources on cell growth and nisin concentration. Treatment 1, Treatment 2, Treatment 3 and Treatment 4 stand for CM medium, CM medium without yeast extract, CM medium without peptone and CM medium without yeast extract and peptone, but supplied with 2.0% corn syrup and 0.2% NH_4NO_3, respectively. Cells were grown in different media, respectively at a shaker speed of 180 rpm and 30 °C anaerobically.

3.2. Effects of the different ingredients of CM medium on nisin production

The first step for searching for the optimal conditions is to identify the variables that are of a significant influence on the desired response and to determine the appropriate ranges within which the variables change. In this work, the six components in CM medium were set as six different variables, and the concentration for each ingredient in CM medium was appropriately enlarged as the ranges for the variables.

2^{6-2} fractional factorial design required 16 experiments, and four other experiments at the center of the design were added for analysis of the variances. The experimental design and the results of the FFD are illustrated in Table 1. The nisin concentration varied markedly from 692 to 1875 IU ml^{-1} with the different levels of the components in the media. The concentration of KH_2PO_4 strongly affected nisin production. The high level

Table 1
Experimental design and results of the FFD

Run	x_1	x_2	x_3	x_4	x_5	x_6	OD_{600}[a]		Nisin[a] (IU ml^{-1})	
							Observed	Expected	Observed	Expected
1	−1	−1	−1	−1	−1	−1	2.192	2.16	811	831
2	+1	−1	−1	−1	+1	+1	2.344	2.54	922	895
3	−1	+1	−1	−1	+1	−1	2.688	2.52	692	650
4	+1	+1	−1	−1	−1	+1	2.632	2.82	692	699
5	−1	−1	+1	−1	+1	+1	2.952	2.76	909	867
6	+1	−1	+1	−1	−1	−1	2.704	2.94	896	993
7	−1	+1	+1	−1	−1	+1	3.16	3.08	922	671
8	+1	+1	+1	−1	+1	−1	3.04	3.30	871	858
9	−1	−1	−1	+1	−1	+1	3.296	3.50	1337	1254
10	+1	−1	−1	+1	+1	−1	3.72	3.76	1875	1621
11	−1	+1	−1	+1	+1	+1	3.56	3.86	1253	1299
12	+1	+1	−1	+1	−1	−1	4.392	4.04	1464	1425
13	−1	−1	+1	+1	+1	−1	3.936	3.98	1583	1593
14	+1	−1	+1	+1	−1	+1	4.44	4.28	1583	1566
15	−1	+1	+1	+1	−1	−1	3.912	4.26	1427	1397
16	+1	+1	+1	+1	+1	+1	4.736	4.64	1390	1461
17	0	0	0	0	0	0	3.536	3.40	1120	1146
18	0	0	0	0	0	0	3.6	3.40	1154	1146
19	0	0	0	0	0	0	3.48	3.40	982	1146
20	0	0	0	0	0	0	3.64	3.40	1040	1146

$x_i = (X_i - 10)/5$, $(i = 1, 2, 3, 4)$; $x_5 = X_5 - 2$; $x_6 = (X_6 - 0.2)/0.1$. X_1, X_2, X_3, X_4, X_5, and X_6 stand for natural variables of sucrose, soybean peptone, yeast extract, KH_2PO_4, NaCl, and $MgSO_4 \cdot 7H_2O$ (g l^{-1}), respectively.

[a] OD_{600} and nisin concentrations were determined at 8 h of incubation for the culture.

Table 2
Results of the FFD regression analysis for nisin and OD_{600}

Term	Regression analysis for nisin			Regression analysis for OD_{600}		
	Coefficient	t-value	Significant level	Coefficient	t-value	Significant level
Intercept	1146.15	41.89	0.0001***	3.40	60.74	0.0001***
x_1	47.44	1.55	0.1450	0.14	2.31	0.0379**
x_2	−75.31	−2.46	0.0286**	0.16	2.53	0.0249**
x_3	33.44	1.09	0.2942	0.25	4.05	0.0014***
x_4	324.81	10.62	0.0001***	0.64	10.27	0.0001***
x_5	22.69	0.74	0.4715	0.02	0.25	0.8082
x_6	−38.19	−1.25	0.2339	0.03	0.54	0.6013
	$R^2 = 0.9055$, $F = 20.752 > F_{6,13,0.01} = 4.62$			$R^2 = 0.9116$, $F = 22.341 > F_{6,13,0.01} = 4.62$		

of KH_2PO_4 concentration (15 g l^{-1}) allowed the strain to produce a greater nisin concentration than the low level of KH_2PO_4 concentration (5 g l^{-1}). De Vuyst and Vandamme (1993) reported that potassium dihydrogen phosphate was able to improve cell growth and nisin synthesis. Also, nisin production was affected by the level of soybean peptone, decreasing with the elevated concentration of soybean peptone. The other components in the media did not significantly influence nisin production.

