JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2012. Vol. **6**(4), p. 1507-1515

Optimization of Bacteriocin Production by a New *Lactobacillus* Strain, ZJ317, using Response Surface Methodology

Dafeng Song[1,2], Yue Chen[2], Muyuan Zhu[1] and Qing Gu[2]*

[1]State Key Laboratory of Plant Physiology and Biochemistry, Key Laboratory for Cell and Gene Engineering of Zhejiang Province, College of Life Sciences, Zhejiang University, Hangzhou, China.
[2]Key Laboratory for Food Microbial Technology of Zhejiang Province, Department of Biotechnology, Zhejiang Gongshang University, Hangzhou, China.

(Received: 20 June 2012; accepted: 07 August 2012)

This work presents a sequential approximate optimization method to achieve accurate and efficient optimization of bacteriocin production by *Lactobacillus* strain, ZJ317. Initially, a Plackett–Burman design study and a determination of the path of steepest ascent were effective in identifying the most significant factors and approaching the optimum region of the response. Response surface methodology (RSM) was then applied to evaluate the effects of different variables on bacteriocin production. The experimental results were fitted to a second–order polynomial model equation. The optimal values of each variable to achieve the theoretical maximum of bacteriocin activity (1742.77 U/ml) were predicted to be 2.0% (w/v) glucose, 1.02% (w/v) yeast extract and 2.06% (w/v) inoculum volume. The observed experimental value of bacteriocin activity under the predicted optimal conditions was 1883.61 U/ml compared to a pre–optimization value of 1037.19 U/ml, thus showing a marked improvement in the efficiency of bacteriocin production.

Key words: *Lactobacillus* ZJ317, Plackett–Burman design, Response surface analysis, Bacteriocins, Optimization, Fermentation.

Bacteriocins are ribosomally synthesized antibacterial peptides produced by bacteria. Bacteriocins kill or inhibit other bacteria (they are particularly potent against closely related species), and producer cells have a specific immunity mechanism against their own bacteriocins. Lactic acid bacteria are known to have an antagonistic activity toward a variety of microorganisms. Bacteriocins are of interest for their potential applications in the food industry because of their antimicrobial activity and their technologically favorable properties. Furthermore, bacteriocin–producing strains of lactic acid bacteria protect themselves against the toxicity of their own bacteriocins by expressing a specific immunity protein that is generally encoded in the bacteriocin operon. Therefore, it is hoped that bacteriocins may one day replace antibiotics.

A widely used complex growth medium containing yeast extract and peptone as nitrogen sources and glucose as a carbon source has played an important role in the efficiency and economics of the fermentation process [1]. MRS is the basic medium for cultivating bacteriocin–producing lactic acid bacteria, and it contains abundant materials such as carbon and nitrogen sources, mineral salts, trace elements, peptides, amino acids, vitamins and other factors. However, the yield of bacteriocins is strongly influenced by growth conditions and medium composition. Industrial–scale production

* To whom all correspondence should be addressed.
Tel.:+86 0571 88071024-8599;
E-mail: guqing2002@hotmail.com
Department of Biotechnology, Zhejiang Gongshang University, Jiaogong Load 149#, Hangzhou, China.

of biomolecules often requires the use of complex and inexpensive media containing yeast extract and peptone as nitrogen sources.

Response surface methodology (RSM) is a mathematical and statistical technique for building empirical models. RSM has become important for optimizing the industrial production conditions for high–value products such as chemicals and enzymes and for studying enzyme kinetics[2]. This useful tool has been used in many biotechnological processes, including optimization of culture conditions [3–5], enzyme production[6-9], ethanol production[10-14] and even biomass production[15-18]. In the present work, RSM, a well–established global approximation technique, was combined with a statistical model–building technique, a subdomain representation of the problem, a genetic algorithm and conventional mathematical programming (optimization methods) to determine optimal culturing conditions. This approach constitutes an efficient and effective approximate optimization method for the production of compounds by bacteria[19].

The aim of this study was to improve the production of bacteriocin by *Lactobacillus* strain ZJ317 by optimizing the fermentation conditions and to determine the optimal culture conditions for attaining maximal bacteriocin yield.

MATERIALS AND METHODS

Microorganisms and media

MRS medium was used as a starting medium for optimization and as a routine culture medium for *Lactobacillus* strain ZJ317. *Lactobacillus* ZJ317 is a bacteriocin–producing strain which was recently isolated from human infant feces and identified by 16S rRNA sequencing[20]. The initial pH of the medium was adjusted to 6.5.

