ORIGINAL RESEARCH PAPER

Microbiology

KEY WORDS: *Lactobacillus pentosus*, response surface methodology, Minitab 14, Statistical Design Expert 10

OPTIMIZATION OF NITROGEN SOURCE FOR THE PRODUCTION OF BACTERIOCIN BY *Lactobacillus pentosus* B25 USING RESPONSE SURFACE METHODOLOGY

BHARTI PRAKASH WADEKAR Research Student Microbiology Department Government Institute of Science, Aurangabad

Dr. Mrs. SMITA M. DHARMADHIKARI Associate Professor Microbiology Department Government Institute of Science, Aurangabad

ABSTRACT

Background: Media composition and growth conditions influence the culture growth thus the antibacterial performance. Antibacterial activity of culture can be increased with proper optimized media composition. The Placket-Burman design provides an efficient way of a large number of variables and identifying the most important ones.
Methods: Prepared Placket-Burman Design using Minitab 14. Set 12 run as per given PB Design and put the observed result In Minitab 14. Prepared steepest ascent design using Regression equation. Prepared Central Composite Design using Design Expert version 10. Set 13 run and put the observed result in software.
Result:. From PB design based on low p value run no. 6 for nitrogen source (1680AU/ml). Significant components were found to be peptone (10.9gm/L.) and yeast extract (6.5gm/L.) as nitrogen source which are giving higher antimicrobial activity against *Klebsiella pneumoniae MTCC 535*. Data validation report was obtained comprising expected optimal concentrations of required components of Formulation medium for high yield of bacteriocin.
Conclusion: The components and their concentrations were found to be (gm./L.) peptone-10.8, yeast extract-5.3, 2% NaCl and an initial pH of 7.0.

1. Introduction:

Probiotics, such as lactic acid bacteria,are widely used in dairy, meat and vegetable fermentation [1,2,3]. One major reason for use of production of a wide range of antimicrobial substsnces that efficiently contribute to the preservation of fermented products. Bacteriocin of LAB are biologically active, ribosomally produced antimicrobial peptides that display an antagonistic activity against related species and other bacteria of pathogenic bacteria such as Bacteroides, Candida, Escherichia, Enterococcus, Helicobacter, Gardnerella, Klebsiella, Listeria, Neisseria, Propionibacterium, Staphylococci, Streptococci and Vibrio [4]. These substances have attached increasing research attention owing to potential use in food bio preservation as purified metabolites, usi in starter culture or as adjunct starter culture[5].

Therefore, there has been a continuous need to define the most appropriate condition for bacteriocin production in fermentation media and food systems. Bacteriocin production is usually affected by medium composition and culture conditions such as pH and temperature, as well as the carbon and nitrogen sources and inorganic salts (6,7) . Importantly, for application, it is necessary to optimize the fermentation conditions and medium composition. Bacteriocin production in Lactobacillus sp. may be dependent on multiple factors and is usually a strain specific phenomenon. Thus, these factors need to be optimized in order to achieve higher productivity of microbial metabolites that can be increased by nutritional supplementations and providing congenial physical environment. Nutritional requirements can be manipulated by the conventional or statistical methods. Conventional method involves changing one independent variable at a time keeping the others at fixed level. 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(8). The effect of medium composition on bacteriocin production has been extensively studied and there is general agreement that De man/Rogosa sharp (MRS) broth is one of the most suitable media for maximizing growth and bacteriocin production in LAB(9).

The objective of this research was to apply the Plackett-Burman design, followed by the paths of steepest ascent and response surface methodology to optimize the Nitrogen source for bacteriocin produced by *Lactobacillus pentosus B*25. The major variables affecting the performance of the culture in terms of bacteriocin production were also investigated.

2. Materials and Methodology:

2.1 Bacterial strains: The bacteriocin-producing strain used in this study was Lactobacillus pentosus B25. The strain used as the indicator microorganism was *Klebsiella pneumoniae MTCC 535.* Stock cultures were maintained at -4şC in MRS medium containing 20% glycerol..

