Biocatalysis and Agricultural Biotechnology 22 (2019) 101386

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: http://www.elsevier.com/locate/bab

Improvement of *Lactobacillus plantarum* for the enhanced production of bacteriocin like inhibitory substance using combinatorial approach

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ARTICLE INFO

Keywords:
Acridine orange
Antibacterial potential
BLIS
Listeria monocytogenes ATCC 7644
Plackett-Burman design
Response surface methodology

ABSTRACT

Over the last few decades, emerging cases of chemical additives and food borne pathogens have enforced the demand of natural antimicrobial compounds. Bacteriocins are one of the natural alternatives to the chemical food preservatives. The research on new and improved bacteriocins is in full swing due to some limitations of commercially available bacteriocins. The aim of the study is to improve the indigenously isolated strain for the enhanced production of bacteriocin like inhibitory substance (BLIS) using combinatorial approach. In the current study, the efficiency of BLIS produced by *Lactobacillus plantarum* KIBGE-IB45 was improved by applying a combinatorial strategies. Primarily, production of BLIS was optimized using *one factor at a time* approach. To further enhance the potential of *L. plantarum*, chemical mutagens were used followed by statistical optimization using multivariate approach. The results revealed that synergistic effect of chemical mutagen and a sequential statistical optimization have significantly enhanced the production of BLIS at 35 °C in MRS medium with an initial pH 9.0 after 16 h of incubation. Response surface methodology revealed 2.0 fold increased in the production of BLIS. The ability of BLIS to inhibit the *Listeria monocytogenes* ATCC 7644 has enforced its application as a natural preservative to resolve the uncontrolled issues of food industry.

1. Introduction

Despite recent advances in food science and technology, food deterioration and spoilage are the major issues in food industry. Food borne pathogens not only caused serious safety and quality issues in food industry but due to the increased economic losses, food preservation remains a debated issue. Although, different physicochemical treatments have been employed to preserved food products, these procedures may not completely assured safety of products. Bacteriocins are an alternative approach to satisfy consumer's demands for ready to eat, fresh-tasting, nutrient rich, minimal processed and preserved foods (Cleveland et al., 2001). Bacteriocins produced by lactic acid bacteria (LAB) have been widely investigated and are focus of interest due to their dual use as an alternative therapeutics and natural food preservatives (Kanmani et al., 2011). The application of LAB bacteriocins in foods has significantly increased in order to replace the use of chemical preservatives to enhance the shelf-life and the safety of food products (Chollet et al., 2008; Silva et al., 2015; Chatzidaki et al., 2018). The demand of bacteriocins has increased in the food industries because of its broad inhibitory potential against several food borne pathogens such as *Listeria monocytogenes, Escherichia coli, Staphylococcus aureus, Salmonella typhimurium* and *Shigella dysenteriae* and their toxins (Onwuakor et al., 2014; Arques et al., 2015). Currently, nisin is the only approved bacteriocin by the Food and Agriculture Organization/World Health Organization (FAO/WHO) as a food preservative. Due to some limitations of stability and bacterial resistance to nisin, screening of new LAB bacteriocins with broad inhibitory potential and stability at wide range of pH and temperature is a need of recent years.

Bacteriocin like inhibitory substances (BLIS) are similar to bacteriocins but they are uncharacterized. BLIS/bacteriocins produced by LAB are generally unstable, sensitive to environmental conditions and required complex medium components. Therefore in batch fermentation, optimization of production parameters under controlled environmental conditions play a crucial role in the product yield. Previously, conventional methods were used in various industrial bioprocess. These conventional methods were expensive, quite laborious and may lead to loss in product yield (Adinarayana et al., 2003; Zendo et al., 2005; Morowvat et al., 2015). Therefore, alternative approaches were

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https://doi.org/10.1016/j.bcab.2019.101386
Received 20 August 2019; Received in revised form 25 September 2019; Accepted 9 October 2019
Available online 10 October 2019
1878-8181/

designed to overcome the limitations of conventional methods. Design of experiment (DoE) is an alternative multivariate approach for the optimization of various products. These statistical models can significantly evaluate influential parameters involved in the production of different metabolites (Chenthamarakshan et al., 2017). Response surface methodology (RSM) is the widely used statistical tool for the designing of experiments, estimation of factors that affect the environment, construction of model and searching optimum conditions to optimize the various biological processes (Nespolo and Brandelli, 2010).

Now a days, combinatorial strategies for the enhanced production of bacteriocin/BLIS are a focus of interest. These strategies include synergism of multivariate optimization approach, rational screening, genetic engineering and improvement of bacterial strains. Strain improvement plays a crucial role and make a significant contribution in the commercialization and high yield of various industrially important products. Random screening and mutations are the frequently used methods for the improvement of strains because of its cost effectiveness (Mahrous et al., 2013).

