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Full Length Research Paper

Application of response surface methodology (RSM) for optimization of high-yielding L-lactic acid strains selected by low-energy ion implantation

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In this paper, in order to obtain mutant strains with high yield of L-lactic acid, the original strain *Lactobacillus casei* SB-310 was implanted by nitrogen ions. It was found that the higher positive mutation rate was appeared when the output power was 10keV and the dose of nitrogen ion implantation was $50\times2.6\times10^{13}$ ions/cm^2. A mutant strain which was named as *Lactobacillus casei* NE-19 was screened, its yield of L-lactic acid was about 120 g/L, and it was high increasing compared to the original strain (80.3 g/L). Response surface method was applied to optimize the fermentation medium. The highest yield of L-lactic acid was obtained when the concentration of glucose, peptone, beef extract and yeast extract were 153.68 g/L, 17.27 g/L, 15.89 g/L and 8.28 g/L respectively, and the highest yield was 134.79 g/L.

Keywords: L-(+)-lactic acid, low-energy ion implantation, mutant, optimization, response surface method

INTRODUCTION

Lactic acid and its derivatives had long been used in food, pharmaceutical, leather, textile industries and cosmetic applications (Toshinari et al., 2009; Wang et al., 2009). In the food industry, L-(+)-lactic acid (L-LA) plays more of vital role than D-(–)-lactic acid because the latter will cause acidosis in human beings with short bowel syndrome (Altaf et al., 2006). Furthermore, since lactic acid has an excellent reactivity that stems from it having both a carboxylic acid and hydroxyl group, it can undergo a variety of chemical conversions into potentially useful chemicals such as propylene oxide, propylene glycol, acrylic acid, 2,3-pentanedione, and lactate ester (Yu et al., 2008). Recently, pure L-LA is being studied with great interest as it could be used as a raw material for the production of poly lactic acid, a polymer used as environmental friendly biodegradable plastics, which substitute for synthetic plastics derived from petroleum. For poly L-LA, it can also be applied in the biocompatible and bio-absorbable markets, such as the dental arena, drug delivery systems, sutures and surgical implants (Ding et al., 2006).

Low-energy ion beam technology, usually characterized by ion bombardment, has been widely applied in the field of materials, physics, chemistry, etc. since 1970s, while people have paid no attention to its biological effects until in 1986 when the first report was published by a group of Chinese researchers (Feng et al., 2006; Zhang et al., 2008). As a new mutagenesis technique, low-energy ion beam implantation has attracted much attention in breeding of plants and microorganisms (Liu et al., 2005). This technology induces bio-effects including physical, chemical, biochemical and biological processes. Compared to the traditional mutation methods, low-energy ion beam technology has more advantages, such as low injury rate, higher mutation rate, and wider spectrum of mutations

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(Song et al., 2010). Many high-yield strains have been obtained by ion beam implantation in industrial microorganisms.

Conventional and classical methods of studying a process by maintaining other factors involved at an unspecified constant level does not depict the combined effect of all the factors involved. These methods are also time consuming and require a number of experiments to determine optimum levels, which are unreliable. The statistics-based procedure called response surface method (RSM) is a powerful experimental design tool (Mu et al., 2006; Praveen et al., 2009; Soo et al., 2004) to recognize the performance of composite systems. The RSM represents an assemblage of experimental design and multiple regression-based methods that can be applied to evaluate tribulations where several factors might influence a response. Among the nutritional parameters affecting the fermentative lactic acid production, glucose, beef extract (BE), yeast extract (YE) and peptone were the main factors due to the purveyance of carbon source, nitrogen and growth factors such as purine and pyrimidine bases, B vitamins and so on. In the present investigation different contents of Glucose, BE, YE and Peptone were studied adopting a full range of RSM using central composite design (CCD) model to analyze the effects of yield by the mutant strain. The regression model provides an excellent explanation of the relationship between the independent variables and the response.

MATERIALS AND METHODS

Microorganism

The wild type strain *Lactobacillus casei* SB-310 was maintained on slants containing storage medium (g/L): beef extract 5, yeast extract 5, peptone 10, glucose 10, lactose 5, sodium chloride 5, agar 20; and pH 6.8.