The values of the regression coefficients were calculated and an equation of the first order could be written from the coefficients:

$$Y_{\text{nisin}} = 1146.15 + 47.44x_1 - 75.31x_2 + 33.44x_3 + 324.81x_4 + 22.69x_5 - 38.19x_6. \quad (2)$$

Regression analysis of the FFD in Table 2 showed that potassium dihydrogen phosphate and soybean peptone were significant at the probability levels of 99 and 95% respectively for nisin accumulation and proved to be the two most important components of the media. Sucrose, yeast extract, NaCl, and $MgSO_4 \cdot 7H_2O$ were not found to be significant at the probability level of 90% for nisin production.

The coefficient of determination R^2 of the model was calculated to be 0.91. This indicates that the model explains 91% of the variability in the data. The statistical significance of the model equation was also confirmed by an F-test, which was 20.75. The model was found to be adequate to the data at the probability level of 99%.

3.3. Effects of the various components of CM medium on cell growth

Regression analysis for cell growth based on the FFD is also presented in Table 2. Potassium dihydrogen phosphate, yeast extract, sucrose, and soybean peptone were found to be significant at the probability level of 99 or 95%. Cell growth of *L. lactis* was positively affected by the four components. But NaCl and $MgSO_4 \cdot 7H_2O$ were not found to be significant at the probability level of 90% for cell growth. Therefore, the different components in CM medium had different effects on cell growth and nisin accumulation. De Vuyst and Vandamme (1992) also found that a high concentration of sucrose was able to result in high biomass, but did not increase the bacteriocin production.

3.4. The path of steepest ascent

The path of steepest ascent was determined by Eq. (2) and by the regression analysis for nisin production. Sucrose (x_1), yeast extract (x_3), NaCl (x_5), and $MgSO_4 \cdot 7H_2O$ (x_6) were fixed at the center level of the FFD because they were not significant at the probability level of 90% for nisin production. For the two significant factors, increasing the concentration of potassium dihydrogen phosphate (x_4) and decreasing the concentration of soybean peptone (x_2) according to the signs of their main effects should have a positive consequence for nisin production by *L.*

lactis. Table 3 illustrates the directions of changing the two variables, that is, potassium dihydrogen phosphate was increased serially by 0.5%, while soybean peptone was decreased serially by 0.116%. It is clearly seen that the yield plateau has been reached at medium four, and this medium was chosen for the further optimization.

Table 3
Experimental design of the ascent and corresponding response

Run	X_2	X_4	OD_{600}[a]	Nisin concentration[a] (IU ml^{-1})
1	8.84	15	4.250	1285
2	7.68	20	4.976	1647
3	6.52	25	4.944	1847
4	5.36	30	4.624	1972
5	4.20	35	4.496	1758
6	3.04	40	3.968	1701
7	1.88	45	3.752	1673
8	0.72	50	3.560	1566

X_2 and X_4 stand for the natural variables of soybean peptone and KH_2PO_4 (g l^{-1}).

[a] OD_{600} and nisin concentrations were determined at 8 h of incubation for the culture.

Table 4
Experimental design and results of the 2^2 full factorial central composite design

Run	Factor		Y value [Nisin concentration (IU ml^{-1})][a]	
	x_2	x_4	Observed	Expected
1	−1	−1	1527	1510
2	+1	−1	2058	1964
3	−1	+1	1527	1522
4	+1	+1	1727	1645
5	−1.414	0	1567	1562
6	+1.414	0	1866	1970
7	0	−1.414	1604	1662
8	0	+1.414	1404	1445
9	0	0	1866	1993
10	0	0	1972	1993
11	0	0	2103	1993
12	0	0	1998	1993
13	0	0	2028	1993

$x_2 = (X_2 - 5.36)/(-1.16)$, $x_4 = (X_4 - 30)/5$. X_2 and X_4 stand for the natural variables of soybean peptone and KH_2PO_4 (g l^{-1}).

