➔DOI : 10.15740/HAS/AJBS/9.2/146-150
e ISSN-0976-8343 | ■ Visit us : *www.researchjournal.co.in*
Asian Journal of Bio Science, Volume **9** | Issue 2 | October, 2014 | 146-150
Received : 26.06.2014; **Revised :** 03.08.2014; **Accepted :** 16.08.2014

RESEARCH **P**APER

Selection of influential parameters for bacteriocin production by *Lactococcus lactis* subsp. *lactis* R10 by Plackett- Burman design

P. UMAMAKESH AND K. VIJILA

Department of Agricultural Microbiology, Tamil Nadu Agricultural University, COIMBATORE (T.N.) INDIA
Email: umamakeshmicro@gmail.com

In the present study, Plackett-Burman design was applied to select influential parameters for bacteriocin production by *Lactococcus lactis* subsp. *lactis* R10, an isolate from fermented radish was investigated. Out of the eleven culture media constituents screened, six constituents namely temperature, dipotassium hydrogen phosphate, triammonium citrate, sodium acetate, yeast extract and pH were found to contribute positively to the overall bacteriocin production. Sucrose, peptone, magnesium sulphate, tween 80 and glycerol had a negative effect on bacteriocin production by *L. lactis* subsp. *lactis* R10. Thus, the statistical approach employed in this study allows for rapid identification of important culture media parameters affecting the bacteriocin production.

Key words : *Lactococcus lactis* subsp. *lactis* R10, Bacteriocin, Plackett-Burman design

How to cite this paper : Umamakesh, P. and Vijila, K. (2014). Selection of influential parameters for bacteriocin production by *Lactococcus lactis* subsp. *lactis* R10 by Plackett- Burman design. *Asian J. Bio. Sci.*, **9** (2) : 146-150.

INTRODUCTION

In recent years, the consumption of foods formulated with chemical preservatives has increased consumer-concern due to health effects and created a demand for more natural and minimally processed foods. As a result, there has been a great interest in naturally produced antimicrobial agents for their application in food preservation (Cleveland *et al*., 2001). Lactic acid bacteria (LAB) are an industrially important group of microorganism with GRAS (Generally Recognized As Safe) status and are associated with meat, dairy and vegetable fermentation. LAB produces a wide-variety of antagonistic factors that include metabolic end products such as lactic acid, antibiotic-like substances and antimicrobial proteins or bacteriocins. Bacteriocins are a heterogeneous group of ribosomally synthesized antimicrobial peptides, which display a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative pathogenic bacteria. LAB bacteriocins have attracted growing interest in recent years because of their potential usage as biopreservatives in the food industry to eradicate food spoilage and food-borne pathogenic bacteria (Kanmani *et al*., 2011). The potential could be achieved either by using a bacteriocin-producing starter culture or by applying the bacteriocin itself as a food additive. Both will necessarily require optimization of their production, which may be dependent on multiple factors and are usually strain specific (Mackay *et al*., 1997).

However, optimization of a medium by the classical method involves changing one independent variable at a time while keeping others at a fixed level. This is extremely time-consuming and expensive for a large number of variables and may also result in unacceptable conclusions (Oh *et al*., 1995). In comparison, the statistical methods offer several advantages over conventional methods in being rapid and reliable, and that shortlists significant nutrient, helps understanding the interactions among the nutrients at various concentrations and reduces the total number of experiments tremendously resulting in saving time and material (Chauhan *et al*., 2007). Plackett-Burman design is the most frequently used screening design because of its ability to estimate all main effects with the same precision (Antony, 2008).

Lactic acid bacteria are fastidious and require complex media and well-controlled physical factors such as temperature and pH, to obtain optimal bacteriocin production. In this

context, this study was aimed at selecting the significant media components using MRS media through Plackett-Burman experimental design for bacteriocin production by *L. lactis* subsp. *lactis* R10. This design was selected based on its ability to screen and evaluate the relevant culture media that affect the bacteriocin production, so as to generate reliable and more manageable set of components, as well as indicating how each component affects the overall response.