Media and culture conditions

Seed medium contained (g/L): glucose 50, peptone 15, beef extract 15, yeast extract 7.5, sodium acetate anhydrous 5, $MgSO_4 \cdot 7H_2O$ 0.2, $MnSO_4 \cdot 7H_2O$ 0.125, $CaCO_3$ 10 (sterilized alone), and 1 ml/L tween-80. The isolation medium was based on the seed medium and added 2% agar. The fermentation medium had the following components (g/L): glucose 140, peptone 15, beef extract 15, yeast extract 7.5, sodium acetate anhydrous 5, $MgSO_4 \cdot 7H_2O$ 0.2, $MnSO_4 \cdot 7H_2O$ 0.125, $CaCO_3$ 10 (sterilized alone), and 1 ml/L tween-80. The pH was adjusted to 6.8 and then sterilized at 115°C for 30 min. The seed culture and fermentation culture were carried out in 250 ml Erlenmeyer flasks with the broth content of 50 ml and the rotation speed was 100 r/min with the culture temperature was 40°C.

Low energy ion implantation

Implantation sources were produced by ion beam bioengineering instrument (CN Patent No. ZL 93103361.6). The instrument was devised by ASIPP (Chinese Academy of Sciences, Institute of plasma physics). The ion source was nitrogen ion (N^+).

Broth 100 µl with proper dilution with the concentration was about 10^5 cells per milliliter were spread over sterilized plates when the seed culture reached to the end of the exponential phase of growth. In each plate, the broth smeared with single-cell layer and dried in aseptic workbench and then the plates were implanted by nitrogen ion at the energy of 10 keV and the dose for implantation range of $0\text{-}180\times2.6\times10^{13}$ ions/cm^2. At the same time, in order to evaluate the survival rate of ion beam implantation, two samples were prepared; they were placed sequentially in the target chamber. The upper one was exposed to ion beam implantation as the treated sample, while the lower one was unexposed to ion beam implantation as control.

Cytotoxicity of ion implantation

The sample was washed from the plates with sterile saline after implantation. The washed solution 100 µl containing the mutated strains was smeared on the isolation medium plates. The colonies will formed in the plates after incubation at 40°C for 24 h. The colonies number was counted to determine the survival ratio. The survival ratio was calculated as: $SR= M_0/M$

SR is the survival ratio; M_0 is the colonies number of the treated sample; M is the colonies number of the control.

Mutant selection

The mutant selection procedures were carried out from two steps. For the first step, the colonies from isolation medium were transferred to storage medium slants. For the second step, the isolated strains were cultured in flasks and the L-LA production examined. Then the mutation rate was calculated. The mutation rate criterion was defined as follows: the strains whose production of L-LA were 5% higher/lower than that of the control strain were concerned as positive/ negative mutants, while others were non-mutation.

Mutagenesis and selection procedure

The procedure of mutagenesis and selection was carried out as Figure 1.

Analysis methods of L-LA and media constituents

The concentration of calcium lactate was tested by EDTA titration method (Jin, 1989). The optical purity of L-LA was measured by HPLC using an uBondpak C18 column (Waters, U.S.A.). The mobile phase composition was CH_2OH: H_2O:

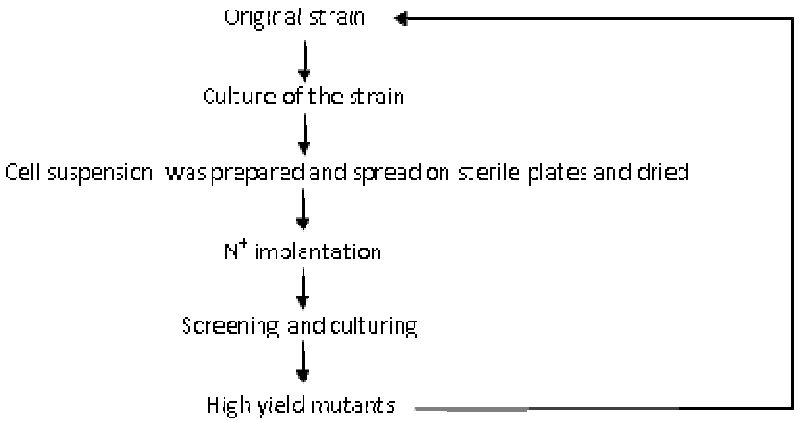

Figure 1. The mutagenesis and selection procedure

Table 1. Real and coded values of variables

Variables	Range and levels				
	- 2	- 1	0	1	2
x1Glucose(g/L)	100	137.5	175	212.5	250
x2 Peptone(g/L)	5	10	15	20	25
x3 BE(g/L)	9	12	15	18	21
x4 YE(g/L)	2.5	5	7.5	10	12.5

H_3PO_4 (10:90:0.3, V:V:V) and the flow rate was 0.8 ml/min. The detection was made in the UV range at 210 nm at the temperature of 25°C. The standard L-LA was purchased from SINGMA (St. Louis, U.S.A.). The residual sugar was quantified by the 3,5-dinitrosalicylic acid method (Miller, 1959).

