Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(4): 194-200
Received: 08-05-2018
Accepted: 12-06-2018

Santosh Kumar Mishra
a) Dairy Microbiology Division, National Dairy Research Institute, Karnal, Haryana, India
b) College of Dairy Science & Technology, GADVASU, Ludhiana, Punjab, India

RK Malik
College of Dairy Science & Technology, GADVASU, Ludhiana, Punjab, India

Rekha Chawla
College of Dairy Science & Technology, GADVASU, Ludhiana, Punjab, India

KK Mishra
College of Veterinary Science and A.H. (NDVSU), Rewa, Madhya Pradesh, India

Correspondence
Santosh Kumar Mishra
a) Dairy Microbiology Division, National Dairy Research Institute, Karnal, Haryana, India
b) College of Dairy Science & Technology, GADVASU, Ludhiana, Punjab, India

Response surface optimization of the cultivation conditions and medium components for maximal reuterin production by *L. reuteri* BPL-36

Santosh Kumar Mishra, RK Malik, Rekha Chawla and KK Mishra

Abstract
A reuterin (3-hydroxy propinaldehyde, 3-HPA) producing strain from a human infant fecal sample was previously isolated and identified in Microbial Metabolite laboratory of Dairy Microbiology Division, National Dairy research Institute as *Lactobacillus reuteri* BPL-36 strain. The organism is known for its broad-spectrum antimicrobial activity and was selected for this present study. An initial attempt in context to optimization study of reuterin production was done for the evaluation of the effect of different growth and environmental factors on reuterin production by *L. reuteri* BPL-36 using linear model. The production of reuterin from BPL-36 strain was optimized in water-glycerol medium using Response surface Methodology considering pH, temperature, incubation time and glycerol content as independent variables. Optimum level of pH (6.0), incubation temperature (37°C), incubation time (2.5h) and Glycerol content (267 mM) were used for maximum reuterin production (58.69mM) in water-glycerol medium. Since this strain showed higher reuterin production to different levels under the optimized conditions compared to their initial values, this justifies the fact that reuterin production is a strain specific attribute of *L. reuteri*.

Keywords: *Lactobacillus reuteri*, reuterin, response surface methodology, optimization

Introduction

Now a days use of natural antimicrobial compounds for bio preservation of foods have gained huge momentum because of increasing consumer demands for the safe and chemical free foods. Reuterin is one of such novel antimicrobial compound produced by certain strains of *Lactobacillus reuteri* during the anaerobic fermentation of glycerol (Mishra *et al.*, 2012; Vu *et al.*, 2017) [9, 13]. Reuterin is also known as β-hydroxypropionaldehyde (Mishra *et al.*, 2011; Mishra *et al.*, 2018) [8, 10].

Application of response surface analysis for optimization is a very fruitful way for the analysis of problems where a response of interest is prejudiced by numerous variables concurrently and the aim is to optimize the response of interest (Myers and Montgomery, 2002) [11]. For optimization, this technique was applied by numerous workers for the optimization of culture conditions (Dagbagli and Goksungur, 2008; Kim *et al.*, 2008) [3, 7] such as pH, temperature, aeration (Wang *et al.*, 2008) [14] and feeding rates (Narsaiah *et al.*, 2014) [12]. Response surface methodology has also been successfully applied for bacteriocin production (Dominguez *et al.*, 2007; Delgado *et al.*, 2007; Anthony *et al.*, 2009; Wiese *et al.*, 2010) [16, 4, 1, 15]]. An initial attempt in context to optimization study of reuterin production was done on considering different growth and environmental factors on reuterin production by *L. reuteri* BPL-36 using linear model.

In the present study, firstly as a validation of above optimization study, the factors having significant effect on reuterin production were maintained and the reuterin production by *L. reuteri* BPL-36 was determined to evaluate the overall effect of the optimized factors and conditions on the level of reuterin produced. Furthermore, based on this preliminary investigation, optimization of the cultivation conditions and medium components having significant effect for maximal growth and reuterin production by *L. reuteri* BPL-36 using response surface methodology (RSM) was aimed.

Materials and Methods

Bacterial strains and culture conditions

Lactobacillus reuteri BPL-36 strain cultured from human infant fecal source (Mishra *et al.* 2012) [9], was maintained and propagated in MRS broth (Hi-Media labs, Mumbai, India). Bacterial strain was maintained as stock cultures at -80°C in MRS broth supplemented with 15% glycerol. The organism was propagated twice before use in the experiments.