2.2 Molecular identification of *Lactobacillus sp*. B25

*Lactobacillus sp.*B25 strain with potent inhibitory activity was characterized to the species level using 16S rRNA sequencing. The 16S rRNA gene of the isolate was sequenced (ABI 3100sequencer and genotyper; (Genei) after the DNA isolation and PCR amplification. The sequence obtained was compared to the GenBank nucleotide database with BLAST (10) and phyloge netically analyzed using MEGA 5.03 software. (Agarkar Research Institute, Pune, Maharashtra, India)

2.3 Production of crude bacteriocin: The isolated strains was grown in MRS broth, pH7 seeded with 1% inoculum of overnight culture and maintained anaerobically at 30oC for 18 hrs. After incubation, cells were removed from the growth medium by centrifugation (12,000 rpm for 15 min, 4oC).The cell free supernatant was adjusted to pH 7.0 using 0.1 N NaOH and it was used as crude bacteriocin (11).

2.4 Bacteriocin assay: The antibacterial spectrum of the bacteriocin from *Lactobacillus sp*. was determined using the well diffusion method. The supernatant from 18 hrs. culture of Lactobacillus pentosus B25 was filter sterilized by passage through a 0.45 um pore size membrane filter. Aliquots (100ul) of the sterile supernatant were placed in 7mm diameter wells that had been cut in MH agar (Himedia lab. Pvt. Ltd. Mumbai, India) plates previously seeded with indicator bacteria. After 24 hrs. of incubation, the diameter of the zone of inhibition were measured. Antibacterial activity was expressed in arbitrary units (AU/ml). 1 AU was defined as the reciprocal of the highest level of dilution resulting in a clear zone of inhibition. (12)

2.5 Statistical Optimization of Nitrogen Source for Production of Bacteriocin :

2.5.1. Plackett-Burman Design (PBD):

PBD was used to screen the most important factors influencing bacteriocin production. The experimental design with the name, symbol code, and level of the variables is shown in table 1, 2 (for nitrogen sources). Each independent variable is represented in two levels, high and low, which are denoted by (+) and (-) respectively. Then the fermentation process was carried out in triplicate and the average value was taken as the response. Usually, the variable with p-value of < 0.05 was considered to have a significant effect on the response and was selected for further optimization.

2.5.2. The Path of the Steepest Ascent Experiment:

To find the neighborhood 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 Placket-Burman design.

2.5.3. 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.

2.5.4. 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.

Table 1: Actual values of nitrogen source variables for PBD

Sr. No.	Nitrogen Source	Code	Low Level (+1)	High Level (-1)
1	Peptone	A	5	15
2	B.E.	B	5	15
3	Y.E.	C	1	10
4	M.E.	D	5	15
5	Malt extract	E	5	15
6	Ammonium nitrate	F	1	3
7	Ammonium citrate	G	1	3
8	Sodium acetate	H	1	10
9	Tween 80	I	0.5	1.5

Table 2: PBD for optimization of nitrogen sources

Run No.	A	B	C	D	E	F	G	H	I
1	+1	+1	-1	+1	-1	-1	-1	+1	+1
2	+1	-1	-1	-1	+1	+1	+1	-1	+1
3	-1	-1	-1	+1	+1	+1	-1	+1	+1
4	-1	+1	+1	-1	+1	-1	-1	-1	+1
5	+1	+1	-1	+1	+1	-1	+1	-1	-1
6	+1	+1	+1	-1	+1	+1	-1	+1	-1
7	-1	+1	+1	+1	-1	+1	+1	-1	+1
8	-1	-1	+1	+1	+1	-1	+1	+1	-1
9	-1	-1	-1	-1	-1	-1	-1	-1	-1
10	-1	+1	-1	-1	-1	+1	+1	+1	-1
11	+1	-1	+1	-1	-1	-1	+1	+1	+1
12	+1	-1	+1	+1	-1	+1	-1	-1	-1

3. Result:

3.1 Molecular identification of Lactobacillus sp. B25: The bacterial strain b25, isolated from Calangute beach, Goa (water) , this had the maximum inhibitory potential and was characterized by 16S rRNA sequencing. The B25 strain revealed 97% similarity with *Lactobacillus pentosus* (T) (Accession Number: D79211) based on nucleotide homology and Phylogenetic analysis by Neighbour Joining method (Fig. 1) and hence designated as *Lactobacillus pentosus.*

3.2 .Statistical optimization of nitrogen sources:

The highest concentration from nitrogen affected on synthesis of sensitive products involved in primary and secondary metabolites as well as the utilization of different carbon sources from fermentation medium. In the present studies, from the preliminary experiments, nine nitrogen sources viz., (A-peptone, B- beef extract, C-yeast extract, D-meat extract, E-ammonium nitrate, F-ammonium citrate, G-sodium acetate, H-tween 80) were selected for statistical optimization procedure for maximum production of Bacteriocin by *Lactobacillus pentosus* (B25). PBD, path of the steepest ascent experiment and CCD were used for statistical optimization of nitrogen sources.