Response surface method

RSM was based on CCD. According to this design, 31 experiments were conducted containing seven replications at the center point for estimating the purely experimental uncertainty variance in triplicates. The Glucose, BE, YE and Peptone were screened as variables for investigation by previous work. Table 1 showed the range and the levels of the variables investigated in this work.

The variables of the experiments were coded according to the following equation:

$$xi = \frac{(Xi - Xcp)}{\Delta Xi}$$

where xi is the coded value of an independent variable, Xi is the real value of an independent variable, Xcp is the real value of an independent variable at the center point, and ΔXi is the step change value.

The relationships and interrelationships of the variables were determined by fitting the second order polynomial equation to data obtained from 31 experiments using mean values of the triplicates of each experiment conducted thrice at different occasions. The behavior of the system was explained by the following equation:

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j$$

In which Y is the predicted response (i.e. L-LA production); b_0 is the constant term; b_i is the linear effect; b_{ij} are the quadratic effects when $i = j$ and interaction effects when $i < j$; b_{ii} are the

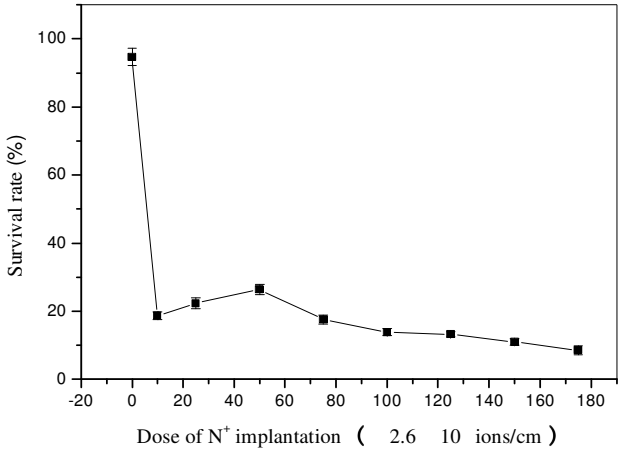

Figure 2. Effect of N^+ implantation doses on survival rate of *Lactobacillus casei* SB-310

squared terms; x_i the ith variable which are called as independent variables.

RESULTS AND DISCUSSION

N^+ implantation and mutant screening

Figure 2 showed the dose-dependent survival curve of *Lactobacillus casei* SB-310 under the energy of 10 keV. The survival rate firstly decreased with increasing dose, then slightly increased with doses in the range of 10~50×2.6×10^{13} ions/cm^2, and decreased again as the dose in the range of 50~180×2.6×10^{13}ions/cm^2. The curve shape trend looks like a "saddle". This pattern of survival is different from the others mutagen such as UV, diethyl sulfate (DES) and ^{60}Co. Therefore, the interaction between low-energy ions and organisms may be more complex than that of other radiation. The biological effect may not only be induced by energy absorption, but also results from mass deposition and charge exchange. At the beginning of ion implantation, more ions reach the cellular cytoplasm; deposition of energy and mass in the cytoplasm might play an important role in breakage of the cytoskeleton and indirect induction of nucleolus damage, which could lead directly to the death of a cell. As the fluence of ion beam is sequentially increased, more deposition of both energy and mass taking place in the nucleolus is likely to be the main factor that causes cellular damage, and activates the cell repair system. No process other than the activation could bring about an increase in the survival fraction. As a result, the survival rate gradually decreases with the increasing ion beam fluence. This was just the characteristic survival curve of N^+ implantation (Wu et al., 2005; Yu, 2007). To study the relationship between dose-dependent survival rate and mutation rate, the mutation rates were recorded (Figure 3) when cells were exposed to the various doses of N^+ with the energy of 10 keV. It was found that the positive and negative mutation rates were all low at the lower doses (30×2.6×10^{13}ions/cm^2) of ions. The highest mutation rate was observed when dose was 50×2.6×10^{13}ions/cm^2. But the mutation rates were lower under higher implantation doses (70×2.6×10^{13}ions/cm^2, 100×2.6×10^{13} ions/cm^2 , 120×2.6×10^{13} ions/cm^2 and 160×2.6×10^{13} ions/cm^2) than that of when the dose was 50×2.6×10^{13} ions/cm^2. It was an interesting phenomenon that the highest mutation rate was appeared just at the ridge of the dose-dependent survival rate curve. So, in order to obtain higher mutation rate, the dose of 50×2.6×10^{13} ions/cm^2 was chose for further mutation.