Experimental design

To describe the nature of the response surface in the optimum region, a two factor (five levels at each factor) second order central composite rotatable design (CCRD) was adopted. The independent factors viz: initial pH (x_1), incubation temperature (x_2), incubation time (x_3) and glycerol concentration (x_4) were considered for optimization of processing variables for growth and reuterin production. The selected range for the variables for water-glycerol medium was kept 4-6 initial pH, 30-42 ºC for incubation temperature, 1-4 h of incubation period and 200-300 mM glycerol content. For the three factors, this design was made up a full 2^4 factorial design with its four cube points, augmented with six replications of the center points and the eight axial designs, i.e., points having for one factor an axial distance to the center of ±α, whereas the other factor is at level 0. The axial distance α was chosen to be 1.68 to make this design rotatable. A center point is a point in which all variables are set at their mid value. Three center experiments were included in factorial designs as repetition so as to minimize the risk of missing non-linear relationships in the middle of the intervals, and also for the determination of confidence intervals. The response function (Y) were the final pH, growth (OD at 620 nm) and reuterin produced (mM). These responses were related with the coded factors by a second- degree polynomial equation Eq. (5) using the least square method.

$$y= \beta_o + \beta_1A + \beta_2B+ \beta_3C +\beta_4D+ \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 +\beta_{44}D^2+ \beta_{12}AB + \beta_{13} AC + \beta_{14}AD+\beta_{23}BC+\beta_{24}BD+\beta_{34}CD+\varepsilon \qquad (1)$$

where, β_0 is the intercept, β_1, β_2, β_3, β_4 are the first order coefficients, β_{12}, β_{13}, β_{14} β_{23}, β_{24}, β_{34} are cross product coefficients, and β_{11}, β_{22}, $\beta_{33,}$ β_{44} are the second order coefficients and ε (random error). Thus the optimization of reuterin production was achieved using a 2^4 central composite design and surface modeling method.

Culture preparation and process parameters

Active culture of *L. reuteri* BPL-36 was prepared by 3 successive subcultures in 10 ml of sterile MRS at 2%, incubated at 37 °C for 24 h, anaerobically. This active culture was used at a constant volume of 2% inoculum volume (v/v) in 10 ml sterile water medium having 4-6 initial pH, 200-300 mM glycerol content and kept at a temperature range of 30-42 ºC, 1-4 h of incubation period. The fermentation was conducted in anaerobic conditions (Gaspak, India), without agitation. The relation between final pH, growth (OD at 620 nm) and reuterin production with time was determined for the strain before and after the optimization procedure.

Estimation of growth and reuterin production

The reuterin concentrations of the samples were determined by Colorimetric assay as described by Circle *et al*. (1945) [2]. Growth was measured by taking OD_{620} of the medium after fermentation was over and final pH was measured using pen pH meter.

Data analysis

Design-Expert (Trial Version 8.0.7.1) was used for the regression analysis of the experimental data obtained. The fitness of the polynomial model equation was calculated by the coefficient of determination (R^2), and its statistical significance was checked by using F-ratio. The significance of the regression coefficient was tested by t-test. The level of significance was given as values of Prob > F less than 0.1. A differential calculation was then employed for predicting the optimum point.

Results and Discussions

Optimization of the cultivation conditions for maximal reuetrin production in water-glycerol media

The optimization of different parameters for maximum reuterin production was carried out using Central Composite Rotatable Design (CCRD) in realistic vicinity to locate the true optimal value of multiple compositional variables. Besides optimization, combined effect of these variables on reuterin production was observed. The central values of four factors/variables i.e. pH (5.5), incubation temperature (36 ºC), incubation time (24 h), Glycerol content (325 mM) were coded as A, B, C and D respectively. A total number of 30 experiments were carried out in randomized order which includes; 16 factorial, 8 star and 6 replicate points.

The design matrix representing different combinations of the four factors along with responses are delineated in Table. 1. Regression coefficient and ANOVA of fitted quadratic model for reuterin production are shown in Table. 2.

Effect of different variables (pH, temperature, incubation time and glycerol content) on pH in water-glycerol media

The average pH after growth varied from 2.2 to 6.8 (Table 1). The minimum value of pH was found in the reuterin produced in Experiment No. 28, whereas, the reuterin produced in Experiment No. 10, had the highest pH.