Keeping all these facts in view, the aim of this study is to improve the *Lactobacillus plantarum* KIBGE-IB45 for the enhanced production of bacteriocin like inhibitory substance (BLIS) using combinatorial approach against *Listeria monocytogenes* ATCC 7644 for its plausible applications in food industry.

2. Materials and methods

2.1. Isolation and screening of bacteriocin like inhibitory substance (BLIS)

Initially, 40 different strains were isolated for the screening of bacteriocin like inhibitory substance (BLIS) from various food sources (Fig. 1). Among 40 isolated strains, 11 strains showed BLIS production in which only FI-10 strain showed broad antibacterial potential against various food borne pathogens. Therefore, FI-10 was selected and subjected to identification. After taxonomic and molecular identification, the strain was confirmed as *Lactobacillus plantarum* KIBGE-IB45 [GenBank Accession number: **MG814034**] and used for further study (Ibrahim et al., 2019).

2.2. Production of BLIS

For the production of BLIS, *L. plantarum* KIBGE-IB45 was cultivated in MRS (DeMan Rogosa Sharpe) medium (Oxoid). Briefly, log phase culture of *L. plantarum* was standardized using 0.5 MacFarland turbidity

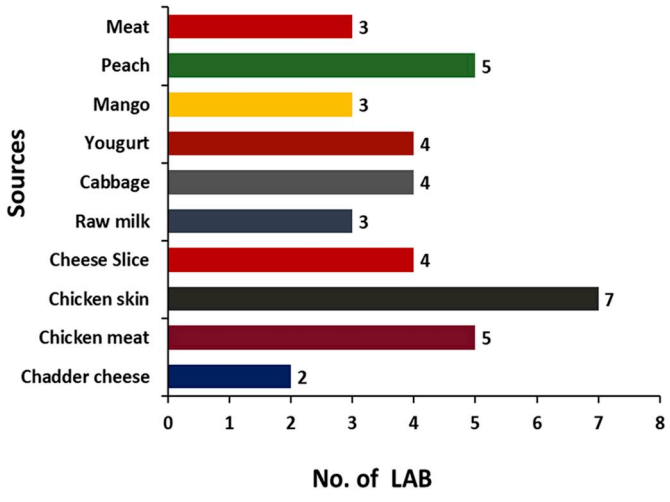

Fig. 1. Isolation of various Lactic Acid Bacteria (LAB) from fermented food sources.

index. Inocula (10^8 CFU mL^{-1}) was transferred into 90 mL MRS medium and after 24 h, the cultivated cells were transferred into 900 mL MRS medium and again incubated at 37 °C for 24 h. Cell free supernatant (CFS) was collected after centrifugation at 3000 × *g* for 15 min at 4 °C and the pH was adjusted to 6.0 by sterilized 5N NaOH to exclude the effect of organic acids. To rule out the inhibitory effect of hydrogen peroxide neutralized CFS was treated with catalase (1 mg mL^{-1}) at 25 °C for 30 min. Afterwards, CFS was sterilized through 0.22 μm membrane filters (Millipore, USA) and the antibacterial activity of CFS was determined by agar well diffusion assay.

2.3. Agar well diffusion assay

Antibacterial activity of BLIS was determined through agar well diffusion assay. In this method, *L. monocytogenes* ATCC 7644 was used as an indicator strain. Briefly, standardized inoculum of *Listeria monocytogenes* ATCC 7644 (10^6 CFU mL^{-1}) was spread on preincubated nutrient agar plates. BLIS (100 μL) was added in 5 mm wells and the plates were incubated at 37 °C for 24 h. After incubation, the antibacterial activity of BLIS was determined against *L. monocytogenes* by measuring zones of inhibitions in millimeters (mm).

2.4. Optimization of BLIS production

2.4.1. Selection of production medium

Initially, four different reported media were used for the production of BLIS which includes MRS medium, BHI (Brain Heart Infusion) medium, LB (Luria Bertani) medium and nutrient broth. For this, standardized inoculum of BLIS producing *L. plantarum* KIBGE-IB45 (10^8 CFU mL^{-1}) was inoculated in media flasks and incubated for 24 h at 37 °C.

2.4.2. Primary optimization (one factor at a time)

One factor at a time (OFAT) approach is a non-statistical technique used to recognize the optimum values of different variables, where the variables cannot be optimize simultaneously. In this study, the effect of various physical parameters including agitation, incubation time, temperature and initial medium pH on the production of BLIS was optimized. The effect of agitation was determined by incubating culture flasks under both shaking (135 rpm) and non-shaking conditions. To examine the optimum cultivation time required for the maximum production of BLIS, MRS medium inoculated with *L. plantarum* KIBGE-IB45 and incubated for different time intervals (2–72 h). Moreover, to determine the effect of incubation temperature, flasks were incubated for 24 h at different temperatures ranges from 20 to 45 °C. Production of BLIS was also monitored at various pH values (pH-4.0 to pH-9.0). However, inoculum was grown in pH 6.5 for 24 h at 35 °C.