Nitrogen ions (10keV, 50×2.6×10^{13} ions/cm^2) were implanted into *Lactobacillus casei* SB-310. The mutation pedigree of the strain was showed in Table 2. A high-yield L-LA mutant *Lactobacillus casei* NE-19 was obtained by several times recurrent mutation and fermentation. The yield reached to 121.3g/L, which was 51.1% higher than that of original strain. Through six-generation investigation, the L-LA production by *Lactobacillus casei* NE-19 was shown in Table 3. The standard deviation was 2.96 which indicated that the L-LA production ability of mutant *Lactobacillus casei* NE-19 was stable.

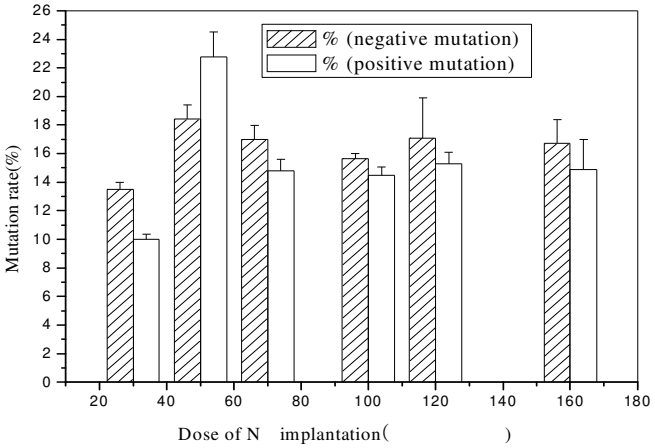

Figure 3. Effect of N^+ implantation doses on mutation rate of *Lactobacillus casei* SB-310

Table 2. Mutation pedigree of *Lactobacillus casei* SB-310

Mutation frequency	Screened strains	Yield of L-LA (g/L)
0	*Lactobacillus casei* SB-310	80.3
1	*Lactobacillus casei* NA-8	90.5
	Lactobacillus casei NA-26	86.4
2	*Lactobacillus casei* NB-2	100.8
	Lactobacillus casei NB-48	99.2
3	*Lactobacillus casei* NC-4	108.2
	Lactobacillus casei NC-11	105.7
4	*Lactobacillus casei* ND-1	112.7
	Lactobacillus casei ND-5	111.9
5	*Lactobacillus casei* NE-19	121.3
	Lactobacillus casei NE-43	116.4

Table 3. Stability of mutant *Lactobacillus casei* NE-19 during generation

Generations	1	2	3	4	5	6
Yield (g/L)	121.3	119.6	120.2	124.6	116.5	123.8

Response surface optimization of the fermentation medium

Experiments were carried out according to the design, and the average yield of L-LA was obtained after 96 hours fermentation with 31 experiments in triplicate. The results were showed in Table 4.

The following regression Eq. (3) was obtained by the application of multiple regression analysis methods according to the experimental data.