3.3. PBD for optimization of nitrogen sources:

Based on the nine nitrogen sources, a Placket-Burman design of the 12 experimental trials with their outcomes is given in Table 3.. The significant levels of each nitrogen source variable were determined by using t test and regression analysis and are shown in Table 4.

Table 3: PBD for evaluating nitrogen sources influencing production of Bacteriocin by *Lactobacillus pentosus* (B25). :

Trials	Nitrogen Sources (gm/L.)									Diameter of zone of inhibition (mm)	
	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	Experimental	Predicted
1	15	15	1	15	5	1	1	10	1.5	14.5	13.92
2	15	5	1	5	15	3	3	1	1.5	15.5	14.92
3	5	5	1	15	15	3	1	10	1.5	10.5	11.08
4	5	15	10	5	15	1	1	1	1.5	19.0	19.00
5	15	15	1	15	15	1	3	1	0.5	13.0	13.58
6	15	15	10	5	15	3	1	10	0.5	21.0	21.00
7	5	15	10	15	5	3	3	1	1.5	18.5	18.50
8	5	5	10	15	15	1	3	10	0.5	17.0	16.42
9	5	5	1	5	5	1	1	1	0.5	12.5	12.50
10	5	15	1	5	5	3	3	10	0.5	11.0	11.00
11	15	5	10	5	5	1	3	10	1.5	20.0	20.58
12	15	5	10	15	5	3	1	1	0.5	22.0	22.00

Table 4: Estimated effect on Bacteriocin production from the results of PBD for nitrogen sources:

Factors	Effect	Coefficient	Standard Error Coef.	T – value	P - value	Confidence Level (%)
A	0.5833	0.2917	0.05833	5.00	0.038	96.2
B	-0.0167	-0.0083	0.05833	-0.14	0.899	10.1
C	1.5000	0.7500	0.06481	11.57	0.007	99.3
D	-0.1167	-0.0583	0.05833	-1.00	0.423	57.7
E	-0.0833	-0.0417	0.05833	-0.71	0.549	45.1
F	0.4167	0.20083	0.29167	0.71	0.549	45.1
G	-0.7500	-0.3750	0.29167	-1.29	0.327	67.3
H	-0.2407	-0.1204	0.06481	-1.86	0.204	79.6
J	0.5000	0.2500	0.05833	0.43	0.710	29

The analysis (Table 4) showed that A (Peptone) and C (Yeast Extract) 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 pentosus* (B25). A and C both showed positive effect on Bacteriocin production i.e. the concentration of both must be on the higher side in the fermentation broth. According to Table 5, on the basis of statistical testing and the F-test applied for the analysis of variance (ANOVA), the *F*-value was 18.47. The *F*-test with a very low probability value (0.052) shows the high statistical significance of the regression model. High value of correlation coefficient (R^2 = 98.81%; Adj. R2 = 93.46%) explains an excellent correlation between the independent variables. The Pareto plot best demonstrates results of Placket-Burman design that illustrate the absolute relative significance of variables independent of their nature (Figure 2). The ranking of factor estimates in a Pareto chart which displays the magnitude of each factor estimate and it is a convenient way to view the results of a Placket-Burman design.

In the Pareto chart, the maximal effect was presented in the upper portion and then progress down to the minimal effect. In addition, it directly shows that the most important factors determining Bacteriocin production from *Lactobacillus pentosus* (B25) were A and C concentrations while the other factors have insignificant influence.

Table 5: ANOVA of PBD for optimization of nitrogen sources:

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	9	169.688	169.688	18.854	18.47	0.052
Residual error	2	2.042	2.042	1.021		
Total	11	171.729				

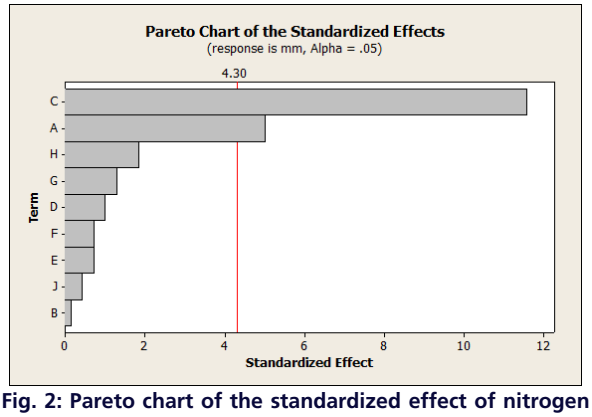

Fig. 2: Pareto chart of the standardized effect of nitrogen sources

3.4. Optimization of nitrogen sources 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 6. 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 (22.5 mm) at run 2 i.e. at 10.9 gm/L. of A and 6.5 gm/L. of C. 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.