Micrococcus luteus 10209 was obtained from the China Center of Industrial Culture Collection (CICC) and was used as a sensitive indicator organism for detection of bacteriocin activity. It was grown on Luria–Bertani (LB) medium and stored at –20°C.

Inocula preparation

A single colony of *Lactobacillus* ZJ317 growing on MRS agar (Oxoid) was inoculated into 20 ml of MRS broth at 37°C in a rotary shaker at 130 rpm and grown to an optical density at 600 nm (OD_{600}) of 1.0 [21]. The cells were harvested by centrifugation at 10,000×g for 10 min at 4°C [10]. The cell pellet was washed and resuspended directly into the cultivation broth (200 ml), the composition of which was varied according to the experimental design described in this work. This procedure was used as the standard inoculum preparation for all experiments.

The indictor bacterium *Micrococcus luteus* 10209 was streaked on an LB plate and cultivated for 24 h. A single colony was then inoculated into 10 ml of LB broth and grown at 37°C to an OD_{600} of 0.5.

Bacteriocin activity determination

Bacteriocin, obtained in the crude enzymatic extract, was measured using a bioassay by spotting 200 ìl of cell–free culture supernatant onto indicator lawns. Indicator lawns were prepared by overlaying 10 ml MRS agar (1.5%, w/v) with 10 ml of MRS soft agar (0.7%, w/v) that was inoculated with 300 ìl of a fresh culture of *Micrococcus luteus* 10209 grown to an OD600 of 0.5. The indicator lawn was placed at 4°C for 30 min, than cultivated at 37°C for 12 h. Zones of inhibition were measured and recorded.

Plackett–Burman design

Experimental designs such as the Plackett–Burman design (PB) and factorial designs are good methods for screening and optimizing medium composition and culture conditions for fermentation processes while requiring a minimal number of experiments [22].

In this study, the same technique was employed to determine the most significant factor affecting the production of bacteria. Eight factors were chosen, and PB was used to find the three most significant factors. The experimental designs with their names, symbol codes and actual factor levels at coded factor levels are shown in Table 1.

Steepest–ascent methods

Based on the PB methodology, the three main factors affecting bacterial production were identified to be glucose, yeast extract and inoculum volume. The steepest ascent in the growth curve will help to reveal the approximate optimum region of the response, which shows the relative amounts by which the factors must vary to attain a maximum increase in response. The design of the steepest ascent coordination path was shown in Table 2.

Response surface methodology

RSM was used to study the combined interactions among different physiological variables[23]. The design of the RSM is based on the results of PB, using the three main factors with the strongest effects on bacteriocin production by *Lactobacillus strain* ZJ317. RSM analysis uses data from experiments using three levels of each of three factors[24]. After the experiments, the bacteriocin activity was taken as the dependent variable or response (Y). A second–order polynomial equation was then fitted to the data by multiple regression analysis. The quadratic equation for the variables was as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i \chi_i + \sum_{i=1}^{k} \beta_{ii} \chi_i \chi_j + \sum_{i<j} \beta_{ij} \chi_i \chi_j \quad ...(1)$$

Where Y is the response variable, β_0, β_i, β_{ii} and β_{ij} are constants, and χ_i and χ_j are the coded levels of variables χ_i and χ. The quadratic equation above was used to plot surfaces for the variables.

RESULTS AND DISCUSSION

Plackett–Burman design

The experimental PB design and results are given in Table 3 and 4. The bacteriocin activity varied considerably within the tested conditions over a range of 379.22–1450.49 U/ml. This result suggested that the tested variables strongly affected bacteriocin production. Based on the main effect factors analysis shown in Table 4, we identified the three main factors as inoculum volume (+0.89), glucose (+0.80) and yeast extract (–1.14). Factors with a confidence level greater than 95% ($P<0.05$) were considered to have a significant effect on the response and were selected for further studies.

According to the PB design experiment, the production of bacteriocin was influenced by inoculum volume ($P=0.012$), glucose ($P=0.017$) and yeast extract ($P=0.004$). The PB design indicated that the three main factors affecting production are inoculum volume (+0.89), glucose (+0.80) and yeast extract (–1.14). We optimized lactic acid fermentation by lowering the yeast extract concentration in the medium, increasing the starting glucose concentration and increasing the inoculum volume of the culture[25].