The regression analysis of the data presented in Table 3 reveals that the coefficient of determination (R^2) was 0.99 (a value of $R^2 > 0.75$ indicates aptness of the model). These values ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that model could explain 99 % of the variability in the response. The ANOVA of quadratic model indicated that model F value of 343.35 was more than the tabulated F value. Furthermore the adequate precision value (APV) was found to be 76.17, which was higher than the minimum desirable (4) for high prediction ability. All this showed that the model can be used to describe the effect of variables on pH.

Final pH after growth of *L. reuteri* BPL-36 was significantly ($p<0.01$) positively affected by the pH of broth, significantly negatively ($p<0.05$) affected by incubation temperature, incubation time. Final pH of broth, and negatively affected by glycerol content at linear level, but not statistically significant. At quadratic level, pH ($p<0.01$) had negative effect. However, incubation temperature ($p<0.01$), time ($p<0.05$) had positive effect on final pH. Whereas, glycerol content had positive effect, though it is statistically non-significant. The interactive effect of all parameters was found to be non-significant except pH*Temperature and pH* Incubation time (Figure 1). Multiple regression equation generated to predict the reuterin production as affected by different factors in terms of coded factors is as follows:

$$pH = +4.90+1.08A-0.065*B-0.24*C-5.417E-003*D-0.078*AB-0.22*AC-4.375E-003*AD+6.875E-003*BC-0.016*BD+0.011*CD-.09A^2+0.094*B^2+0.043*C^2+5.729E-003*D^2$$

Effect of different variables (pH, temperature, incubation time and glycerol content) on growth (optical density) in water-glycerol media

The average OD after growth varied from 0.9 to 10.02 (Table. 1). The minimum value of OD was found in case of reuterin produced in Experiment No. 28, whereas, the reuterin produced with Experiment No. 17 showed the highest overall acceptability score.

The regression analysis of the data presented in Table 2 reveals that the coefficient of determination (R^2) was 0.93. The ANOVA of quadratic model indicated that model F value of 15.47 was more than tabulated F value. Furthermore the adequate precision value (APV) was found to be 15.746, which was higher than the minimum desirable (4) for high prediction ability. All this showed that the model can be used to describe the effect of variables on maximum reuterin production.

Optical density after growth was significantly positively ($p<0.05$) affected by pH. However, non-significantly negatively affected by incubation temperature, glycerol content negatively significantly ($p<0.01$) affected by incubation time, but was statistically non-significant at linear level. At quadratic level, pH ($p<0.01$) and incubation temperature ($p<0.05$) and incubation time (statistically non-significant) had negative effect, whereas, glycerol content had positive non-significant effect. Also, the interactive effect of all parameters was found to be non-significant (Figure 4.22).

Multiple regression equation generated to predict the reuterin production as affected by different factors in terms of actual factors is as follows:

$$OD = +7.82 +0.99*A -0.21*B -1.61*C -0.022*D -0.014*AB +0.18*AC +0.24*AD -0.19*BC -7.500E-003*BD +0.13*CD -1.02*A^2 -0.61*B^2 -0.19*C^2 +0.11*D^2$$

Effect of different variables (pH, temperature, incubation time and glycerol content) on reuterin concentration in water-glycerol media

The average reuterin concentration in the growth media varied from 0 to 58.3 mM (Table. 1). The minimum value was found in the reuterin produced in Experiment No. 17, whereas, the reuterin produced in Exp. No. 6 had the highest overall acceptability score.

The regression analysis of the data presented in Table 2 reveals that the coefficient of determination (R^2) was 0.90. The ANOVA of quadratic model indicated that model F value of 10.23 was more than tabulated F value. Furthermore the adequate precision value (APV) was found to be 10.23, which was higher than the minimum desirable (4) for high prediction ability. All this showed that the model can be used to describe the effect of variables on maximum reuterin production.

Reuterin concentration after growth was positively significantly ($p<0.01$) affected by pH of the broth and incubation time, temperature and glycerol content. However, was non-significant at linear level. At quadratic level, all parameters had negative effect and except glycerol content all other parameters were significant ($p<0.01$). The interactive effect of all parameters was found to be non-significant, except pH and incubation time which had positive significant effect ($p<0.05$) (Figure 3).