Table 4. Experimental design and the results of the CCD

Runs	X1	X2	X3	X4	Yield of L-LA (mg/L)
1	-1	-1	-1	-1	96.38
2	-1	-1	-1	1	99.09
3	-1	-1	1	-1	119.21
4	-1	-1	1	1	105.39
5	-1	1	-1	-1	104.19
6	-1	1	-1	1	120.41
7	-1	1	1	-1	118.00
8	-1	1	1	1	127.61
9	1	-1	-1	-1	67.56
10	1	-1	-1	1	93.08
11	1	-1	1	-1	78.07
12	1	-1	1	1	88.28
13	1	1	-1	-1	80.77
14	1	1	-1	1	93.98
15	1	1	1	-1	78.07
16	1	1	1	1	91.58
17	-2	0	0	0	103.89
18	2	0	0	0	64.56
19	0	-2	0	0	95.18
20	0	2	0	0	115.30
21	0	0	-2	0	98.18
22	0	0	2	0	112.60
23	0	0	0	-2	88.88
24	0	0	0	2	119.81
25	0	0	0	0	123.21
26	0	0	0	0	133.95
27	0	0	0	0	131.10
28	0	0	0	0	127.07
29	0	0	0	0	132.09
30	0	0	0	0	130.12
31	0	0	0	0	129.49

$$Y=129.5757 - 12.39792X_1 + 4.49125X_2 + 3.31625X_3 + 5.792917X_4 - 2.045625X_1X_2 - 3.095625X_1X_3 + 2.983125X_1X_4 - 1.183125X_2X_3 + 1.745625X_2X_4 - 2.384375X_3X_4 - 11.70362X_1^2 - 6.449866X_2^2 - 6.412366X_3^2 - 6.673616X_4^2 \quad (3)$$

Where Y is the predicted response, that is, the yield of L-LA, X_1, X_2, X_3 and X_4 are the coded values of the optimum variables glucose, peptone, BE and YE, respectively.

The response surface quadratic model was performed

Table 5. ANOVA for the quadratic model

Source	DF	SS	MS	F-value	P
Model	14	11704.15	836.0108	32.74427	0.0001
Error	16	408.5042	25.53151		
Lack of fit	10	333.6202	33.36202	2.673097	0.120562
Pure Error	6	74.88397	12.48066		
Total	30	12112.66			

R^2 = 0.9663; adjusted R^2 = 0.9368; CV = 4.794436; DF, Degree of freedom; SS, Sum of squares; MS, Mean square; F, variance ratio; P, probability.

Table 6. Significance of each of the variables

Source	**DF**	**SS**	**MS**	**F**	**P**
X1	1	3689	3689	144.4881	0.0001
X2	1	484.1118	484.1118	18.96135	0.000492
X3	1	263.9403	263.9403	10.33783	0.005402
X4	1	805.3892	805.3892	31.54491	0.0001
X1*X1	1	3916.892	3916.892	153.414	0.0001
X1*X2	1	66.95331	66.95331	2.622379	0.124906
X1*X3	1	153.3263	153.3263	6.005375	0.026142
X1*X4	1	142.3846	142.3846	5.576816	0.031215
X2*X2	1	1189.605	1189.605	46.5936	0.0001
X2*X3	1	22.39656	22.39656	0.877212	0.362889
X2*X4	1	48.75531	48.75531	1.909613	0.185997
X3*X3	1	1175.812	1175.812	46.05338	0.0001
X3*X4	1	90.96391	90.96391	3.562809	0.077363
X4*X4	1	1273.573	1273.573	49.88239	0.0001

in the form of analysis of variance (ANOVA) and the results were summarized in Table 5. The model of the quadratic regression was highly significant (Pr>F=0.0001). The goodness of fit of the model was checked by determination coefficient (R^2). In this case, the value of the R^2 (0.9663) for the model indicates that the sample variation of 96.63% for lactic acid was attributed to the independent variables, and only 3.37% of the total variation cannot be explained by the model. The value of the adjusted determination coefficient (adjusted R^2 = 0.9368) is also high, which advocates a high significance of the model. At the same time, a relatively lower value of the coefficient of variation (CV = 4.794436) indicates better precision and reliability of the experiments carried out. In addition, the value of lack of fit for regression model was not significant at the 5% level (P = 0.120562 > 0.05), indicating the good predictability of the model.