Steepest–ascent methods

The path of steepest ascent for bacteriocin production was determined using the data shown in Table 2 and by regression analysis. As indicated by the PB design, increasing the concentration of glucose, increasing the inoculum volume and decreasing the concentration of yeast extract, according to the signs of their main effects, should positively affect bacteriocin production by *Lactobacillus* ZJ317 [26]. Fig 1 illustrates the effects of changing the three variables. Glucose concentration and inoculum volume were serially increased by 1.75% and 4%, respectively, while yeast extract concentration was serially decreased by 1.2%. It was clear that the yield plateau had been reached at group four, and this group was chosen for further optimization[27]. The steepest–ascent method established that varying glucose by 1.75%, yeast extract by 1.2% and inoculum volume by 4% were best for determining the optimal growth conditions of *Lactobacillus* ZJ317.

Response surface

Based on the above steepest–ascent method, response surface methodology was employed to determine the optimal conditions of the three most significant factors, namely glucose, yeast extract and inoculum volume. Randomized experimental runs were carried out to minimize the error due to matching setup. The ranges of glucose concentrations, yeast extract concentrations and inoculum volume used in the experiments are listed in Table 5. Table 6 shows the experimental plan and details the experimental run order and coded values of the process parameters.

Error! No bookmark name given.

This analysis resulted in a proposed second–order polynomial equation as follows:

$$Y = 1742.24 + 19.58A - 4.86B + 29.60C + 242.38AB - 174.82AC - 140.63BC - 472.30A^2 - 283.84B^2 - 592.40C^2 \quad ...(2)$$

Error! No bookmark name given.

where Y is the response (bacteriocin activity) and A, B and C represent the variables glucose yeast extract and inoculum volume, respectively. The model using all coefûcients was very reliable, with an R^2 value of 94.07, meaning that 94.07% of the total variation is explained by the model[28].

The regression equation was represented

using 3–D response surface plots, and each contour curve corresponds to an infinite number of combinations of two test variables, while the third concentrations of the variables can also be predicated from the respective response surface plots (Figs. 2A, 2B, 2C). In these figures, each row of the plot corresponds to a factor and each column of the plot corresponds to a response, each plot

Table 1. Experimental variables at different levels used for the production of *Lactobacillus* ZJ317 using Plackett–Burman design

Variables	Symbol code	Actual factor level at coded factor levels		
		Lower (–1)	Center (0)	Higher (–1)
Inoculum volume (%)	A	1.5	3	4.5
Culture volume (ml)	B	10	20	30
Glucose (%)	C	0.75	1.5	2.25
Maltose (%)	D	0.75	1.5	2.25
Yeast extractÿ (%)	E	0.75	1.5	2.25
Peptone (%)	F	0.25	0.5	0.75
Sodium dihydrogen Phosphate (%)	G	0.1	0.2	0.3
Tween 80 (%)	H	0.1	0.2	0.3

Table 2. Design of steepest ascent coordination path

No.	Glucose (%)	Yeast extract (%)	Inoculum volume (%)
1	1	2.1	1
2	1.25	1.8	2
3	1.5	1.5	3
4	1.75	1.2	4
5	2	0.9	5
6	2.25	0.6	6
7	2.5	0.3	7

Table 3. Result of Plackett–Burman design

Runs	Team	A	B	C	D	E	F	G	H	Y(U/ml)
1	1	+	+	–	+	+	–	+	–	379.22
2	1	0	0	0	0	0	0	0	0	474.24
3	1	–	–	–	–	–	–	–	–	663.21
4	1	–	–	+	+	+	–	+	+	424.08
5	1	–	–	–	+	+	+	–	+	480.13
6	1	0	0	0	0	0	0	0	0	474.24
7	1	+	–	+	+	–	+	–	–	999.26
8	1	–	+	+	+	–	+	+	–	550.47
9	1	0	0	0	0	0	0	0	0	474.24
10	1	–	+	+	–	+	–	–	–	593.07
11	1	+	+	+	–	+	+	–	+	688.40
12	1	–	+	–	–	–	+	+	+	453.26
13	1	+	+	–	+	–	–	–	+	799.05
14	1	+	–	+	–	–	–	+	+	1450.49
15	1	+	–	–	–	+	+	+	–	510.94

shows how one response changes as a function of one factor, with all other factors remaining fixed. The maximum predicted yield is indicated by the confined surface in the response surface diagram.