Multiple regression equation generated to predict the reuterin production as affected by different factors in terms of actual factors is as follows:

$$\text{Reuterin concentration} = +55.70 +5.42*A +0.33*B +6.59*C +1.26*D +0.51*AB +6.39*AC +0.14*AD -1.31*BC -0.031*BD -0.32*CD -11.04*A^2 -7.55*B^2 -8.63*C^2 -0.62*D^2$$

Diagnostic check of the quadratic model

The quadratic model for various responses, namely Optical density, pH, and reuterin concentration (mM) was obtained through successive regression analysis. The dependence of these responses with respect to levels of four factors (pH, Temperature, Incubation time and Glycerol content) in the form of correlation is presented in Table. 2. The model F values for all attributes were more than the table F values at 5% level of confidence and it indicated the significance of model terms. The lack of fit test, which measures the fitness of the model obtained, did not result in a significant F value, indicating that the model is sufficiently accurate for predicting the reuterin production by *L. reuteri* BPL-36 from any combination of factor levels within the range evaluated.

Validation of the model by optimization of pH, incubation temperature, incubation time and glycerol content for reuterin production in water-glycerol media

The optimization of levels of pH, incubation temperature, incubation time and glycerol content was attempted using CCRD response surface design and different parameters were set as presented in Table 3.

The optimum solution obtained as a result of numerical optimization was verified and the optimum level of pH (6.0), incubation temperature (37 ºC), incubation time (2.5 h) and Glycerol content (267 mM) were used for maximum reuterin production. The actual values of the optimization were compared with the predicted values given by the software using t-test as shown in Table 4. The t-test indicated that there were non-significant differences between the predicted and the observed values of reuterin produced. This indicated that the model was significant and fitted perfectly, so the reuterin produced was maximum from possible combinations of variables.

The perfect agreement between the observed values and the values predicted by the equation confirms the statistical significance of the model and its adequate precision in predicting the optimum conditions in the domain of levels chosen for the independent variables.

The approach used in this study allowed the determination of the medium components and physical parameters that gave the highest reuterin production. In this case, suitable model was developed to describe the response of the experiment as the values obtained experimentally are in accordance with the predicted values determined by the model. The model was validated by comparing the actual and predicted values at the optimum point, and a deviation of about 6% was observed. The optimization procedure allowed an increase in reuterin production and growth of the culture. In one of the similar study, a very high reuterin concentration of 235 mM was obtained after 45 min of incubation at 30 °C in 400 mM glycerol (Doleyres *et al.*, 2005) [5].

Table 1: Response surface design of the reuterin production from *L. reuteri* BPL-36 cultivated with different levels of pH, temperature, incubation time and glycerol content in water-glycerol media

S. N.	Factors				Responses		
	pH (A)	Incubation temperature (ºC) (B)	Incubation time (h) (C)	Glycerol Conc. (mM) (D)	OD at 620 nm	Final pH	Reuterin Conc. (mM)
1	7	42	3	200	4.1	5.4	46.7
2	7	42	1	200	8.8	6.4	26.2
3	5.5	24	2	250	6.97	5.31	39.8
4	5.5	36	2	250	7.82	4.9	55.7
5	5.5	36	2	250	7.82	4.9	55.7
6	5.5	36	2	350	7.82	4.9	58.3
7	4	42	1	300	7.11	3.93	29.2
8	5.5	36	2	250	7.82	4.9	55.7
9	7	30	1	200	7.88	6.6	15.7
10	8.5	36	2	250	6.7	6.8	23.2
11	4	42	3	300	3.15	3.88	23.2
12	7	30	3	300	6.12	5.7	41.7
13	5.5	36	4	250	4.2	4.6	43.2
14	4	30	3	300	3.27	3.89	18.1
15	5.5	36	2	250	7.82	4.9	55.7
16	5.5	36	2	150	8.82	4.9	49
17	5.5	36	0	250	10.02	5.5	0
18	7	42	3	300	5.87	5.4	47.2
19	7	30	1	300	7.91	6.7	16.8
20	4	30	3	200	3.47	3.88	18.3
21	4	30	1	300	7.12	3.92	21.2
22	5.5	36	2	250	7.82	4.9	55.7
23	4	42	3	200	3.89	3.87	22.1
24	7	42	1	300	8.71	6.2	29.7
25	4	30	1	200	7.07	3.97	17.9
26	5.5	36	2	250	7.82	4.9	55.7
27	5.5	48	2	250	3.92	5.2	12
28	2.5	36	2	250	0.9	2.2	0.7
29	7	30	3	200	5.2	5.7	39.8
30	4	42	1	200	7.37	3.93	28.7

Table 2: Regression coefficients and ANOVA of fitted quadratic model for maximum reuterin production in water-glycerol media

