



OPTIMIZATION OF MEDIUM FOR ENHANCING BACTERIOICIN PRODUCTION BY MARINE *Lactobacillus rhamnosus* L43 USING STATISTICAL DESIGN.

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ABSTRACT:

Media composition and growth conditions influence the culture growth of *Lactobacillus rhamnosus* L43 and the antibacterial performance. The Plackett-Burman design, steepest ascent path design and central composite design provides an efficient way of a large number of variables and identifying the most important ones. Different statistical designs prepared and carried step by step, observed and predicted result using software Minitab 14 and design Expert Version 9. According to PB design and based on low p value, significant components were found to be lactose, peptone, yeast extract, MgSO₄, KH₂PO₄, K₂HPO₄, NaCl had a significant influence (at 95% significant level) on Bacteriocin production. The optimization medium included lactose of concentration of 20 gm/L, peptone of concentration of 10 gm/L and MgSO₄ of concentration of 0.10gm/L. Predicted value of the diameter of zone of inhibition against *Escherichia coli* was 19.66 mm by RSM method and data validation report. The mMRS medium gave the highest yield of bacteriocin as well as reduced the cost of medium.

Keywords: *Lactobacillus rhamnosus* L43, *Escherichia coli* (1687), MINITAB 14, Design Expert 9, PBD, RSM.

INTRODUCTION:

The biological antimicrobial substances have increased research attention to their potential in food bio preservation as purified metabolites, through the use of their producer strains in the starter culture (Chen and Hoover, 2003). Therefore, there has been a continuous need to define the most appropriate condition for bacteriocin production in fermentation media and food systems. The optimization is a process which includes the adjustment of internal and external parameters for the maximum production. Growth media compositions and environmental factors such as pH, temperature have some influence for production of antibacterial compounds. MRS broth remains the

most widely used culture medium for growth of LAB (Todorov and Dicks, 2006). Certain components, such as carbohydrates, salts, nitrogen sources, surfactants or oxygen tension reducing agents added to the MRS broth can interfere with bacteriocin production (Keren *et al.*, 2004; Mataragas *et al.*, 2004; Trinetta *et al.*, 2008; Todorov, 2008; Abo-Amer *et al.*, 2011, Castro *et al.*, 2011). These factors need to evaluate and achieve optimized bacteriocin production for industrial applications. Bacteriocin production in *Lactobacillus* sp. is dependent on multiple factors and is usually a strain specific phenomenon. Nowadays, statistical models are available for the optimization process such as Factorial, Fractional factorial, Plackett- Burman, Taguchi,

Response Surface Methodology (RSM), Polynomial and rational modelling, Box-Menken and Central composite design. The use of Plackett Burman Design (PBD) and response surface methodology (RSM) has a history of successful application in many fields, especially biotechnology, and suited for the study of the effects of distinct factors on bacteriocin production (Cladera-Olivera *et al.*, 2004; Kumar *et al.*, 2012, Suganthi and Mohanshrinivasan, 2015). Response surface methodology is one of the effective statistical and mathematical technique used for developing, improving and optimizing the complex process in the experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable responses (Leal-Sanchez *et al.*, 2002).

In this study, we have reported optimization of nutritional parameters of which have direct influence on the growth of bacterial strain *Lactobacillus rhamnosus* L43 and production of bacteriocin by statistical method.

MATERIAL & METHODS:

1.a Bacterial strains:

The bacteriocin-producing strain used in this study was *Lactobacillus rhamnosus*. L43 which was isolated from Gopalpur beach, Orrissa (water). The strain used as the indicator microorganism was *Escherichia coli* MTCC 1687. Stock cultures were maintained at -4°C in MRS medium containing 20% glycerol.

1.b Bacteriocin assay:

The antibacterial spectrum of the bacteriocin from *Lactobacillus sp.* was determined using the well diffusion method. (Ogunbanawo *et al.*, 2003)

2. Statistical Optimization of medium for Production of Bacteriocin:

The nutrients selection was done according to the flasks which were supporting

good growth of the organism. Thus accordingly glucose (A), Fructose (B), lactose (C), sucrose (D), peptone (E), beef extract (F), yeast extract (G), meat extract (H), KH₂PO₄ (I), K₂HPO₄ (J), MgSO₄ (K), MnSO₄ (L), Sodium potassium tartrate (M) were selected for statistical optimization process.