The regression equation, maximized using Design Expert 7.1 (Stat–Ease, Inc.), allowed us to obtain the following optimal coded units for test variables: A=0.02, B=0.0102 and C=0.0206. Under

Table 4. Main effect factors analysis

Item	Effect	Coefficient	Standard error	T	P value
Constants		6.02	0.11	52.43	0.000
Inoculum volume	0.89	0.45	0.11	3.88	0.012
Culture volume	–0.53	–0.27	0.11	–2.31	0.069
Glucose	0.80	0.40	0.11	3.49	0.017
Maltose	–0.49	–0.24	0.11	–2.12	0.088
Yeast extract	–1.14	–0.57	0.11	–4.97	0.004
Peptone	–0.22	–0.11	0.11	–0.96	0.382
Na_2HPO_4	–0.54	–0.27	0.11	–2.36	0.065
Tween 80	0.28	0.14	0.11	1.22	0.277
Ct	Pt	4.28	0.26	16.66	0.000

Table 5. The factor and level of Box–Behnken design for optimization of culture medium to product bacteriocin by *Lactobacillus* ZJ317

Symbol code	Variables	Actual factor levels at coded factor levels		
		–1	0	1
A	Glucose (%)	1.55	1.75	1.95
B	Yeast extract (%)	1	1.2	1.4
C	Inoculum volume (%)	3	4	5

Table 6. Response surface Box–Behnken design and corresponding response

Std R1	Run	A	B	C	Results
1	7	–1	–1	0	1297.07
2	4	1	–1	0	688.40
3	10	–1	1	0	799.05
4	14	1	1	0	1159.88
5	5	–1	0	1	1562.72
6	3	1	0	–1	635.58
7	8	–1	0	1	612.41
8	6	1	0	1	627.29
9	9	0	1	–1	872.17
10	16	0	1	–1	893.56
11	13	0	1	–1	674.83
12	11	0	1	1	893.56
13	17	0	0	0	1747.57
14	15	0	0	0	1682.69
15	12	0	0	0	1644.38
16	2	0	0	0	1450.49
17	1	0	0	0	1531.53

Error! No bookmark name given

these optimized conditions, the predicted response for bacteriocin activity was 1742.77 U/ml, and the observed experimental value was 1882.79 U/ml, 140.02 U/ml higher than predicted. The increased amount of bacteriocin production in the validation experiment might be due to *Lactobacillus* strain ZJ317 having positively adapted to produce bacteriocin after long periods of cultivation.

The response surface methodology using the second–order regression equation was a successful tool for the optimization of bacteriocin production by *Lactobacillus* ZJ317 [29]. The optimal conditions found using RSM were 2.0% glucose, 1.02% yeast extract and 2.06% inoculum volume.

Lactic acid bateria has become an important topic in research work, because of its probiotic function, and abundant natural resources in China. The bacteriocin produced by lactic acid bacteria is considered to be the most effective antibiotic alternatives and synthetic preservatives due to its non–toxic, non–residual, non–drug–resistant. Optimization of bacteriocin production by *Lactobacillus* ZJ317 in a laboratory fermentor as a preliminary step to producing bacterioncin for usa as feed additive substituting antibiotics was

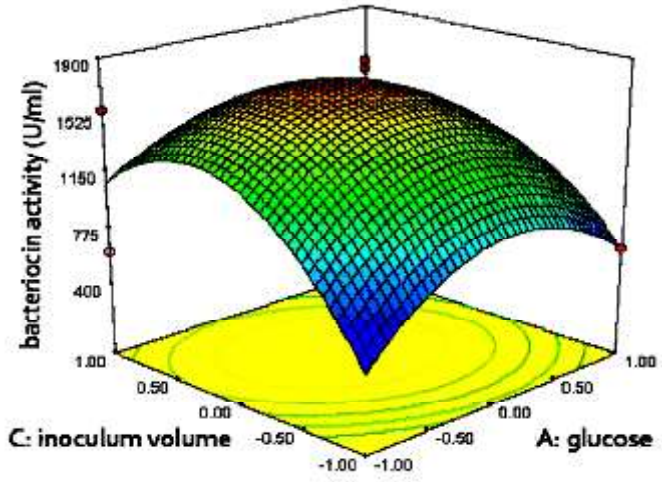

Fig. 1.