RESEARCH METHODOLOGY

Bacterial strains, culture media and growth conditions:

The bacteriocinogenic strain *L. lactis* subsp. *lactis* R10 was isolated from fermented radish (*Raphanus sativus)*. The strain was identified by sequencing of 16S rRNA gene followed by blast homology search. Modified sucrose MRS media containing, sucrose 12 g/l, peptone 10 g/l, beef extract 10 g/l, yeast extract 5 g/l, dipotassium hydrogen phosphate 2 g/l, tween 80 1 ml/l, triammonium citrate 2 g/l, sodium acetate 5 g/l, magnesium sulphate 0.2 g/l, manganese sulphate 0.05 g/l and pH 6.5 were used for maximum bacteriocin production by *L. lactis* subsp. *lactis* R10. *Bacillus subtilis* was used as an indicator organism and grown in Nutrient agar media.

Bacteriocin assay :

Bacteriocin activity was determined by using agar well diffusion assay (Tagg and McGiven, 1971). The supernatant of 36 h grown culture (from modified sucrose MRS broth) was centrifuged at 12,000×g for 15 min at 4^0C, then supernatant was neutralized with sterile 5M NaOH. Aliquots (50µl) of culture supernatants were applied to disks on agar plates previously inoculated with a cell suspension of *B. subtilis* (10^6 cfu/ml). The agar plates were incubated for 24 to 48 h and the diameter of inhibition zone around the wells was measured with a calliper. Bacteriocin activity was expressed as arbitary units (AU/ml) and defined as the reciprocal of the highest two fold serial dilution showing a distinct zone of inhibition.

Bacteriocin activity (AU/ml) = 2^n×100 n- Highest dilution showing growth inhibition zone

Selection of influential parameters by Plackett-Burman design :

The Plackett-Burman design (Plackett and Burman, 1946; Haaland, 1989) was used to evaluate the main effects of various nutritional and physical factors on bacteriocin production during 2011. The design considers the main effect of these variables but not their interaction effects. It can be represented by first-order polynomial equation :

$$Y = {}_0 + p^n{}_{i=1}\ i\ ¡\ i \qquad \text{.....(1)}$$

where, Y represents the response (bacteriocin), β_0 is the model co-efficient, βi is the liner co-efficient, x*i* is the variables and n is the number of parameters (variables).

The total number of experiments to be carried out according to Plackett-Burman design is n+1, where n is the number of variables. The each variable was prepared in two levels, -1 for low level and +1 for high level based on Plackett-Burman design using Design Expert software (8.0.6, Stat-Ease, Minneapolis, Minn. USA) (Table A). Each column should contain equal number of positive and negative signs. Thus, the effect of each variable is the difference between the average of the measurements made at the high and the average of the measurements made at the low level of that factor, which was determined by the equation :

$$E(Xi) = \frac{2(\sum Pi + -\sum pi-)}{N} \qquad \text{.....(2)}$$

where, E(*xi*) is the concentration effect of the tested variables. Pi^+ and Pi^- represent the bacteriocin activities of the trials where the variable (*Xi*) measured was present at the high or low level, respectively and the N is the number of trials (experiments).

Standard error (SE) of the concentration effect was the square root of the variance of an effect and the significance level (*P*-value) of the effect of each constituent was determined

Table A : Factors and levels in Plackett-Burman design applied in this study

Factors	Abbreviation	Low level (-1)	High level (+1)
Sucrose (g/L)	A	8	12
Peptone (g/L)	B	3	8
Yeast extract (g/L)	C	3	8
Sodium acetate (g/L)	D	3	8
Dipotassium hydrogen phosphate (g/L)	E	1	3
Triammonium citrate (g/L)	F	1	3
Magnesium sulphate (g/L)	G	0.2	0.6
Tween 80 (ml/L)	H	0.5	2
Glycerol (ml/L)	J	0	1
Temperature (°C)	K	28	37
pH	L	5.5	6.5

using student's t-test as given by the equation :

$$t(X_i) Z \frac{E(x_i)}{SE} \quad \text{.....(3)}$$

where, E (*xi*) is the effect of variable *Xi*

RESEARCH FINDINGS AND ANALYSIS

Plackett-Burman design offers an effective screening procedure and computes the significance of a large number of factors in one experiment, which is time- saving and maintains convincing information on each component (Sharma and Satyanarayana, 2006). The expermental data of bacteriocin production in the screening Plackett- Burman experiments were listed in Table 1, and the results illustrated a wide variation of bacteriocin activity from 593.60 to 761.60 AU/ml, which reflected the importance of medium optimization to attain higher yields.