2.a Plackett-Burman Design (PBD):

PBD was used to screen the most important factors influencing bacteriocin production. The fermentation process was carried out in triplicate by using an experimental design with symbol code and level of the variables and the average value was taken as the response. Plackett-Burman design had carried by Plackett-Burman, (1946); Djekrif-Dakhmouche *et al.*, (2006). 2.b The Path of the Steepest Ascent Experiment:

To find the neighbourhood of the optimum condition quickly, the method of the steepest ascent was used. The experiments were applied to determine a suitable direction by increasing or decreasing the variables according to the results obtained from the Plackett-Burman design. This experiment was designed by Rodrigues *et al.*, (2006); Han *et al.*, (2011).

2.c Central Composite Design (CCD):

To describe the nature of the response surface in the optimum region, a central composite design and response surface methodology was performed. This experimental design was carried by Montgomery, (1997); Leaes *et al.*, (2011).

2.d Statistical analyses:

The PBD, analysis of variance (ANOVA) for the PBD data and the model coefficients were computed with Minitab 14.0 (Minitab Inc., Pennsylvania, USA) software. The Design Expert software (Version 9.0.0, Stat-Ease, Minneapolis, USA) was used for the RSM experimental design and the analysis of variance (ANOVA) for the data. The quality of the polynomial model equation was judged statistically by the coefficient of determination R², and its statistical significance was tested by an F-test. The

significance of the regression coefficients was determined by a t-test.

RESULT & DISCUSSION:

1. Statistical Optimization of medium for Production of Bacteriocin

1.a PBD for optimization of medium:

Plackett-Burman statistical design offers an effective screening procedure and computes the significance of a large number of factors in a few experiments; based on the variables, a Plackett-Burman design of the 16 experimental trials with their outcomes is given in Table 1. The significant levels of each variables were determined by using t test and regression analysis.

The experimental yields of bacteriocin at varied levels of different variables as per Plackett-Burman design are given in Table 2. The analysis showed that lactose, peptone, yeast extract, MgSO₄, KH₂PO₄, K₂HPO₄ had a significant influence (at 95% significant level) on bacteriocin production, while other factors had insignificant influence (below 95% significant level) on antibacterial activity by *Lactobacillus rhamnosus* L43. On the basis of statistical testing and the F-test applied for the analysis of variance (ANOVA), the F-value was 24.14. The F-test with a very low probability value (0.040) shows the high statistical significance of the regression model. The Pareto plot best demonstrates results of Plackett-Burman design that illustrate the absolute relative significance of variables independent of their nature (Figure 1). The ranking of factor estimates in a Pareto chart which displays the magnitude of each factor estimate, it directly shows that the most important factors determining Bacteriocin production from *Lactobacillus rhamnosus* L43 were Lactose, Peptone, Yeast extract, MgSO₄, KH₂PO₄, K₂HPO₄ concentrations while the other factors have insignificant influence.

1.b Optimization of medium by the path of the steepest ascent experiment:

A steepest ascent experiment was performed before the second-order polynomial model. The design and the steepest ascent experiment are shown in Table 3. The centre point of PBD has been considered as the origin of the path and the path was moved along in which the concentration of peptone and MgSO₄ were increasing. Steepest ascent path design was prepared by using regression equation which was obtained by PBD. The regression equation is

$$\begin{aligned} \text{mm} = & 7.39 + 0.0812 \text{ Glucose} + 0.0562 \text{ Sucrose} - \\ & 0.0187 \text{ Lactose} - 0.219 \text{ Fructose} \\ & + 0.244 \text{ Peptone} - 0.0563 \text{ Beef extract} + \\ & 0.160 \text{ Malt extract} \\ & + 0.906 \text{ KH}_2\text{PO}_4 + 0.719 \text{ K}_2\text{HPO}_4 + 24.4 \\ & \text{MgSO}_4 + 9.03 \text{ MnSO}_4 \\ & + 14.6 \text{ Pot.Sod.tartarate} + 0.187 \text{ yeast} \\ & \text{extract} \end{aligned}$$

The response for this point was determined as the average of responses for all the runs. From the results of the path of the steepest ascent, it is clearly seen that the yield profile shows a maximum diameter of zone of inhibition (20.5 mm) at run 15 i.e. at 7.5gm/L of Lactose, 7.5 gm/L of peptone, 0.5 gm/L of yeast extract, 0.5 gm/L of KH₂PO₄, 1.5gm/L of K₂HPO₄ and 0.075 gm/L of MgSO₄. It is suggested that this point might be near the region of the maximum antibiotic yield response. Consequently, this point was chosen as the central point of CCD.

1.c Optimization of Medium by Response Surface Methodology:

Based on the identification of variables by the PBD and the steepest ascent method, the experiments were performed according to a CCD experimental plan. The CCD experimental plan together with results is given in Table 4.