[a] Nisin concentrations were determined at 8 h of incubation for the culture.

3.5. Optimization of the medium

As seen above, by determining the path of steepest ascent, the neighborhood of the optimum response seems to be approached. The levels of the two significant variables, potassium dihydrogen phosphate (x_4) and soybean peptone (x_2), were further optimized using a central composite design. The levels of the two variables, the experimental design and the results are presented in Table 4.

The experimental results of the CCD were fitted with a second-order polynomial function. The results of the regression analysis are shown in Table 4 and the fitted equation for estimation of nisin production had the following form:

$$Y = 1993.39 + 144.25x_2 - 76.74x_4 - 82.75x_2x_4 - 113.57x_2^2 - 219.86x_4^2. \quad (3)$$

From Eq. (3), it was shown that the signs of b_{11} and b_{22} were both negative, so the parabola would be open downward and indicated to be of a maximum point.

The model adequacy was checked by an F-test and the determination coefficient R^2. $F = MS_R/MS_e = 14.202 > F_{5,7,0.01} = 7.46$, $F_{LF} = MS_{LF}/MS_{pe} = 10664.33/7479.75 = 1.426 < F_{3,4,0.1} = 4.19$. Therefore, the obtained model was adequate. The goodness of fit of the model was expressed by the coefficient of determination R^2, which was calculated to be 0.91, indicating that 91% of the variability in the response could be explained by the model. This proves that the model equation as expressed in Eq. (3) provides a suitable model to describe the response of the experiment pertaining to nisin production. Fig. 2 shows the contour plot of the model equation and this plot indicates a rather broad plateau region in which the nisin concentrations change relatively little when the nutrient concentrations vary.

From equations derived by differentiation of Eq. (3), we can obtain the maximum point of the model, which was 4.49 g l^{-1} of soybean peptone and 28.42 g l^{-1} of potassium dihydrogen phosphate. The model predicted a maximum response of 2060 IU ml^{-1} for this point. In order to confirm the predicted results of the model, experiments using the medium representing this maxi-

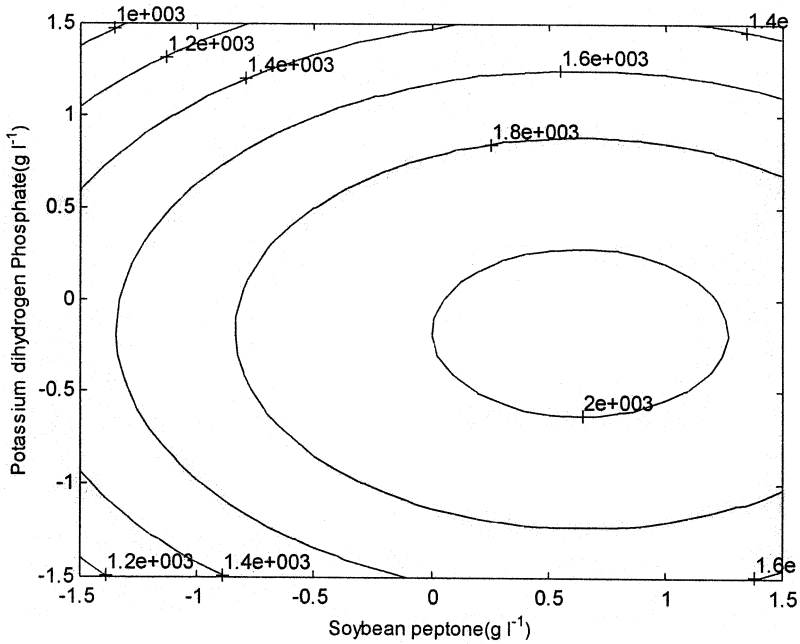

Fig. 2. Contour plot of the model equation fitted to the data of the central composite design experiment. On the x and y axes, the concentrations of soybean peptone and potassium dihydrogen phosphate are given in their coded forms as listed in Table 4 respectively.

mum point were performed and a value of 2150 ± 121 IU ml^{-1} ($N = 3$) was obtained. The good correlation between these two results verifies the validity of the response model and the existence of an optimal point.