On analyses of regression co-efficients, *F*- value and *p*-value of 11 ingredients (Table 2), *p*-value less than 0.05 indicate that the model terms are significant. When the concentration effect value ($E_{(Xi)}$) of the tested variable was positive, the influence of the variable was greater at the high concentration tested and when negative, the influence of the variable was greater at low concentration. The factors with positive effect for bacteriocin production are temperature, dipotassium hydrogen phosphate, triammonium citrate, sodium acetate, yeast extract and pH. This indicates that the bacteriocin production was enhanced by adding higher concentration of these ingredients. Sucrose, peptone, magnesium sulphate, tween 80 and glycerol had a negative effect which indicate that increasing the concentration of these ingredients, decrease the production of bacteriocin.

The most important factor was determined by the *P*-value and *F*- value evaluation of each individual effect. Of the 11 variables, temperature, concentration of dipotassium hydrogen phosphate and triammonium citrate were regarded as the most significant factors, with contributions of 30.20 per cent, 11.08 per cent and 9.84 per cent to bacteriocin production, respectively (Fig.1). Generally, bacteriocin production by LAB is reported as a temperature-sensitive

Table 1 : The Plackett-Burman design matrix representing the coded values for 11 independent variables with response

Standard order	Variables											Bacteriocin activity (AU/ ml)
	A	B	C	D	E	F	G	H	J	K	L	
1.	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	761.60
2.	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	673.12
3.	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	658.00
4.	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	593.60
5.	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	728.00
6.	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	672.00
7.	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	700.00
8.	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	616.00
9.	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	602.00
10.	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	714.00
11.	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	658.00
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	658.00

Table 2 : Results of regression analysis for the Plackett-Burman design for bacteriocin production from *Lactococcus lactis* subsp. *lactis* R10

Variables	Main effect	Co-efficient	Standard error	T- value	F-value	p-value
A.	-7.18667	-3.59	2.08	-1.72596	2.98	0.2264
B.	-18.9467	-9.47	2.08	-4.55288	20.72	0.0450
C.	5.32	2.66	2.08	1.278846	1.63	0.3295
D.	13.34667	6.67	2.08	3.206731	10.28	0.0850
E.	32.38667	16.19	2.08	7.783654	60.54	0.0161
F.	30.52	15.26	2.08	7.336538	53.76	0.0181
G.	-39.48	-19.74	2.08	-9.49038	86.96	0.0109
H.	-2.52	-1.26	2.08	-0.60577	0.22	0.6824
J.	-49.1867	-24.59	2.08	-11.8221	139.63	0.0071
K.	53.48	14.86	1.16	12.81034	165.07	0.0060
L.	5.32	2.66	2.08	1.278846	1.63	0.3295

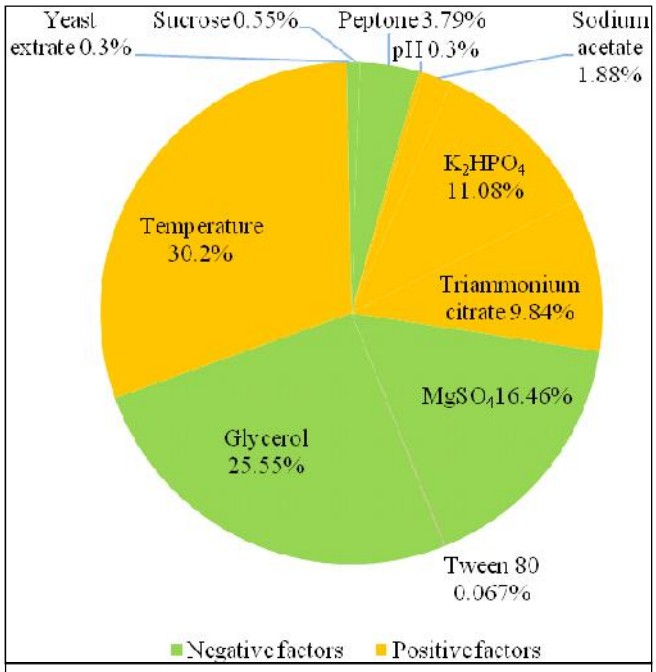

Fig. 1 : Percentage contribution of the variables tested on bacteriocin production by *Lactococcus lactis* subsp. *lactis* R10