The Fisher F-test and the probability (P) values serve as a tool to check the significance of each of the variables. The smaller of the P-value, the more significant was the corresponding variable (Reddy et al., 2008; Garg et al., 2008). It was evident from Table 6 that the linear, quadratic were significant (P<0.05). This suggested that the concentrations of glucose, peptone, BE and YE had a

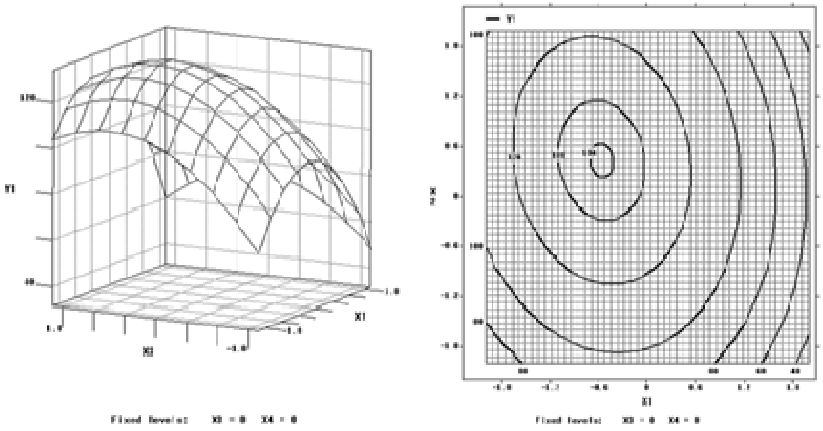

Figure 4 Response surface plot for L-LA production as a function of glucose (X_1) and peptone (X_2) concentration while keeping BE (X_3) and YE (X_4) at their respective zero level

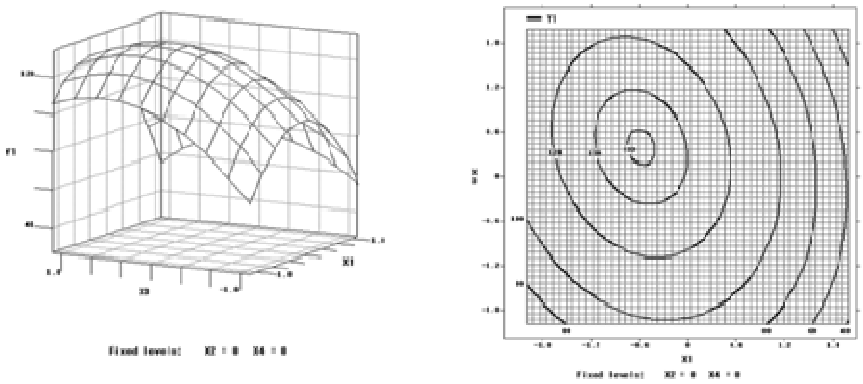

Figure 5. Response surface plot for L-LA production as a function of glucose (X_1) and BE (X_3) concentration while keeping peptone (X_2) and YE (X_4) at their respective zero level

direct relationship with the production of L-LA in the fermentation medium, i.e. any minor change in these variables from their zero level values may cause a great change in the L-LA production. The interactive effects of glucose and BE (X_1X_3), glucose and YE (X_1X_4) were also significant, while the other variables were not significant ($P>0.05$), this indicated that, the interaction effects of glucose to BE and YE were significant for L-LA production.

DF, Degree of freedom; SS, Sum of squares; MS, Mean square; F, variance ratio; P, probability.

To study the interaction of the variables and to locate the optimum level of each variable for maximum response, the 3-dimension response surface and the 2-dimension contour plots were drawn based on the graphical representations of the regression equation. Each response surface and contour plotted for L-LA production represents the different combinations of two test variables at one time while keeping the other two variables at their respective zero level. There were 6 pairs of response surfaces (Figures. 4-9) and corresponding contour plots in this work. The convex response surfaces suggested that there were well-defined optimum variables. If the response surfaces were rather symmetric and flat near