3.5 Optimization of nitrogen sources by Response Surface Methodology:

The real values of significant variables used in CCD model are given in Table 7. The CCD experimental plan together with results is given in Table 8.

Table 6: The path of steepest ascent experiment for optimization of nitrogen sources.

Run	Nitrogen Sources (gm/L.)		Diameter of zone of inhibition (mm)
	A	C	
1	10	5.5	17.5
2	**10.9**	**5.53**	**22.5**
3	11.8	5.56	12
4	12.7	5.59	10.5
5	13.6	5.63	12.5
6	15.5	5.65	14.5
7	15.4	5.68	0
8	16.3	5.71	0
9	17.2	5.75	0

Table 7: Real values of the significant factors used in CCD for optimization of nitrogen sources.

Component	Real Values				
	-1.414	-1	0	+1	+1.414
A (gm/L.)	9.06152	9.6	10.9	12.2	12.7385
B (gm/L.)	5.48757	5.50	5.53	5.56	5.57243

Table 8: CCD for optimization of nitrogen sources and the corresponding responses

Run No.	Factor A Peptone (gm/L.)	Factor B Yeast Extract (gm/L.)	Diameter of zone of inhibition (mm)
1	12.2	5.50	19.0
2	10.9	5.53	22.5
3	12.738	5.53	22.5
4	10.9	5.53	22.5
5	10.9	5.53	22.5
6	10.9	5.53	22.5
7	12.2	5.56	19.5
8	10.9	5.48757	17.0
9	9.6	5.56	18.5
10	10.9	5.57243	22.5
11	10.9	5.56	21.0
12	9.0615	5.53	16.5
13	9.6	5.50	16.0

Table 8 shows the observed values of the antibiotic 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, was the predicted response (Bacteriocin yield), $\beta 0$, $\beta 1$, $\beta 2$, $\beta 11$, $\beta 12$ and $\beta 22$ were the regression coefficients, and X*1* and X*2* were the coded levels of the independent variables.

(Diameter of zone of inhibition) = 22.50 +1.30*A +1.26*B -0.50 *AB -2.09*A^2 – 1.72*B^2

Furthermore, the results of the second-order response surface model in the form of analysis of variance (ANOVA) were shown in Table 11. The P-value was used as a tool for checking the significance of each coefficient. 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. Among the model terms, A, B, AB, A^2 and B^2 had significant effects on Bacteriocin production with a probability of not less than 95%.

Table 9: ANOVA for response surface quadratic model for optimization of nitrogen sources

Source	Sum of Squares	DF	Mean Square	F-value	P-value
Model	*72.39*	*5*	*14.48*	*23.56*	<0.0001
A-A (Peptone)	*13.43*	*1*	*13.43*	*21.85*	0.0023
B-B (Y.E.)	*12.68*	*1*	*12.68*	*20.63*	0.0027
AB	*1.00*	*1*	*1.00*	*1.63*	0.2428
A2	*30.50*	*1*	*30.50.*	*49.63*	0.0007
B2	*20.55*	*1*	*20.55*	*33.44*	0.2428

Residual	*4.30*	*7*	*0.61*		0.0002
Lack of fit	*4.30*	*3*	*1.43*		0.0007
Pure Error	*0.000*	*4*	*0.000*		
Core Total	*76.69*	*12*			

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 R2 was 0.9439, implying that 94.39% of the variability in the response could be explained by the model. The value of the Adj R2 was 0.9038 and it was also high enough to advocate for the significance of the model. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. In the present experiment, the lower value of CV (3.89%) indicates a better precision and reliability of the experiments performed.

The 3D response surface curve and 2D contour plot are generally the graphical representation of the regression equation. Figure 2 shows the 3D response surface plot and Figure 3 shows 2D contour plot for interaction between A and B. Analysis of response surface plot indicated that A and B have slight variation in concentrations from zero code value led to an increase in Bacteriocin production.