process, whereby the optimal temperature for bacteriocin production does not necessarily coincide with the optimal growth temperature (Leroy and De Vuyst, 1999). It has been suggested that bacteriocin production by LAB is enhanced by suboptimal temperatures (De Vuyst *et al.*, 1996; Delgado *et al.*, 2005). Inorganic phosphate was also previously shown to be a positive factor for lacticin LLC518 production by *L. lactis* subsp. *lactis* LLC518 using response surface methodology (Meng *et al.*, 2012). De Vuyst and Vandamme (1993) reported that potassium dihydrogen phosphate was able to improve cell growth and nisin synthesis. Nilsson *et al.* (2002) demonstrated that acetate induced the antilisterial bacteriocin production by *Carnobacterium piscicola* A9b. Previously Kumar and Srivastava (2010) screened sodium acetate, K_2HPO_4 and triammonium citrate had positive factors for enterocin production from *Enterococcus faecium* LR/6. Glycerol is not used by LAB as a carbon source, and the decrease in bacteriocin production may be due to changes in osmotic stress (Todorov and Dicks, 2005). Earlier Settanni *et al.* (2008) reported that minor components of MRS (tween 80, triammonium citrate, K_2HPO_4, sodium acetate, $MgSO_4$ and $MnSO_4$ did not affect bacteriocin like substance production. In contrast to our results, Hugas *et al.* (2002) found that manganese enhances sakacin K activity by *Lactobacillus sakei* CTC 494.

The co-efficient of determination (R^2) was 0.9963, indicating that 99.63 per cent of variability in the response could be explained by the model and it indicates good agreement between experimental and predicted values and implies that the mathematical model is very reliable for bacteriocin production in the present study. The overall linear regression equation showing the empirical relationship between bacteriocin activity and eleven test variables in actual units is represented by Eq. (1).

Final equation in terms of actual factors :

Bacteriocin activity (AU/ml) = + 469.777 - 1.796 * Sucrose - 3.789 * Peptone + 1.064 * Yeast extract + 2.669 * Sodium acetate + 16.193 * Di-potassium hydrogen phosphate + 15.26 * Tri-ammonium citrate - 9 8.700 * Magnesium sulphate- 1.680 * Tween 80 -49.186 * Glycerol + 5.942 * Temperature + 5.320 * pH ————————————1

Conclusion :

The findings of this study showed the importance of using Plankett-Burman experimental design as a preliminary optimization technique, which aids in screening and evaluating the medium components affecting the bacteriocin production using MRS medium by *L. lactis* subsp. *lactis* R10. Among all the tested components, temperature was the most contributing. Based on the results, bacteriocin production from *L. lactis* subsp. *lactis* R10 which is among the microorganisms generally recognized as safe (GRAS) make, this process worthy of future investigation. As such, further statistical optimization using response surface methodology of medium and process parameters needs to be conducted.

LITERATURE CITED

Antony, J. (2008). *Design of experiments for engineers and scientists*. Butterworth Heinemann, U.K., pp. 44-53.

Chauhan, K., Trivedi, U. and Patel, K.C. (2007). Statistical screening of medium components by Plackett-Burman design for lactic acid production by *Lactobacillus* sp. KCP01 using date juice. *Biores. Technol.*, **98**(1): 98-103.

Cleveland, J., Montville, T.J., Nes, I.F. and Chikindas, M.L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *Internat. J. Food Microbiol.*, **71**(1): 1–20.

De Vuyst, L. and Vandamme, E.J. (1993). Influence of the phosphorus and nitrogen source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentation using a complex medium. *J. Appl. Microbiol. Biotechnol*., **40**(1): 17-22.