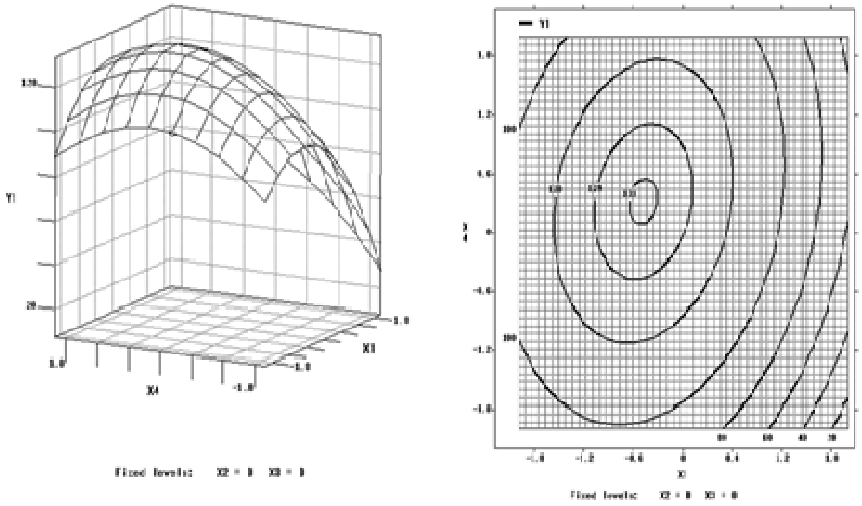

Figure 6. Response surface plot for L-LA production as a function of glucose (X_1) and YE (X_4) concentration while keeping peptone (X_2) and BE (X_3) at their respective zero level

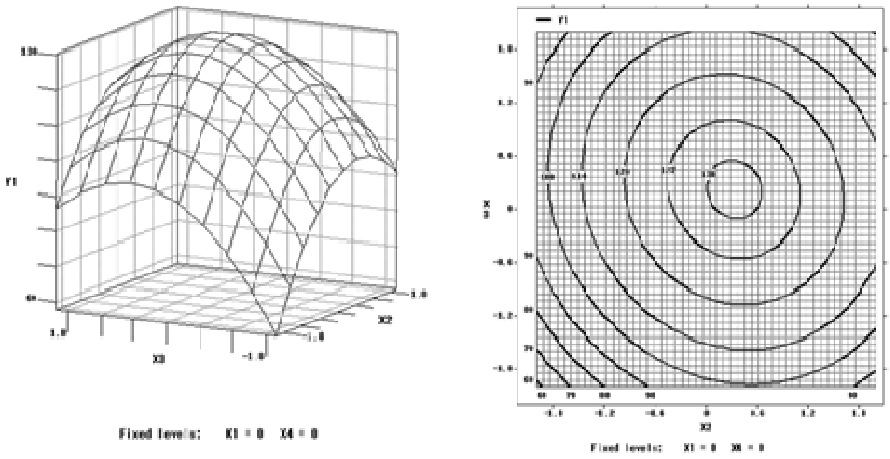

Figure 7. Response surface plot for L-LA production as a function of peptone (X_2) and BE (X_3) concentration while keeping glucose (X_1) and YE (X_4) at their respective zero level

the optimum, the optimized values may not vary widely from the single variable conditions. The interactions between the variables can be inferred from the shapes of the contour plots. Circular contour plots indicated that the interactions between the variables were negligible. In contrast, elliptical ones indicate the evidence of the interactions.

Glucose was the single substrate for L-LA production by *Lactobacillus casei* in this study, so the concentration variation of glucose containing in the fermentation medium will significantly affect the L-LA yield. Lactic acid bacteria are nutritionally fastidious, requiring various amino acid and vitamins for growth, so, various types of the nitrogen source appeared to be very important. The graphical representation of the response shown in figures helped to visualize the effect of glucose, peptone, BE and YE on production of L-LA. Figure 4 explained that higher concentration of glucose and peptone and lower concentration of peptone might cause inhibition of lactic acid production in the experiment design range. This

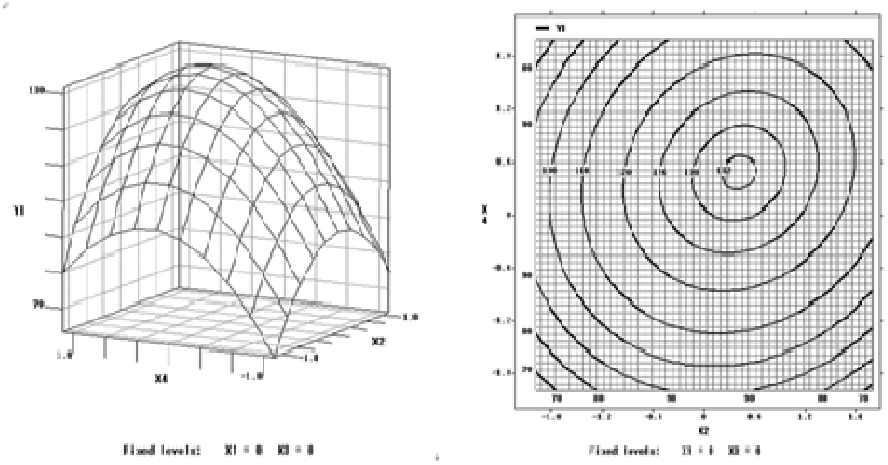

Figure 8. Response surface plot for L-LA production as a function of peptone (X_2) and YE (X_4) concentration while keeping glucose (X_1) and BE (X_3) at their respective zero level