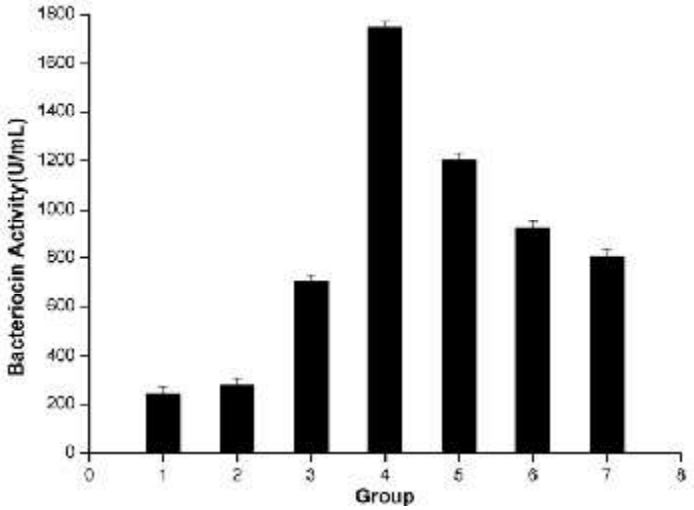

Fig. 2.

accomplished. As large amounts of bacteriocin are necessary to test their antibactericin activity, establishment of the factors and levels influencing maximal production would lead to a more effective recovery of these antimicrobial compounds from a defined laboratory culture medium.

The study was accomplished in three major steps. First, a Plackett–Burman design study was performed to identified the most significant factors that could affect bacteriocin production by *Lactobacillus* ZJ317. According to the PB design experiment, the production of bacteriocin was influenced by inoculum volume, glucose and yeast extract. Second, a determination of the path of steepest ascent was effective in approaching the optimum region of the response. The steepest–ascent method established that varying glucose by 1.75%, yeast extract by 1.2% and inoculum volume by 4% were best for determining the optimal growth conditions of *Lactobacillus* ZJ317. Third, Response surface methodology (RSM) was then applied to evaluate the effects of different variables

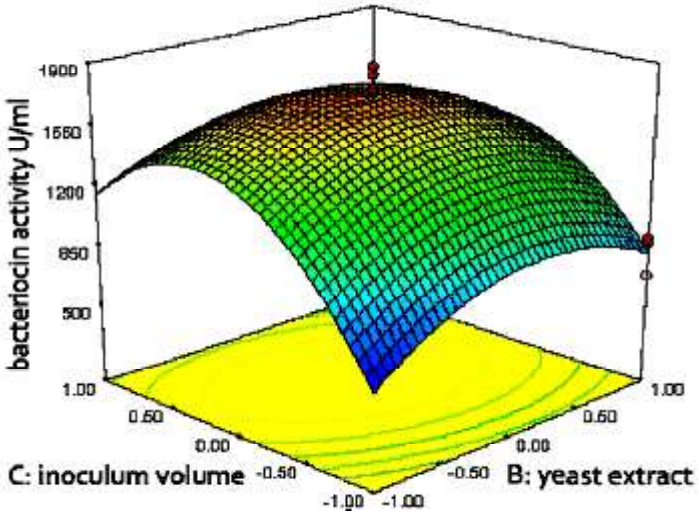

Fig. 3.

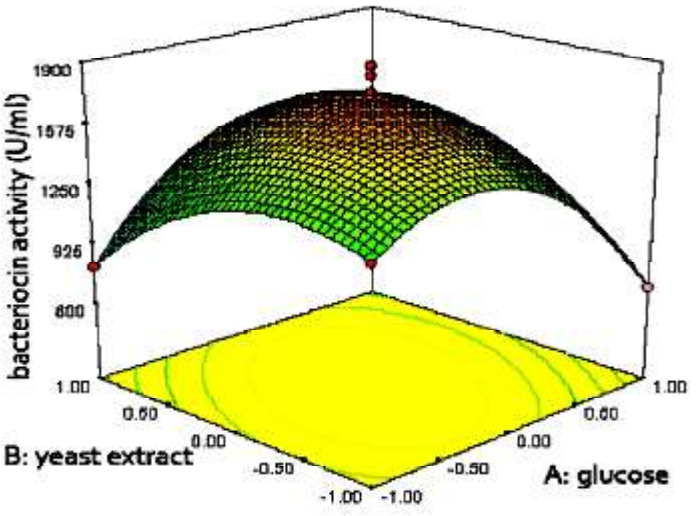

Fig. 4.

on bacteriocin production. The optimal conditions found using RSM were 2.0% glucose, 1.02% yeast extract and 2.06% inoculum volume and predicted response for bacteriocin activity of 1742.77 U/ml.