Partial coefficients	Final pH	OD	Reuterin concentration
Intercept	4.90	7.82	55.70
A-Initial pH	1.08^{*}	0.99^{*}	5.42^{**}
B-Incubation temperature	-0.065^{**}	-0.21^{NS}	0.33^{NS}
C-Inoculation time	-0.24^{*}	-1.61^{*}	6.59^{**}
D- Glycerol content	$-5.417E-003^{NS}$	-0.022^{NS}	1.26^{NS}
A^2	-0.094^{*}	-1.02^{*}	-11.04^{*}
B^2	0.094^{*}	-0.61^{**}	-7.55^{*}
C^2	0.043^{**}	-0.19^{NS}	-8.63^{*}
D^2	$5.729E-003^{NS}$	0.11^{NS}	-0.62^{NS}
AB	-0.078^{**}	-0.014^{NS}	0.51^{NS}
AC	-0.22^{*}	0.18^{NS}	6.39^{**}
AD	$-4.375E-003^{NS}$	0.24^{NS}	0.14^{NS}
BC	$6.875E-003^{NS}$	-0.19^{NS}	-1.31^{NS}
BD	-0.016^{NS}	$-7.500E-003^{NS}$	-0.031^{NS}
CD	0.011^{NS}	0.13^{NS}	-0.32^{NS}
Model F value	343.35^{*}	15.47^{*}	10.00^{*}
R^2	0.99	0.93	0.90
APV	76.174	15.746	10.23
Lack of fit	0.097^{NS}	0.87^{NS}	86.93^{NS}

**Significant at 1% level ($p<0.01$) *Significant at 5% level ($p<0.05$)

Table 3: Conditions during optimization of reuterin production in CCRD

Name	Goal	Low limit	Upper limit
pH	In range	4	7
Incubation temperature	In range	30	42
Incubation time	In range	1	3
Glycerol content	In range	200	300
pH	In range	2.2	6.8
OD	In range	0.9	10.02
Reuterin content	Maximize	0	58.3

Table 4: Optimized Values of pH, Growth (OD) and reuterin concentration as compared to the predicted values in water-glycerol media

Responses	Predicted score*	Observed score@	t-value#
pH	5.08	5.1	2.32NS
OD	7.12	7.22	0.72NS
Reuterin concentration	58.72	58.69	0.65NS

*predicted values of Design Expert 8.0.7.1 package @actual values (mean of three trials) #p<0.05, NSnonsignificant