2.5. Improvement of L. plantarum for the enhanced production of BLIS

2.5.1. Treatment with chemical mutagens

After primary optimization, *L. plantarum* KIBGE-IB45 was treated with chemical mutagens to further enhance the BLIS production. For this, two chemical mutagens were used including ethyl methane sulfonate (EMS) and acridine orange (AO). *L. plantarum* KIBGE-IB45 was grown in MRS broth, cells from the log phase were transferred into a fresh medium and exposed to various concentrations of EMS (0.01, 0.025, 0.05, 0.1, 0.5 mg mL^{-1}) and acridine orange (0.1, 0.125, 0.25, 0.5, 0.75 mg ml^{-1}) for 15 min (Ghani et al., 2013). Afterwards, the cells were serially diluted (10^{-1}-10^{-6}) in sterilized normal saline and 0.1 mL from each dilution was spread on MRS agar plates and incubated at 35 °C for 24 h. After incubation, bacterial colonies were counted from the selected dilution (10^{-4}) and assayed for antibacterial activity of treated *L. plantarum* KIBGE-IB45 using stab and overlay method (Maricic and Dawid, 2014).

2.6. Optimization of BLIS using multivariate approach

The selected strain capable of producing maximum amount of BLIS was subjected to statistical optimization using multivariate approach. For this purpose, a statistical model was constructed using different physical parameters obtained from primary optimization and a two-step model was designed. A sequential statistical approach was employed in which a factorial Plackett-Burman Design (PBD) was performed primarily for the selection of significant parameters. Furthermore, Response Surface Methodology (RSM) was employed to select the optimal levels of variables selected by PBD and also to check the interactions among the selected variables.

2.6.1. Screening of production parameters using Plackett-Burman Design

Plackett-Burman design (PBD) is a two-factorial design, mostly used for the rapid identification of the significant variables. The total number of experiments were n+1 according to Plackett-Burman, where n is the number of variables selected for the bacteriocin production (Zhao et al., 2013). In this study, 4 variables including agitation, incubation time, temperature and initial medium pH were screened in 12 runs experimental design and response was measured in terms of zones of inhibition (mm). Each variable is characterized as high and low levels symbolized by (+1) and (−1), respectively. Levels selection depend on the results of primary optimization which was performed earlier for the enhanced production of BLIS. Along with all the selected variables, seven dummy variables were also analyzed in this design to compute the standard error. After successful experimentation each response were calculated using first degree polynomial equation:

$$Y = \beta_o + \sum \beta_i X_i \quad (1)$$

where, *Y* refers to the response (BLIS production) β_o, βi and *Xi* represent the model intercept, the linear coefficient, the level of the independent variables (factors), respectively.

2.6.2. Optimization of variables using response surface methodology

Response surface methodology (RSM) was employed to identify the optimal levels of significant variables identified from PBD for the enhanced production of BLIS. It is a useful tool to detect the significant parameters and their interactions with less number of experiments (Namal and Shahidi, 2002). In this study, RSM was carried out using central composite design (CCD). CCD contains 5 different levels of each factor. In this experiment, 20 experimental runs were carried out with 3 independent variables which are represented as X_1 (time), X_2 (pH), X_3 (temperature) at 5 coded levels (−α, −1, 0, +1, +α). To interpret the results second order polynomial equation was employed (Wang and Liu, 2008; Miao et al., 2015). The equation was used to interpret the correlation between the variables and the response. The second degree polynomial equation is as follows:

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where, *Y* is the predicted response (BLIS production). β_o is the model interception, β_i is the linear coefficient, β_{ii} is the squared coefficient, β_{ij} is the coefficient for the interaction effect. Whereas, X_i and X_j are the coded value of independent variable.

2.7. Statistical analysis

Design-Expert® software (version 10.0, Stat-Ease Inc., Minneapolis, MN, USA) was used for the analysis of data. 3D surface plots were constructed using STATISTICA® (Version 10.0, StatSoft Inc., Tulsa, USA) statistical software. All the experiments were carried out in triplicates of three experiments and the results were expressed in mean ± S. D.

3. Results and discussion

The efficacy of BLIS/bacteriocin against undesirable microorganisms has unlocked the interminable potentials for innovative research. Over the last decade, the LAB bacteriocins were focused of interest in various food applications. Generally, microbes which are isolated from natural sources to produce BLIS/bacteriocin are considered safe in its utilization for the preservation of foods. In the current study, *Lactobacillus plantarum* KIBGE-IB45 was improved for the enhanced production of BLIS using combinatorial approach. Improvement of microbial strains provide economic feasibility for commercial applications of biological products during upstream bioprocessing.