Under the optimized conditions, the model predicted 1742.77 U/ml bacteriocin activity, while validation experiments indicated the enzymatic activity of bacteriocin at 1882.79 U/ml. This result established that the tested conditions were truly optimal for the production of bacteriocin. Thus, results obtained here clearly indicated the importance of this study in the production of bacteriocin from *Lactobacillus* ZJ317 and the significant for the further development of industrial processes for bacteriocin production and application in feed additive.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (No. 31071513, No. 30970108), the Natural Science Foundation of Zhejiang Province (No. Z3110399), the Program for Science and Technology from Zhejiang Province (No. 2007C12037), the Scientific Research Project for the Education Department of Zhejiang Province, China (No.Y201120078) and the Undergraduate Scientific and Technology Innovation Project of Zhejiang Province, China (No.2011R408018).

REFERENCES

1. Brinques, G., do Carmo Peralba, M., Ayub, M. Optimization of probiotic and lactic acid production by Lactobacillus plantarum in submerged bioreactor systems. *Journal of Industrial Microbiology & Biotechnology.,* 2010; **37**(2): 205-212.
2. Callewaert, R., De Vuyst, L. Bacteriocin Production with Lactobacillus amylovorus DCE 471 Is Improved and Stabilized by Fed–Batch Fermentation. *Applied and Environmental Microbiology*., 2002; **66**(2): 2606-2613.
3. Calado, CRC., Monteiro, SMS., Cabral, JMS., Fonseca, LP. Effect of pre–fermentation on the production of cutinase by a recombinant S*accharomyces cerevisiae*. *Journal of Bioscience and Bioengineering.,* 2002; **93**(4): 354–359.
4. Ergun, M., Ferda Mutlu, S. Application of a statistical technique to the production of ethanol from sugar beet molasses by *Saccharomyces cerevisiae*. *Bioresource Technology*., 2000; **73**(3): 251–255.
5. Ismael, A., Soultani, S., Ghoul, M. Optimization of the enzymatic synthesis of butyl glucoside using response surface methodology. *Biotechnol Prog*., 1998; **14**(9): 874–878.
6. Kim V, S., Min, K., Choi, I., Pyo, S., Rhee, D. Optimization of culture conditions for production of pneumococcal capsular polysaccharide type IV. Archives of Pharmacal Research., 1996; **19**(3): 173-177.
7. Kim, WS., Hall, RJ., Dunn, NW. Improving nisin production by increasing nisin immunity/ resistance genes in the producer organism *Lactococcus lactis*. Applied Microbiology and Biotechnology., 1998; **50**(4): 429-433.
8. Kotzamanidis, C., Roukas, T., Skaracis, G. Optimization of lactic acid production from beet molasses by *Lactobacillus delbrueckii* NCIMB 8130. *World Journal of Microbiology and Biotechnology*., 2002; **18**(5): 441-448.
9. Kalil, SJ., Maugeri, F., Rodrigues, MI. Response surface analysis and simulation as a tool for bioprocess design and optimization. *Process Biochemistry*., 2000; **35**(6): 539–550.
10. Kennedy, M., Krouse, D. Strategies for improving fermentation medium performance: a review. *Journal of Industrial Microbiology & Biotechnology*., 1999; **23**(6): 456-475.
11. Lotfy, WA., Ghanem, KM., El–Helow, ER. Citric acid production by a novel *Aspergillus niger* isolate: II. Optimization of process parameters through statistical experimental designs. *Bioresource Technology*., 2007, 98(18): 3470-3477.
12. Liu, C., Liu, Y., Liao, W., Wen, Z., Chen, S. Application of statistically–based experimental designs for the optimization of nisin production from whey. *Biotechnology Letters*., 2003; **25**(11): 877–882.
13. Liu, GQ., Wang, XL. Optimization of critical medium components using response surface methodology for biomass and extracellular polysaccharide production by *Agaricus blaze*. Applied Microbiology and Biotechnology., 2007; **74**(1): 78-83.
14. Leal–Sánchez, MV., Jiménez–Díaz, R., Maldonado–Barragán, A., Garrido–Fernández, A., Ruiz–Barba, JL. Optimization of Bacteriocin Production by Batch Fermentation of *Lactobacillus plantarum* LPCO10. *Applied and Environmental Microbiology.,* 2002; **68**(9): 4465–4471.
15. Laxman, RS., Sonawane, AP., More, SV., Rao, BS., Rele, MV., Jogdand, VV., Deshpande, VV., Rao, MB. Optimization and scale up of