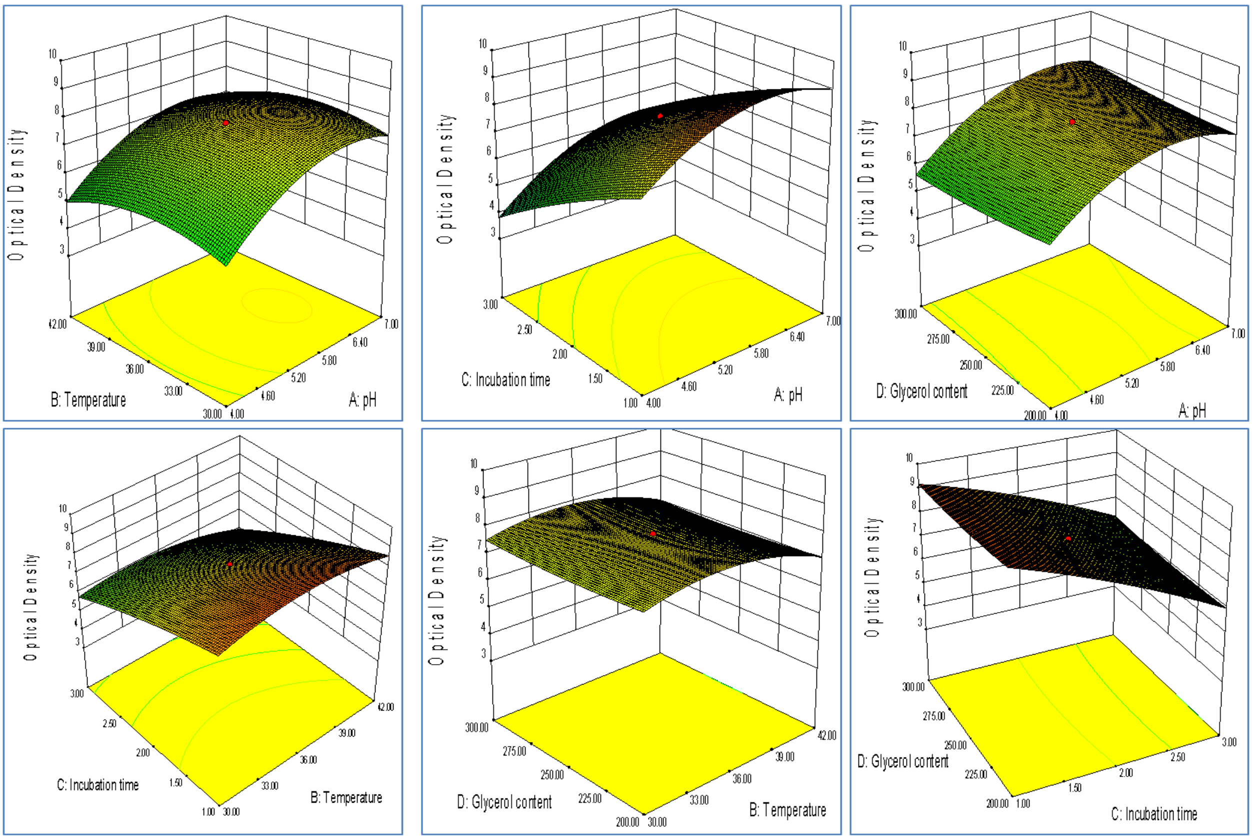

Fig 1: Response surface plot indicating the effect of pH, Temperature, Incubation time and Glycerol content interaction on final pH

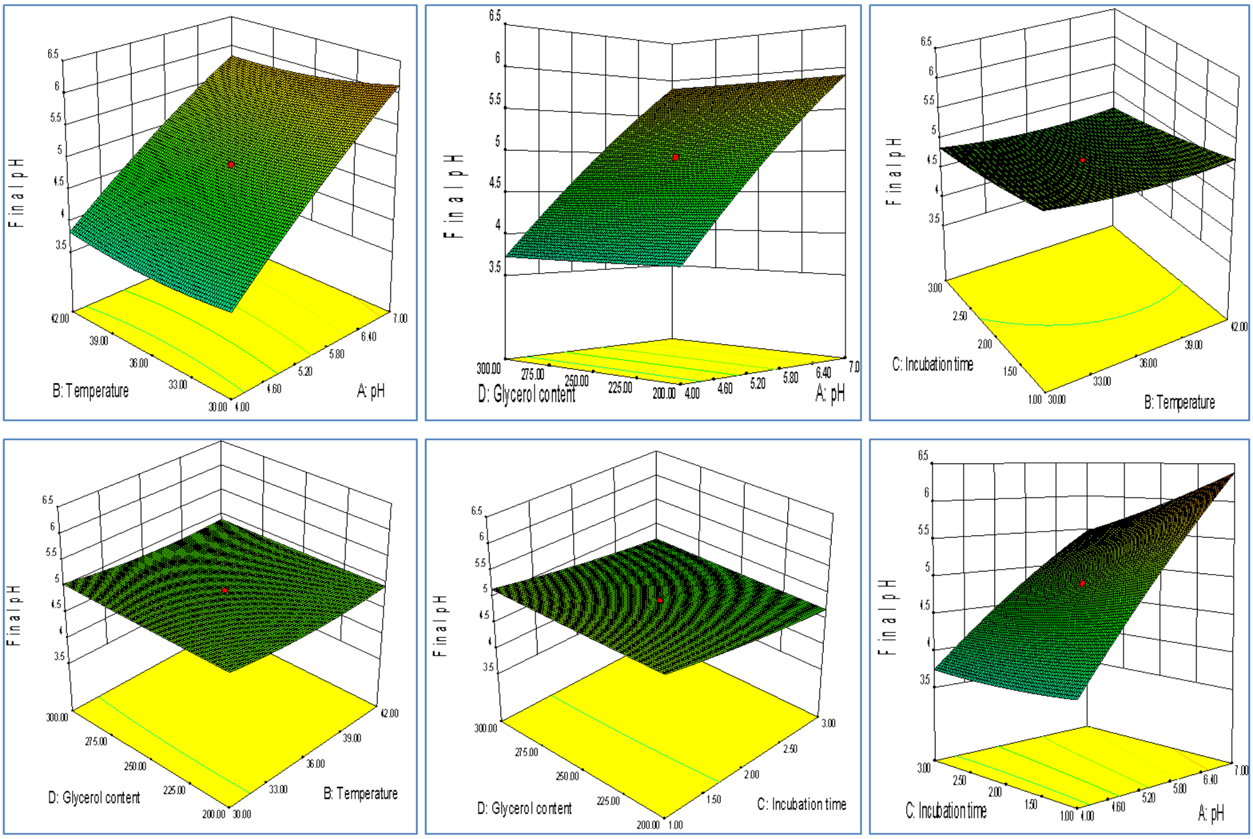

Fig 2: Response surface plot indicating the effect of pH, temperature, incubation time and glycerol content interaction on optical density