3.1. Production of BLIS

Initially, the BLIS was produced in MRS (DeMan Rogosa Sharpe) medium at 37 °C for 24 h. After 24 h of incubation, the activity of neutralized CFS was screened using agar well diffusion assay. Result revealed that the BLIS was found to be active and showed 10 mm zone of inhibition against *Listeria monocytogenes* ATCC 7644 which is the most common food borne pathogen. This formidable food borne pathogen causes listeriosis which can results in high mortality rate (Hernandez--Milian and Payeras-Cifre, 2014). Therefore, *L. monocytogenes* ATCC 7644 was selected as an indicator strain throughout the current study. After primary screening, the production of BLIS was optimized using various strategies.

3.2. Primary optimization of BLIS

Optimization of production parameters during upstream bioprocessing is a valuable approach towards the enhancement of any product yield. Production of bacteriocin from LAB is greatly influenced by formulation of medium and culture conditions therefore, optimization of bacteriocin production is important in lowering the production cost by increasing its productivity and efficacy (Mahrous et al., 2013). Initially, *one factor at a time* (OFAT) approach was used for the production of BLIS in which influence of physical parameters were tested on the production. At first, four different previously reported media were used to select an appropriate medium for maximum BLIS production by *L. plantarum* KIBGE-IB45. Results revealed that maximum production of BLIS was observed in MRS medium based on the maximum zone of inhibition against an indicator strain (Fig. 2a). This result was consistent with the report by Yang et al. (2018).

Afterwards, physical parameters including agitation, cultivation time, temperature and initial pH of medium were studied. The results revealed that the production of BLIS under agitation (135 rpm) was high as compared to the production under static condition. It is due to the microaerophilic nature of *Lactobacillus* species. Determination of optimum production time is also an important factor for BLIS production. Results indicated that the production of BLIS is a time dependent factor. *L. plantarum* KIBGE-IB45 produced BLIS significantly in the late logarithmic phase (18 h) and production reached to maxima around 24 h of cell multiplication which is a stationary phase of bacterial growth cycle (Fig. 2b). A continuous decline in BLIS production was noticed after 24 h of incubation. These observations showed that BLIS exhibited secondary metabolite kinetics and after stationary phase, both the cell mass and BLIS started to decline. This drastic decline in the antibacterial activity could be due to the induction of extracellular endogenous proteolytic enzymes during the decline phase or due to the protein aggregation. It could also be due to the low pH of the medium after stationary phase. Similar observations were obtained when *Lactococcus lactis* isolated from marine environment was used to produce bacteriocin like inhibitory substance (BLIS) (Rajaram et al., 2010). Alam et al. (2011) also stated that a BLIS from *Bacillus subtilis* BS15 was produced at the early stationary-phase or late exponential phase of producer strain.