Table 4 shows the observed values of the bacteriocin yield obtained using CCD equation as follows:

$$\hat{Y} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{12} X_1 X_2 + \beta_{22} X_2^2$$

Where, \hat{Y} was the predicted response (antibiotic yield), β_0 , β_1 , β_2 , β_{11} , β_{12} and β_{22} were the regression coefficients, and X_1 and X_2 were the coded levels of the independent variables.

In order to predict the maximum production corresponding to the optimum levels of the two variables, a second-order polynomial model was proposed to calculate the optimum levels of these variables. By applying the multiple regression analysis on experimental data, a second order polynomial model in coded unit explains the role of each variable and their second-order interactions in producing Bacteriocin.

Equation:

$$R1 = +19.49 - 0.17 * A - 0.59 * B - 0.53 * C + 1.38 * AB + 0.88 * AC - 1.37 * BC + 0.25 * A^2 - 1.87 * B^2 - 2.14 * C^2$$

Furthermore, the results of the second-order response surface model in the form of analysis of variance (ANOVA) were shown in Table 5. The smaller the P -value, the more significant is the corresponding coefficient. The Fisher's F test with a very low probability value demonstrated that the model was highly significant (Myers and Montgomery 2002). Among the model terms, A , B , AB , A^2 and B^2 had significant effects on production with a probability of not less than 95%.

The fitness of the model can be checked by the determination coefficient (R^2) and the adjusted determination coefficient (Adj R^2). Here, the value of the R^2 was 0.9777, implying that 97.77% of the variability in the response could be explained by the model. The value of the Adj R^2 was 0.950 and it was also high enough to advocate for the significance of the model. In the present experiment, the lower value of CV (3.53%) indicates a better precision and reliability of the experiments performed. The 3D response surface curve and 2D contour plot (figure 2 and 3) are generally the graphical representation of the regression equation. Analysis of response surface

plot indicated that lactose and peptone have slight variation in concentrations from zero code value led to an increase in bacteriocin production.

1.d Validation of the optimized condition:

. The optimized medium included Lactose 20 gm/L, Peptone 10 gm/L and $MgSO_4$ 0.10gm/L . Predicted value of the diameter of zone of inhibition against *Escherichia coli* was 19.48 mm (maximum response) (Table 6).

Under the optimized conditions, average diameter of zone of inhibition against *Escherichia coli* was 19.66 mm which was very closer to the predicted response, in fact, higher than the predicted response. It confirms the authenticity of the model. These results indicate that the optimization of medium through RSM favoured enhanced Bacteriocin production.

The composition of the medium was also shown to have an important role in bacteriocin production (Li et al., 2002). RSM has been successfully used in many studies for optimization of bacteriocin production. However, since factors varied among different strains, this work differs from previous studies (Cladera-oliveria et al., 2004) in choosing factors before RSM. However, studies to reduce the cost of the medium have only been recently conducted (Dominguez et al., 2007). In the present study, some nitrogen sources in MRS were used to reduce costs viz., peptone and yeast extract. Earlier, Li and co-workers 2002 showed that peptone and KH_2PO_4 are the two significant factors for bacteriocin production and have the positive effect.

CONCLUSION:

The bacterial suspension of *Lactobacillus rhamnosus* L43 has grown in MRS and mRS Broth had the best inhibitory activity against wide spectrum of bacteria. The present study demonstrated the production of bacteriocin by

Lactobacillus rhamnosus L43 which has grown at nutrient medium. RSM was used to determine the effects of three important factors of peptone, lactose, MgSO₄ on bacteriocin production from broth. Linear, quadratic and interaction effects of these variables on bacteriocin production were determined. The statistical approach proved to be beneficial in optimizing a medium for bacteriocin production by *Lactobacillus rhamnosus* L43.. The model generated in this study satisfied all the necessary arguments for its use in optimization. By fitting the experimental data to a second-order polynomial equation, the optimum levels of important response variables were determined. Using the optimum levels of fermentation parameters, a maximum bacteriocin production of 19.66 mm was obtained. This study indicates that the medium design using statistical technique such as Plackett-Burman Design, RSM can be very useful in improving the production of bacteriocin production by *Lactobacillus rhamnosus* L43. and in similar bioprocesses. The optimized medium not only allowed the increase in bacteriocin activity, but also reduced the cost of the medium.