4. Conclusion

Response surface methodology proved to be a powerful tool in optimizing a medium for bacteriocin production by *L. lactis*. The optimal medium made soybean peptone decreased by 0.55% and allowed bacteriocin yield to increase from 1074 to 2150 IU ml^{-1} compared to CM medium.

The different ingredients of CM medium have different effects on bacteriocin yield and cell growth. For cell growth, sucrose, soybean peptone, yeast extract, and potassium dihydrogen phosphate were positively significant factors, whereas for bacteriocin production, only soybean peptone and potassium dihydrogen phosphate were significant factors, the effect of the former being negative and of the latter positive.

It was found from the experiments that higher biomass concentration could not necessarily result in higher nisin production.

References

Bogovic-Matijasic, B., Rogeli, I., 1998. Bacteriocin complex of *Lactobacillus acidophilus* LF221—production studies in MRS-media at different pH-values and effect against *Lactobacillus helveticus* ATCC 15009. Process Biochem. 33, 345–352.

Carolissen-Mackay, V., Arendse, G., Hastings, J.W., 1997. Purification of bacteriocins of lactic acid bacteria: problems and pointers. Int. J. Food Microbiol. 34, 1–16.

De Man, J.C., Rogosa, M., Sharpe, E., 1960. A medium for cultivation of *Lactobacilli*. J. Appl. Bacteriol. 23, 130–135.

De Vuyst, L., 1995. Nutritional factors affecting nisin production by *Lactococcus lactis* subsp. *lactis* NIZO 22186 in a synthetic medium. J. Appl. Bacteriol. 78, 28–33.

De Vuyst, L., Vandamme, E.J., 1992. Influence of the carbon source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentations. J. Gen. Microbiol. 138, 571–578.

De Vuyst, L., Vandamme, E.J., 1993. Influence of the phosphorus and nitrogen source on nisin production in *Lacto-*

coccus lactis subsp. *lactis* batch fermentation using a complex medium. J. Appl. Microbiol. Biotechnol. 40, 17–22.

Escamilla, E.M.S., Dendooven, L., Magaña, I.P., Parra, R.S., De la Torre, M., 2000. Optimization of gibberellic acid production by immobilized *Gibberella fujikuroi* mycelium in fluidized bioreactors. J. Biotechnol. 76, 147–155.

Ismail, A., Soultani, S., Ghoul, M., 1998. Optimization of the enzymatic synthesis of butyl glucoside using response surface methodology. Biotechnol. Prog. 14, 874–878.

Kim, W.S., Hall, R.J., Dunn, N.W., 1997. The effect of nisin concentration and nutrient depletion on nisin production of *Lactococcus lactis*. Appl. Microbiol. Biotechnol. 48, 449–453.

Kiran, K.R., Karanth, N.G., Divakar, S., 1999. Preparation of stearoyl lactic acid ester catalyzed by lipases from *Rhizomuco miehei* and porcine pancreas optimization using response surface methodology. Appl. Microbiol. Biotechnol. 52, 579–584.

Li, C., Ouyang, F., Bai, J., 2000. Extractive cultivation of *Lactococcus lactis* using a polyethylene glycol/$MgSO_4 \cdot 7H_2O$ aqueous two-phase system to produce nisin. Biotechnol. Lett. 22, 843–847.

Oh, S., Rheem, S., Sim, J., Kim, S., Baek, Y., 1995. Optimizing conditions for the growth of *Lactobacillus casei* YIT 9018 in tryptone-glucose medium by using response surface methodology. Appl. Environ. Microbiol. 61, 3809–3814.

Ooijkaas, L.P., Wilkinson, E.C., Tramper, J., Buitelaar, R.M., 1999. Medium optimization for spore production of *Coniothyrium minitans* using statistically-based experimental designs. Biotechnol. Bioeng. 64, 92–100.

Li, C., Bai, J., Li, W., Cai, Z., Ouyang, F., 2001. Optimization of conditions for bacteriocin extraction in PEG/salt aqueous two-phase systems using statistical experimental designs. Biotechnol. Prog. 17, 366–368.

Terzaghi, B.E., Sandine, W.E., 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29, 807–813.

Tramer, J., Fowler, G.G., 1964. Estimation of nisin in foods. J. Sci. Food Agric. 15, 522–528.