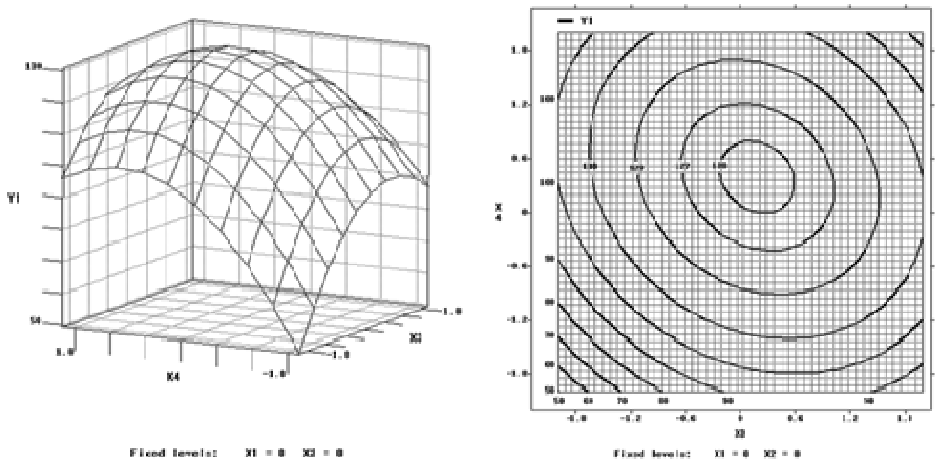

Figure 9. Response surface plot for L-LA production as a function of BE (X_3) and YE (X_4) concentration while keeping glucose (X_1) and peptone (X_2) at their respective zero level

suggested that the concentration of glucose have to be decreased and the peptone concentration have to be determined at an appropriate value for higher yields of L-LA. Figure 5 showed the interaction effect of glucose and BE in L-LA production and Figure 6 showed the interaction effect of glucose and YE. From the two figures, it was found that, the interaction effects of glucose to BE and glucose to YE were significant. At the same time, it was also found that, higher concentration of glucose was negative for L-LA production and the appropriate concentration of BE and YE was required for higher yield of L-LA. Figure 7 showed the L-LA production as a function of peptone and BE. It indicated that increase in concentration of both peptone and BE will increase the production yield, while the optimum concentration of the two variables were appeared near their zero level. As Figure 8 showed, when the concentration of peptone and YE at lower level the L-LA production was low, however

Table 7. The coded and real values of the optimum concentrations of the four variables

Factor name	Coded	Real value (g/L)
Glucose (X_1)	-0.56846	153.68
Peptone (X_2)	0.45361	17.27
BE (X_3)	0.29567	15.89
YE (X_4)	0.31347	8.28

The real value was calculated by Eq. (1)

when the concentration at higher level the L-LA production was low too, so, to obtain high yield of L-LA, the appropriate concentration of the two variables were chose. In addition to provide necessary nitrogen source, YE and BE provide a lot of growth factors such as B vitamins, purine, pyrimidine and so on. So, the concentrations of the two variables were very important in L-LA production. Figure 9 showed the BE and YE effects on the L-LA production. It was clearly demonstrated that with the concentration of BE and YE increased the yield of L-LA increased significantly, while when it increased to a maximum the yield didn't increase with the concentration of BE and YE increased, instead of decreased.

The optimum concentrations of the four variables were calculated by SAS 9.1 and they were showed in table 7. The results indicated when the concentrations of glucose, peptone, BE and YE were 153.68 g/L, 17.27 g/L, 15.89 g/L and 8.28 g/L respectively, the predicted yield of L-LA will reached to 135.52 g/L and the standard error of predicted value was 1.89. This result was proved by six times fermentation experiments using the optimum medium which the yield was as average of 134.79 g/L and the standard error was 1.02. Compared to the yield before optimization (yield of L-LA was 120 g/L) it was increased by 12.33%. This indicated that the RSM was suitable for the fermentation medium optimization of the mutant.

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