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Table 1: PBD for evaluating variables influencing Bacteriocin

Run	A	B	C	D	E	F	G	H	I	J	K	L	M	Zone of inhibition (mm)	Predicted values
1	0.75	0.75	0.75	1.25	0.25	0.25	0.5	0.5	0.15	0.05	0.0075	0.0005	0.005	16	16.56
2	1.25	0.75	1.25	1.25	0.75	0.75	0.05	0.5	0.05	0.05	0.0075	0.0005	0.0005	13	13.12
3	0.75	0.75	0.75	0.75	0.25	0.25	0.05	0.05	0.05	0.05	0.0025	0.0005	0.0005	17.5	17.43
4	1.25	0.75	1.25	0.75	0.75	0.75	0.5	0.05	0.15	0.05	0.0025	0.0005	0.005	17	17.12
5	0.75	0.75	1.25	0.75	0.25	0.75	0.5	0.5	0.05	0.15	0.0025	0.005	0.005	14	14.43
6	1.25	1.25	0.75	1.25	0.25	0.75	0.5	0.5	0.15	0.15	0.0025	0.0005	0.0005	10	9.8
7	0.75	1.25	1.25	0.75	0.75	0.25	0.5	0.05	0.15	0.15	0.0075	0.0005	0.0005	20	20.56
8	1.25	1.25	1.25	0.75	0.25	0.25	0.5	0.5	0.05	0.05	0.0075	0.005	0.0005	15.5	15.93
9	1.25	1.25	1.25	1.25	0.25	0.25	0.05	0.05	0.15	0.05	0.0025	0.005	0.005	18.5	18.8
10	1.25	1.25	0.75	0.75	0.25	0.75	0.05	0.05	0.05	0.15	0.0075	0.0005	0.005	16.5	16.12
11	0.75	0.75	1.25	1.25	0.25	0.75	0.05	0.05	0.15	0.15	0.0075	0.005	0.0005	15.0	16.3
12	0.75	1.25	0.75	1.25	0.75	0.75	0.5	0.05	0.05	0.05	0.0025	0.005	0.0005	14.5	14.06
13	1.25	0.75	0.75	0.75	0.75	0.25	0.05	0.5	0.15	0.15	0.0025	0.005	0.0005	13	15.06
14	0.75	1.25	1.25	1.25	0.75	0.25	0.05	0.5	0.05	0.15	0.0025	0.0005	0.005	18	18.43
15	1.25	0.75	0.75	1.25	0.75	0.25	0.5	0.05	0.05	0.15	0.0075	0.005	0.005	20.5	20.87
16	0.75	1.25	0.75	0.75	0.75	0.75	0.05	0.5	0.15	0.05	0.0075	0.005	0.005	19.5	19.6

Table 2: Estimated effect from the results of PBD

Estimated Effects and Coefficients For Mm (Coded Units)					
Term	Effect	Coef	SE Coef	T	P
Constant	15.844	0.1563	101.40	0.000	
Glucose	1.188	0.594	0.1563	3.80	0.063
Sucrose	0.812	0.406	0.1563	2.60	0.122
Fructose	0.062	0.031	0.1563	0.20	0.860
Lactose	-2.187	-1.094	0.1563	-7.00	0.020
Peptone	2.687	1.344	0.1563	8.60	0.013
Beef Ext	-0.438	-0.219	0.1563	-1.40	0.296
Malt Ext	1.062	0.531	0.1563	3.40	0.077
KH₂PO₄	2.187	1.094	0.1563	7.00	0.020
K₂HPO₄	1.688	0.844	0.1563	5.40	0.033
MgSO₄	2.562	1.281	0.1563	8.20	0.015
MnSO ₄	0.687	0.344	0.1563	2.20	0.159
Pot.Sod.	1.313	0.656	0.1563	4.20	0.052
Yeast Ex	1.687	0.844	0.1563	5.40	0.033

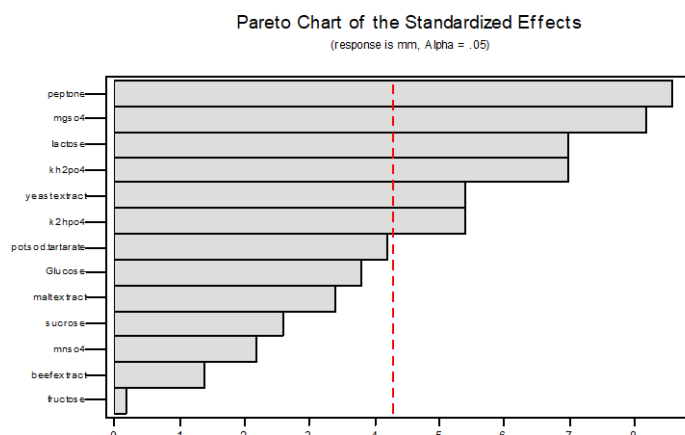


Fig 1 : Pareto chart

Table 3: The path of steepest ascent experiment for optimization of efficient variables