However, the optimal temperature for the production of BLIS was

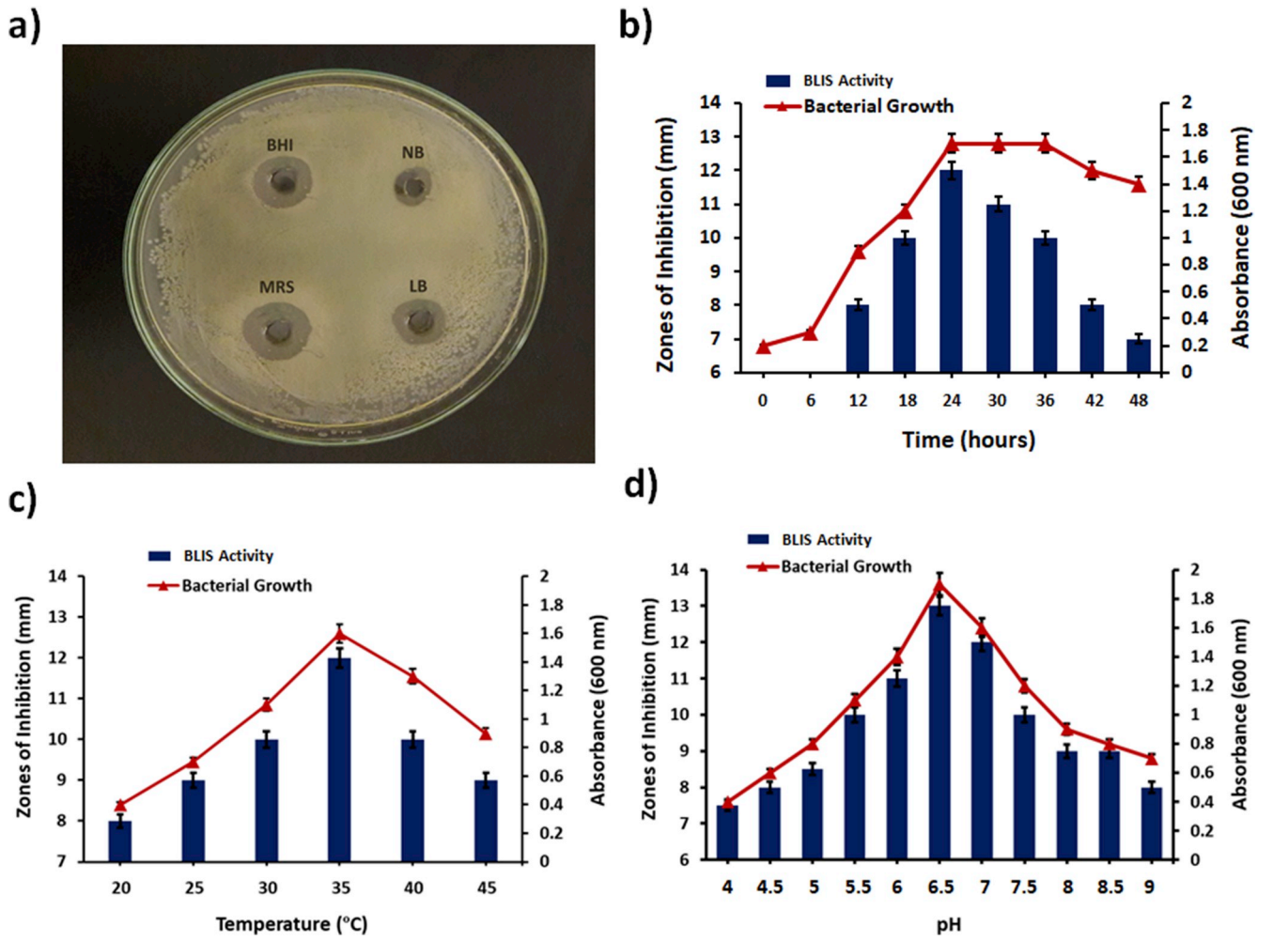

Fig. 2. Optimization of BLIS production using *one factor at a time* approach. **a**: Effect of four different media on the BLIS production, **b**: Effect of incubation time, **c**: Effect of different temperatures, **d**: Effect of initial medium pH on the production of BLIS.

35 °C as *L. plantarum* are mesophilic bacterium and usually grow best at moderate temperatures (Fig. 2c). Gautam and Sharma (2009) reported the production of bacteriocin in between 30 °C to 37 °C for most of the *Lactobacillus* strains. Moreover, initial pH of the medium also plays an important role in the production of BLIS. The pH of the medium decreases the production of BLIS either by effecting the growth of bacteria or by creating undesirable environment that leads to the inactivation of BLIS. In this study, maximum BLIS production was obtained at pH 6.5 however, gradual decrease in the activity was observed as pH increases (Fig. 2d). Lee et al. (2012) reported that initial pH had a greater impact on the production of bacteriocins by *Lactobacillus brevis* DF01. After OFAT optimization various approaches were implemented to further enhance the production of BLIS for its future applications.

3.3. Improvement of Lactobacillus plantarum

Several mutational strategies have been reported to enhance the biosynthesis of various microbial metabolites (Knapp et al., 2016; Ziemons et al., 2017). In the current study, chemical mutagens were used to further enhance the production and anti-listerial potential of BLIS. Results indicated that ethyl methane sulfonate (EMS) found to be lethal for *L. plantarum* even at lower concentration (0.01 mg mL^{-1}). This lethal effect of EMS on bacteria might be due to the DNA damage because bacterial cells lost their efficient DNA repair mechanism after exposure to EMS (Sega, 1984). However, in case of acridine orange (AO) bacterial growth was observed only after exposure to lowest concentration (0.1 mg mL^{-1}) While, at higher concentrations the bacterial growth was inhibited. In another study, it was reported that the exposure of bacterial cells to ultraviolet irradiations for long time period and high concentrations of acridine orange found to be lethal for bacteria. Though, sub lethal concentrations of mutagens can allow the bacteria to survive and resist the effect of mutagens (Khanam and Prasuna, 2014). Therefore, in this study the survived mutant strains were screened for anti-listerial activity. Out of 75 colonies, only 15 (14%) were showed improved antibacterial activity as compared to the parent strain. AO is an aromatic compound with an efficient biological activity and frequently used as an effective mutagen in numerous genetic studies (Kusuzaki et al., 2000; Botelho et al., 2004). Muzzamal and Latif (2016) also generated a mutant after exposure to acridine orange that exhibited 3.0 fold increase in polygalacturonase activity as compared to the parental strains. Similarly, enhancement in antibiotic activity with exposure to acridine orange has also reported in *Bacillus subtilis* (Bernal et al., 2002). In contrast, Ghani et al. (2013) used ultraviolet irradiation and ethidium bromide for the improvement of strain which leads to the enhanced production of glucoamylase. In the current study, the strain which showed improved anti-listerial activity (18 mm) as compared to primary optimization after exposure to chemical mutagen was selected for further study. The selected strain was further optimized for BLIS production by applying a sequential statistical model.