De Vuyst, L., Callewaert, R. and Crabbe, K. (1996). Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. *Microbiol.*, **142**(4): 817–827.

Delgado, A., Brito, D., Peres, C., Noe-Arroyo, F. and Garrido-Fernandez, A. (2005). Bacteriocin production by *Lactobacillus pentosus* B96 can be expressed as a function of temperature and NaCl concentration. *Food Microbiol.,* **22**(6): 521–528.

Guo, W.I., Zhang, Y.B., Lu, J.H., Jiang, L.Y., Teng, L.R., Wang, Y. and Liang. Y.C. (2010). Optimization of fermentation medium for nisin production from *Lactococcus lactis* subsp. *lactis* using response surface methodology (RSM) combined with artificial neutral network- genetic algorithm (ANN-GA). *Afr. J. Biotechnol.,* **9**(38): 6264-6272.

Haaland, P.D. (1989). *Experimental design in biotechnology*. Marcel Dekker Inc., New York.

Hugas, M., Garriga, M., Pascual, M., Aymerich, M.T. and Monfort, J.M. (2002). Enhancement of sakacin K activity against *Listeria monocytogenes* in fermented sausages with pepper or manganese as ingredients. *Food Microbiol.,* **19**(5): 519–528.

Kanmani. P., Sathishkumar, R., Yuvaraj, N., Paari, K.A., Pattukumar, V. and Arul, V. (2011). The role of environmental factors and medium composition on bacteriocin production by an aquaculture probiotic *Enterococcus faecium* MC13 isolated from fish intestine. *Korean. J. Chem. Engg.,* **28**(3): 860-866.

Kumar, M. and Srivastava, S. (2010). Enhanced production of enterocin from *Enterococcus faecium* LR/6 by statistical optimization of the growth medium. *Trakia J. Sci.,* **8**(4): 12-21.

Leroy, F. and De Vuyst, L. (1999). Temperature and pH conditions that prevail during the fermentation of sausages are optimal for production of the antilisterial bacteriocin sakacin K. *Appl. Environ. Microbiol.,* **65** (3) : 974–981.

Mackay. V.C., Arendse, G. and Hastings, J.W. (1997). Purification of bacteriocins of lactic acid bacteria: problems and pointers. *Internat. J. Food Microbiol.,* **34** (1) : 1-16.

Meng, Q., Cai, Q., Shi, B., Fu, R., Li, J., Chen, X., Qi, K. and Zhang, M. (2012). Optimization of medium composition for production of lacticin LLC518 by *Lactococcus lactis* subsp. *lactis* LLC518 using response surface methodology. *J. Food Agric. Environ*., **10**(1): 137-142.

Nilsson, L., Nielson, M.K., Yin, Ng and Lone, Gram. (2002). Role of acetate in production of an autoinducible class IIa bacteriocin in *Carnobacterium piscicola* A9b. *Appl. Environ. Microbiol.,* **68**(5): 2251-2260.

Oh, S., Rheem, S., Sim, J., Kim, S. and 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**(11): 3809-3814.

Plackett, R.L. and Burman, J.P. (1946). The design of optimum multifactorial experiments. *Biometrika*, **33**(4): 305-25.

Settanni, L., Valmorri, S., Suzzi, G. and Corsetti, A. (2008). The role of environmental factors and medium composition on bacteriocin like inhibitory substances (BLIS) production by *Enterococcus mundtii* strains**.** *Food Microbiol*., **25**(5): 722– 728.

Sharma, D.C. and Satyanarayana, T. (2006). A marked enhancement in the production of a highly alkaline and thermostable pectinase by *Bacillus pumilus* dcsr1 in submerged fermentation by using statistical methods. *Bioresour. Technol.,* **97**(5): 727–733.

Tagg, J.R. and Mc Given, A.R. (1971). Assay system for bacteriocins. *Appl. Microbiol.,* **21**(5): 943-949.

Todorov, S.D. and Dicks, L.M.T. (2005). *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram Negative bacteria. *Enzyme Microb. Technol.,* **36**(2-3): 318–326.