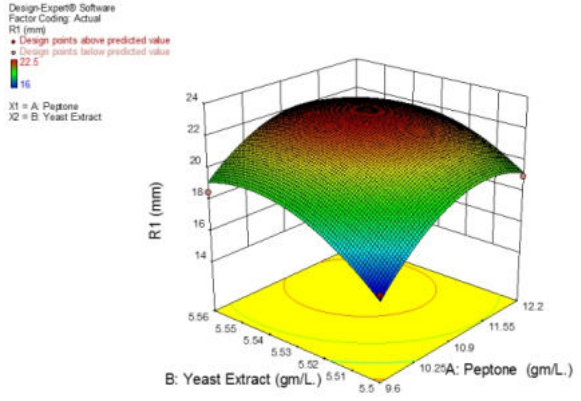

Fig. 3: Response surface plot of bacteriocin production by Lactobacillus pentosus B25 as a function of A and B concentration

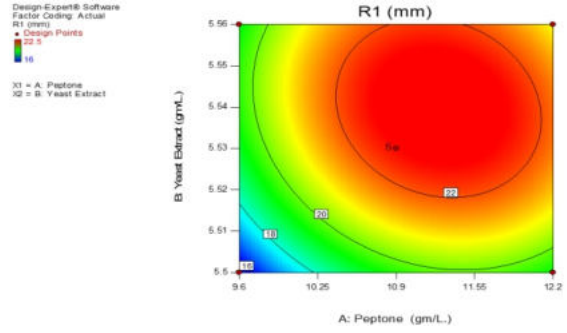

Fig. 4: 2D contour plot for optimization of nitrogen sources by RSM

3.6 Validation of the optimized condition:

To consider the antibacterial activity, a numerical method was used to solve the regression equation for high activity yields. The optimization solution included a A concentration of 10.80 gm/L. and a B concentration of 5.53 gm/L.. Predicted value of the diameter of zone of inhibition was 22.83 mm (maximum response) (Table 12).

Table 10: Confirmation report for optimization for nitrogen sources by RSM

Factor	Name	Level	Low level	High level	Std. Deviation	Coding
A	Peptone	10.80	9.60	12.20	0.000	Actual
B	Y.E.	5.53	5.00	5.56	0.000	Actual
Response		Prediction		Std. Dev.		S.E. (n=1)
Diameter of zone of inhibition (mm)		22.83		0.944521		1.03

Validation under optimized conditions was performed in a 250 ml conical flask containing 50 ml of reaction medium. The experiments were conducted in triplicate. Under the optimized conditions, average diameter of zone of inhibition against Klebsiella pneumonia(535) was 22mm which was very closer to the predicted response, in fact, higher than the predicted response. It confirms the authencity of the model. These results indicate that the optimization of nitrogen sources through RSM favored enhanced Bacteriocin production.

4. Discussion :

The composition of the medium was also shown to have an important role in bacteriocin production (13). However, studies to reduce the cost of the medium have only been recently conducted (14). In the present study, some nitrogen sources in MRS were used to reduce costs viz., peptone and yeast extract. Tween 80 used in Placket-Burman design did not significantly affect bacteriocin production, similar to reports for other bacteriocins (15). In contrast, surfactant could stimulate the production of bacteriocins in other studies (16,17). Earlier, Li and co-workers 18 shows that peptone and KH2PO4 are the two significant factors for bacteriocin production and have the positive effect. The optimal medium made peptone decreased by 0.5% and allowed bacteriocin yield to increase from 1074 to 2150 IU ml-1 compared to Cooked Meat Medium. It was well known that NaCl is required by many bacteria, for Na+ is important to the osmotic pressure to the cells. But NaCl was not needed for other bacteriocin production (19). In this study, peptone and yeast extract played an important part to the growth of the bacteria.

5. Conclusion

RSM was used to determine the effects of two important factors of nitrogen source (peptone, yeast extract) 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 pentosus B25. 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 1760AU/ml was obtained. This study indicates that the medium design using statistical technique such as Placket-Burman Design, RSM can be very useful in improving the production of bacteriocin by Lactobacillus pentosus B25 and in similar bioprocesses. The optimized medium not only allowed the increase in bacteriocin activity, but also reduced the cost of the medium.

6. Acknowledgement: The authors are extending their thanks to Director, Government Institute of Science, Aurangabad, and Maharashtra. We express our gratitude to DST, Delhi for accepting project (Ref No: SR/WOS-A/LS-210/2013).