Table 1
Production of BLIS by Placket Burman Design with observed and predicted values along with the coded and actual values.

Run No	Coded Levels of Independent Variables											BLIS Activity (mm)	
	A	B	C	D	Dummy 1	Dummy 2	Dummy 3	Dummy 4	Dummy 5	Dummy 6	Dummy 7	Actual Value	Predicted Value
1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	14.00	13.92
2	-1	1	-1	1	1	-1	1	1	1	-1	-1	11.00	10.08
3	-1	-1	-1	1	-1	1	1	-1	1	1	1	12.00	11.92
4	1	1	-1	1	1	1	-1	-1	-1	1	-1	15.00	14.92
5	1	-1	1	1	-1	1	1	1	-1	-1	-1	14.00	14.52
6	1	-1	-1	-1	1	-1	1	1	-1	1	1	15.00	15.25
7	-1	-1	1	-1	1	1	-1	1	1	1	-1	16.00	16.25
8	1	-1	1	1	1	-1	-1	-1	1	-1	1	14.00	15.25
9	-1	1	1	-1	1	1	1	-1	-1	-1	1	12.00	13.08
10	1	1	-1	-1	-1	1	-1	1	1	-1	1	14.00	14.92
11	-1	1	1	1	-1	-1	-1	1	-1	1	1	11.00	12.08
12	1	1	1	-1	-1	-1	1	-1	1	1	-1	10.00	11.92

Codes	Variables	Low Level -1	High Level 1
A	Cultivation time	16	24
B	Initial pH	7	9
C	Production temperature	30	35
D	Agitation	0	135

A: Cultivation time, ***B***: Initial medium pH, ***C***: Production temperature, ***D***: Agitation.
The "−1" sign represents minimum value and "1" sign represents maximum value of the input parameters range.

3.4. Plackett-Burman Design (PBD)

The selected strain was subjected to statistical model for the initial screening of significant production variables. For this, PBD was employed to screen the influential parameters for the production of BLIS by *L. plantarum* KIBGE-IB45. The experimental design comprises of coded levels with the actual and predicted values is presented in Table 1. A large distinction in BLIS production was noticed in both experimental (10–16 mm) and theoretical values (10–16 mm) which revealed that the initial screening of production parameters by statistical approach is important for the selection of influential parameters. Maximum activity of BLIS was noticed in the 7th experimental run (16 mm) with an optimized condition (production time, 16 h; initial medium pH, 7.0; temperature, 35 °C with no agitation). Hegde et al. (2013) also reported that these three parameters are essential to enhance the productivity and efficiency of bioactive microbial metabolites. Pareto chart predicted the order and significance of all variables. In Pareto analysis variables were placed from largest to smallest orders (Fig. 3) and two reference lines (Bonferroni limit and t-value limit) were constructed to interpret the results. The factors which are above the Bonferroni limit (7.70406) are considered more significant whereas, those which are above the t-value limit (3.18245) are less significant. The current results showed the effect of three parameters: cultivation time > temperature > pH in subsequent order on the production of BLIS. Moreover, the analysis of variance (ANOVA) was estimated in which the sum of square, mean square, *F*-value and *P*-value were analyzed (Table 2). The *F*-value (43.00) suggested that the current model is significant and there is only a 0.52% chance that an *F*-value this large could occur due to any experimental noise. Probability measures the significance and the factors with probability less than 0.05 ($P < 0.05$) showed that the model terms are statistically significant. It can be estimated through the data that three variables (cultivation time, initial medium pH and temperature) were considered to be significant for the production of BLIS. Whereas, the remaining one variable (agitation) was found to be insignificant in this statistical experimental design. Therefore, these three significant variables which showed significant probability were selected to further optimize the production of BLIS however, the insignificant factors were not considered (Kohli et al., 2017).