Run	Lactose	Peptone	KH ₂ PO ₄	K ₂ HPO ₄	MgSO ₄	Yeast extract	Zone of inhibition (mm)
1	1	0.5	0.1	0.1	0.05	0.25	18.5
2	1.025	0.554	0.1485	0.1105	0.015	0.2504	16
3	1.05	0.6085	0.1975	0.1215	0.020	0.2508	13
4	1.075	0.6625	0.246	0.1325	0.0317	0.2512	10.5
5	1.1	0.7165	0.295	0.143	0.042	0.2516	12
6	1.125	0.771	.0.34	0.1515	0.053	0.252	13
7	1.15	0.825	0.3925	0.165	0.064	0.2524	9

Table 4: CCD for optimization of medium and the corresponding responses

Run	Factor 1 A: Lactose gm/L	Factor 2 B: Peptone gm/L	Factor 3 C: MgSO ₄ gm/L	Response 1 R1 MM
1	22	12	0.12	16
2	20	10	0.1	19.5
3	20	10	0.1	19.5
4	20	10	0.1	19.5
5	22	8	0.12	17
6	20	10	0.1	19.5
7	20	6.63641	0.1	15.5
8	20	10	0.133636	12
9	22	8	0.08	13
10	18	12	0.08	16.5
11	18	8	0.08	17.5
12	20	10	0.1	19.5
13	18	12	0.12	11.5
14	22	12	0.08	18
15	18	8	0.12	17.5
16	23.3636	10	0.1	19
17	20	13.3636	0.1	12.5
18	20	10	0.1	19.5
19	20	10	0.0663641	14.5
20	16.6364	10	0.1	21

Table 5: ANOVA for response surface quadratic model for optimization of medium

Sources	Sum of squares	df	Mean Square	F value	p-Value probe>F
Model	156.57	9	17.40	48.69	<0.0001
A-Lactose	0.41	1	0.41	1.15	0.3097
B-Peptone	4.74	1	4.74	13.27	0.0049
C-MgSO ₄	3.80	1	3.80	10.64	0.0085
AB	15.13	1	15.13	42.34	<0.0001
AC	6.13	1	6.13	17.14	0.0020
BC	15.12	1	15.12	42.34	<0.0001
A ²	0.91	1	0.91	2.54	0.1418
B ²	50.40	1	50.40	141.09	<0.0001
C ²	65.71	1	65.71	183.93	<0.0001
Residual	3.57	10	0.36		
Lack of Fit	3.57	5	0.71		
Pure error	0.000	5	0.000		
Cor Total	160.14	19			

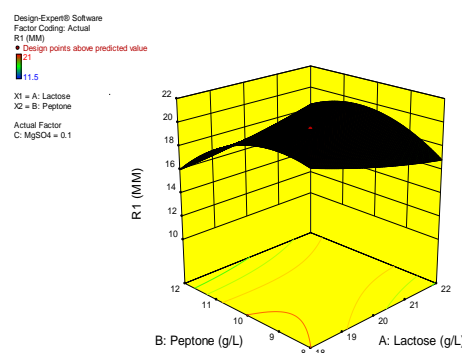
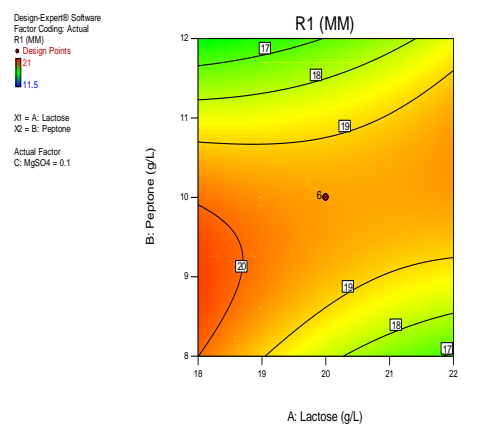


Fig.2 : 2D Contour Plot

Fig.3: 3D Response Surface Curve

1.d Validation of the optimized condition:

Table 6: Confirmation report for optimization for medium by RSM

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding		
A	Lactose	20.00	18.00	22.00	0.000	Actual		
B	Peptone	10.00	8.00	12.00	0.000	Actual		
C	MgSO ₄	0.100	0.080	0.12	0.000	actual		
Response	Predicted Mean	Predicted Median <u>1</u>	Observed	Std. Dev.	N	SE Pred	95% PI Low	Data Mean
R1	19.4886	19.4886	-	0.597703	1	0.65	18.05	