production of alkaline protease from *Conidiobolus coronatus*. Process Biochemistry., 2005; **40**(9): 3152–3158.

16. Liu, C., Sun, ZT., Du, JH., Wang, J. Response surface optimization of fermentation conditions for producing xylanase by *Aspergillus* SL–05. *Journal of Industrial Microbiology & Biotechnology.,* 2008; **35**(7): 703-711.
17. Ooijkaas, LP., Wilkinson, EC., Tramper, J., Buitelaar, RM. Medium optimization for spore production of *Coniothyrium minitans* using statistically–based experimental designs. *Biotechnology and Bioengineering*., 1999; **64**(1): 92-100.
18. Park, YP., Kang, SK., Lee, JL., Hong, SH., Kim, SK. Xylanase production in solid state fermentation by *Aspergillus niger* mutant using statistical experimental designs. *Applied Microbiology and Biotechnology*., 2002; **58**(6): 761-766.
19. Paul, DC., Colin, HR., Paul, R. Bacteriocins, developing innate immunity for food. *Nat Rev Microbio*., 2005; **3**: 777-788.
20. Parente, E., Brienza, C., Ricciardi, A., Addario, G.. Growth and bacteriocin production by Enterococcus faecium DPC1146 in batch and continuous culture. *Journal of Industrial Microbiology & Biotechnology*., 1997; **18**(1): 62-67.
21. Sampaio, P., Calado, C., Sousa, L., Bressler, D., Pais, M., Fonseca, L. Optimization of the culture medium composition using response surface methodology for new recombinant cyprosin B production in bioreactor for cheese production. *European Food Research and Technology.,* 2010; **231**(2): 339-364.
22. Soni, P., Singh, M., Kamble, AL., Banerjee, UC. Response surface optimization of the critical medium components for carbonyl reductase production by *Candida viswanathii* MTCC 5158. *Bioresource Technology*., 2007; **98**(4): 829-833.
23. Toksoy, Öner E. Optimization of ethanol production from starch by an amylolytic nuclear petite *Saccharomyces cerevisiae* strain. Yeast., 2006; **23**(12): 849–856.
24. Todorov, SD., Dicks, LMT. Effect of medium components on bacteriocin production by *Lactobacillus* plantarum strains ST23LD and ST341LD, isolated from spoiled olive brine. *Microbiological Research*., 2006; **161**(2): 102-108.
25. Todorov, SD., Dicks, LMT. Bacteriocin production by Lactobacillus pentosus ST712BZ isolated from boza. *Brazilian Journal of Microbiology*., 2007; **38**: 166-172.
26. Thammajaruk, N., Sriubolmas, N., Israngkul ,D., Meevootisom, V., Wiyakrutta, S. Optimization of culture conditions for mycoepoxydiene production by *Phomopsis* sp. Hant25. *Journal of Industrial Microbiology & Biotechnology.,* 2011; **38**(6): 679-685.
27. Volpato, G., Rodrigues, RC., Heck, JX., Ayub, MAS. Production of organic solvent tolerant lipase by Staphylococcus caseolyticus EX17 using raw glycerol as substrate. *J Chem Technol Biotechnol.,* 2005; **83**(6): 821-828.
28. Wang, XJ., Bai, JG., Liang, YX. Optimization of multienzyme production by two mixed strains in solid–state fermentation. *Applied Microbiology and Biotechnology*., 2006; **73**(3): 533-540.
29. Yu, X., Hallett, SG., Sheppard, J., Watson, AK. Application of the Plackett–Burman experimental design to evaluate nutritional requirements for the production of *Colletotrichum coccodes* spores. *Applied Microbiology and Biotechnology*., 1997; **47**(3): 301-305.