The R-squared (R^2) usually determines the model accuracy by measuring the response explained by the experimental variables (Edwards et al., 2008). In this model, R-squared value (0.9914) suggested that the variation of 99.14% occurs due to independent variables while there is a 0.86% chance that the response could not be explained by the selected experimental model variables. The value of R^2 should be in between 0 and 1, if the value is greater than 0.9 then the model is considered as fit (Liu et al., 2003; Cladera-Olivera et al., 2004; Zafar et al., 2018). Hence, R^2 value supports the validity of this model. The Predicted R^2 value (86.17%) is also in reasonable agreement with the Adjusted R^2 value (96.83%), which confirms a significant correlation between the predicted and experimental value. Adequate precision usually measures the signal to noise ratio, if a ratio is greater than 4 then it is considered as significant. It relates the predicted points with the average prediction error (Singh and Kayastha, 2014). In the current model, the ratio of 20.66 showed an adequate signal. The coefficient of variance (C.V) used to measure the reliability of the experimental performance. Whereas, the lesser value of C.V. (2.0%) shows a greater reliability. The mean and standard deviation of the present model were

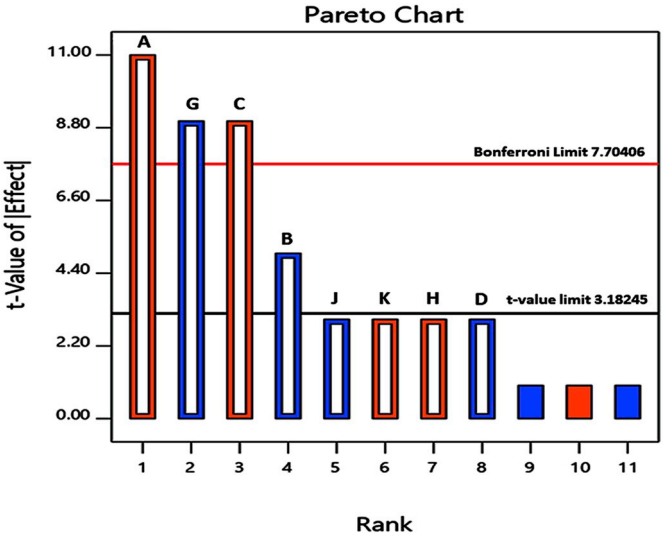

Fig. 3. Pareto chart illustrating the effect of independent variables on bacteriocin production. Blue colored bars represent negative effect of variables while, orange colored bars represent positive effect of variables. A: Cultivation time, B: Initial medium pH, C: Incubation temperature, D: Agitation, G, J, K, H: Dummy variables. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Regression statistics and analysis of variance (ANOVA) of the experimental results for BLIS production.

Sources	Sum of Squares	df	Mean Square	*F*-value	*p*-value Prob > *F*
Model	28.67	8	3.58	43.00	0.0052*
A-time	10.08	1	10.08	121.00	0.0016*
B-pH	2.08	1	2.08	25.00	0.0154*
C-temperature	6.75	1	6.75	81.00	0.0029*
D-agitation	0.7500	1	0.7500	9.00	0.0577
G	6.75	1	6.75	81.00	0.0029
H	0.7500	1	0.7500	9.00	0.0577
J	0.7500	1	0.7500	9.00	0.0577
K	0.7500	1	0.7500	9.00	0.0577
Residual	0.2500	3	0.0833		
Cor Total	28.92	11			
Std. Dev.	0.2887			R^2	0.9914
Mean	14.42			Adjusted R^2	0.9683
C.V. %	2.00			Predicted R^2	0.8617
				Adequate Precision	20.6667

A: cultivation time, B: pH, C: temperature, D: agitation, G, H, J, K: dummy variables.
F-value: Fisher's function, *P*-value: level of significance, Std. dev.: standard deviation, C.V.: coefficient of variance.
* Significant values.

14.42 and 0.288, respectively. The first degree polynomial equation was generated after the regression analysis. The equation is represented as:

$$Y = +14.42 + 0.9167\,A - 0.4167\,B + 0.7500\,C - 0.2500\,D \quad (3)$$

where, *Y* is the predicted response (BLIS activity in terms of zones of inhibition) and *A, B, C* and *D* are the cultivation time, initial medium pH, production temperature and agitation, respectively. These represents the independent variables and their coefficients which shows the effect of each variable on the response.

3.4.1. Validation of the PBD model

The competence of the PBD model was confirmed by performing the experiments under optimized conditions (16 h, 35 °C and initial medium pH 7.0). After optimization using PBD, the close correlation between the experimental (16.0 mm) and predicted (16.25 mm) data represented the accuracy and the relevance of the current experimental design. The selected variables (cultivation time, initial medium pH and temperature) from PBD model have a significant impact on the production of BLIS. Therefore, these variables were selected for further optimization using response surface methodology (RSM) because the current model only screened the influential variables and doesn't provide evidence related to the interactions and the optimum level of the variables.

Table 3
Production of BLIS by central composite design with the observed and predicted values along with the coded and actual levels of independent variables.