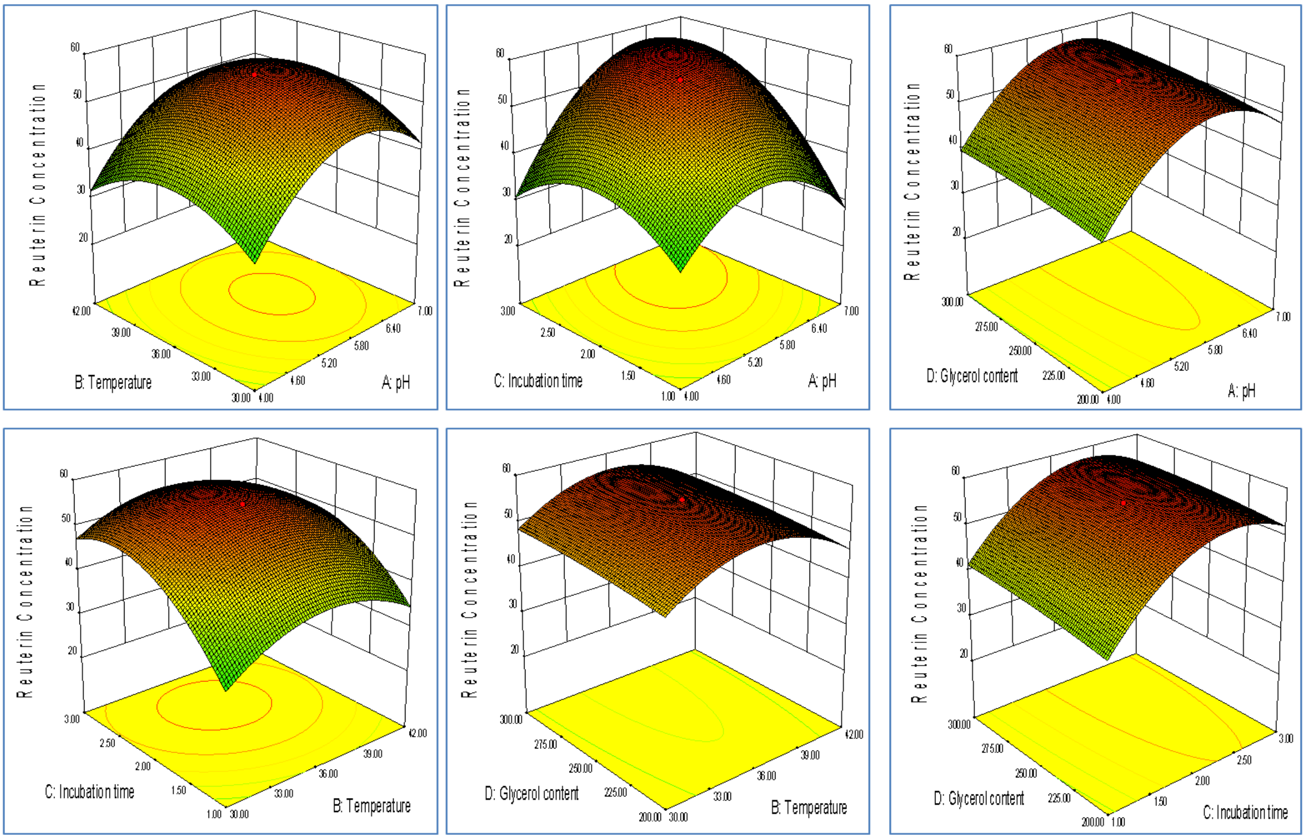

Fig 3: Response surface plot indicating the effect of pH, temperature, incubation time and glycerol content interaction on reuterin production

Conclusions
Using the method of experimental factorial design and response surface analysis, it was possible to determineoptimal operating conditions to obtain a higher reuterin production by *L. reuteri* BPL-36 strain. In the present study it was observed that the growth and production yield of reuterin increased using a response surface optimization of medium components and growth factors. This increase in growth and reuterin production with the optimization procedure can be justified by the fact that it is due to the combined effect of all the four selected parameters besides the influence of the specific strain. Therefore, present study constitutes a step in developing strategies to modulate the reuterin levels more than many folds.