7. References:

1. Martínez-Cuesta, M. C., Bengoechea, J., Bustos, I., Rodríguez, B., Requena, T. and Peláez, C. 2010. Control of late blowing in cheese by adding lacticin 3147-producing Lactococcus lactis IFPL 3593 to the starter. Int. Dairy J. 20:18-24
2. Govaris, A., Solomakos, N., Pexara, A. and Chatzopoulou, P. S. 2010. The antimicrobial effect of oregano essential oil, nisin and their combination against Salmonella enteritidis in minced sheep meat during refrigerated storage. Int. J. Food Microbiol. 137:175-180.
3. Stiles, M. E. 1996. Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek 70:331-345.
4. Kumar B, Balgir PP, Kaur B, Garg N. Antimicrobial and spermicidal activity of native and recombinant pediocin CP2: a comparative evaluation. Arch Clin Microbiol 2012; 3(3:2) doi:10.3823/254.
5. Chen H, Hoover DG. Bacteriocins and their food applications. Compr Rev Food Sci Food Saf 2003; 2: 82-100.

6. Keren, T., Yarmus, M., Halevy, G. and Shapira, R. 2004. Immunodetection of the bacteriocin lacticin RM: Analysis of the influence of temperature and Tween 80 on its expression and activity. Appl. Environ. Microbiol. 70:2098-2104.
7. Mataragas, M., Drosinos, E. H., Tsakalidou, E. and Metaxopoulos, J. 2004. Influence of nutrients on growth and bacteriocin production by Leuconostoc mesenteroides L124 and Lactobacillus curvatus L442. Antonie van Leeuwenhoek 85:191-198
8. Chauhan K, Trivedi U, Patel KC. Statistical screening of medium components by Plackett-Burman design for lactic acid production by Lactobacillus sp. KCP01 using date juice. Biores Technol 2007; 98: 98-103.
9. Zendo T, Eungruttanagorn N, Fujioka S, Tashiroy, Nomura K, Sera Y. Identification and production of a bacteriocin from Enterococcus mundtii Qu 2 isolated from soybean. Appl Microbiol 2005; 99: 1181-1190.
10. Altschul, S. F., Gish, W., Miller, M., Myres, E. W. and Lipman, D.J. Basic local alignment search tool. J. Molecular biology, 1990; 215(3): 403-410.
11. I. P. Ivanova, A. Kabadjova, S. Pantev and X. Danova. Detection, purification and partial characterization of a novel bacteriocin Substance produced by Lactoccous lactis subsp. lactis b14 isolated from Boza-Bulgarian traditional cereal beverage. Biocatalysis. 2000, 41, 47-53
12. S.T. Ogunbanwo, A.I. Sanni, and A.A. Onilude. Characterization of bacteriocin produced by Lactobacillus plantarum F1 and Lactobacillus brevis OG1. Afr. J. Biotechnol. 2003, 2, 219-227.
13. Li C, Bal JH, Cai ZL, Ouyang F (2002). Optimization of a cultural medium for bacteriocin production by Lactococcus lactis using response surface methodology. J. Biotechnol., 93: 27-34
14. Dominguez APM, Bizani D, Cladera-Olivera F, Brandelli A (2007).Cerein 8A production in soybean protein using response surface methodology. Biochem. Eng. J., 35: 238-243.
15. Trinetta V, Rollini M, Manzoni M (2008). Development of a low cost culture medium for sakacin A production by L. sakei. Process Biochem., 43: 1275-1280.
16. Rajaram G, Manivasagan P, Thilagavathi B, Saravanakumar A (2010). Purification and characterization of a bacteriocin produced by Lactobacillus lactis isolated from Marine Environment. Adv. J. Food Sci. Technol., 2: 138-144.
17. Huot E, Barrena-Gonzalez C, Petitdemange H (1996). Tween 80 effect on bacteriocin synthesis by Lactococcus lactis subsp. cremoris J46. Lett. Appl. Microbiol., 22: 307-310.
18. Li C, Li W, Cai Z. Optimization of a cultural medium for bacteriocin production by Lactococcus lactis using response surface, Bai J methodology. J Biotechnol 2001; 93: 27-34.
19. Delgado A, López NAL, Brito D, Peres C, Fevereiro P, Garrido- Fernández A (2007). Optimum bacteriocin production by Lactobacillus plantarum 17.2b requires absence of NaCl and apparently follows a mixed metabolite kinetics. J. Biotechnol., 130: 193-201.