Acknowledgements
Mr. Santosh Kumar Mishra is the recipient of scholarship from National Dairy Research Institute, Karnal, Haryana, India and duly acknowledges the financial assistance received. Funds received from NICRA project for carrying out this work are also acknowledged.

Compliance with ethics requirements
This article does not contain any studies with human or animal subjects. Additional informed consents were obtained from all the parents of infants from whom fecal samples were collected for isolation of lactic acid bacteria.

Conflict of interest
On behalf of all authors, the corresponding author states that there is no conflict of interest.

References
1. Anthony T, Rajesh T, Kayalvizhi N, Gunasekaran P. Influence of medium components and fermentation conditions on the production of bacteriocin (s) by *Bacillus licheniformis* AnBa9. Bioresource Technol. 2009; 100:872-877.
2. Circles SJ, Stone L, Borufff CS. Acrolein determination by means of tryptophane- a colorimetric micromethod. Industrial Eng Chem. 1945; 17(4):259-262.
3. Dagbagli S, Goksungur Y. Optimization of β-galactosidase production using *Kluyveromyces lactis* NRRL Y-8279 by response surface methodology. Process Biotechnol. 2008; 11(4):1-12.
4. Delgado A, López NAL, Brito D, Peres C, Fevereiro P, Garrido- Fernández A. Optimum bacteriocin production by *Lactobacillus plantarum* 17.2b requires absence of NaCl and apparently follows a mixed metabolite kinetics. J Biotechnol. 2007; 130:193-201.
5. Doleyres Y, Beck P, Vollenweider S, Lacroix C. Production of 3-hydroxypropionaldehyde using a two-step process with *Lactobacillus reuteri*. Appl Microbiol Biotechnol. 2005; 68:467-474.
6. Dominguez APM, Bizani D, Cladera-Olivera F, Brandelli A. Cerein 8A production in soybean protein using response surface methodology. Biochem Engineering J. 2007; 35:238-243.
7. Kim JK, Oh BR, Shin, H, Eom, C Kim SW. Statistical optimization of enzymatic saccharification and ethanol fermentation using food waste. Process Biochem. 2008; 43(11):1308-1312.
8. Mishra SK, Malik RK, Kaur G, Manju G, Pandey N, Singroha G. Potential bioprotective effect of reuterin produced by *L. reuteri* BPL-36 alone and in combination with nisin against food borne pathogens. Ind J Dairy Sci. 2011; 64(5):406-411.
9. Mishra SK, Malik RK, Manju G, Pandey N, Singroha G, Bahare P, Kaushik JK. Characterization of a reuterin-producing *Lactobacillus reuteri* BPL-36 strain isolated from human infant fecal sample. Probio Antimicrob Prot. 2012; 4(3):154-161.

10. Mishra SK, Malik RK, Panwar H, Barui A. Microencapsulation of reuterin to enhance long-term efficacy against food-borne pathogen *Listeria monocytogenes*. 3 Bio. 2018; 8:23.
11. Myers RH, Montgomery D. Response surface methodology: process and product optimization using designed experiments, 2nd edn. Wiley, New York, 2002, pp 1-17.
12. Narsaiah K, Jha SN, Wilson RA, Mandge HM, Manikantan MR. Optimizing microencapsulation of nisin with sodium alginate and guar gum. J Food Sci Technol. 2014; 51(12):4054-4059.
13. Vu KD, Salmieri S, Rosa A, Lacroix M. Evaluation of bioconversion conditions on reuterin production using response surface methodology and *Listeria monocytogenes* as target bacteria. J Microbio Biotechnol Food Sci. 2017; 6(4):1007-1011.
14. Wang YH, Feng JT, Zhang Q, Zhang X. Optimization of fermentation condition for antibiotic production by *Xenorhabdus nematophila* with response surface methodology. J Appl Microbiol. 2008; 104(3):735-744.
15. Wiese B, Bru E, Juárez Tomás MS, Nader-Macías MEF. Optimization of low-cost culture media for the production of biomass and bacteriocin by a Urogenital *Lactobacillus salivarius* strain. Probio Antimicrobial Prot. 2010; 2:2-11.