Run No.	Variables			BLIS Activity (mm)	
	X_1	X_2	X_3	Observed values	Predicted values
1	+1	-1	-1	15.00	14.35
2	0	0	0	14.00	13.71
3	0	1.68179	0	16.00	15.29
4	-1	+1	-1	15.00	15.25
5	-1	-1	-1	16.00	15.86
6	0	0	0	14.00	13.35
7	0	0	1.68179	15.00	15.41
8	1	-1	+1	18.00	18.35
9	0	-1.68179	0	15.00	16.04
10	0	0	0	15.00	14.61
11	+1	+1	+1	16.00	15.89
12	-1.68179	0	0	15.00	14.71
13	-1	+1	+1	22.00	22.35
14	-1	-1	+1	14.00	15.01
15	0	0	0	12.00	11.35
16	1.68179	0	0	15.00	14.71
17	0	0	0	16.00	15.29
18	0	0	-1.68179	16.00	15.37
19	+1	+1	-1	16.00	15.86
20	0	0	0	12.00	11.35

Codes	Independent variables	Coded levels				
		-1.68^{α}	−1	0	+1	$+1.68^{\alpha}$
X_1	Time (h)	13.27	16	20	24	26.72
X_2	pH	6.318	7	8	9	9. 68
X_3	Temperature (°C)	28.29	30	32.5	35	36.70

+1, −1 = intermediate; 0 = central and +α, −α = highest concentration levels of range studied for each variable.

3.5. Response surface methodology (RSM)

In RSM, central composite design (CCD) was used for the production of BLIS using 20-run experimental design with the selected influential parameters. All the influential variables were set at 5 different levels. The experiment was designed to determine the parameter ranges and coded levels with actual and predicted values of the three independent variables (Table 3). A wide variation in the activity of BLIS was observed during 20 run experiments from 12.0 mm to 22.0 mm. This variation was because of the change in each experimental condition of 20 run experimental design. It reflects the significance of statistical optimization over conventional method. For the maximum activity of BLIS, optimized levels of the selected parameters were cultivation time: 16 h,

Table 4
Analysis of variance (ANOVA) for response surface quadratic model.

Source	Sum of Squares	*df*	Mean Square	*F-value*	*P-value* Prob > *F*
Model	151.36	9	16.82	14.21	0.0001*
X_1	0.0340	1	0.0340	0.0288	0.8687
X_2	1.60	1	1.60	1.36	0.2713
X_3	0.3063	1	0.3063	0.2588	0.6220
X_1X_2	0.1250	1	0.1250	0.1056	0.7519
X_1X_3	0.1250	1	0.1250	0.1056	0.7519
X_2X_3	0.1250	1	0.1250	0.1056	0.7519
X_1^2	66.09	1	66.09	55.84	<0.0001*
X_2^2	66.09	1	66.09	55.84	<0.0001*
X_3^2	46.07	1	46.07	38.92	<0.0001*
Residual	11.84	10	1.18		
Lack of Fit	6.50	5	1.30	1.22	0.4165
Pure Error	5.33	5	1.07		
Cor Total	163.20	19			

X_1 = Cultivation time, X_2 = Initial medium pH, X_3 = Production temperature, *F*-value: Fisher's function, *P*-value: level of significance.
* Significant value.

temperature: 35 °C and initial medium pH: 9.0. Similarly, Yang et al. (2018) also reported the maximum bacteriocin activity in MRS medium after 16 h of incubation. Comparison of experimental and predicted values indicated a good correspondence suggesting that experimental model obtained from RSM can be used to sufficiently demonstrate the relationship between the variable and response. In this model, mean and standard deviation of the experimental data are 17.20 and 1.09, respectively. The R^2 value shows the accuracy of the model. Mostly, the coefficient of determination for model variable (R^2) is significant when its value is closer to 1, which is 100% (Kaushik et al., 2006). The R^2 value is 0.9275, demonstrating that the 92.75% variation in the response was elucidated by the independent variables. Therefore, in the current study the model was considered significant with over 90% of model variability (R^2-0.9275) explained. The adjusted R^2 with value of (R^2-0.8622) was in accordance with the predictable (R^2-0.6492). Adequate precision analyzes the signal to noise ratio, a ratio greater than 4 is desirable. In this model, ratio of 8.7591 indicated an adequate signal and it was considered acceptable. Moreover, analysis of variance (ANOVA) was also investigated using Fisher's (*F*-test) statistical analysis (Table 4). The model *F*-value of 14.21 implies that the model is significant. X_1^2, X_2^2, X_3^2 were considered as significant with probability <0.0001. The *P*-value (<0.0001) was measured as a tool for assessing the significance of the model. The model terms of Prob > *F* (<0.05) was considered significant whereas, the prob > *F* (>0.10) was insignificant (Salihu et al., 2011). Moreover, to assess the model accuracy the values for 'lack of fit' must be insignificant because it reflects the failure of the model. In the present model, the 'lack of fit' ($P > 0.10$) was not significant and considered good and fitted well to the model. To further elucidate the dependence of BLIS production on physical parameters, a second degree polynomial equation was generated from the CCD model.

$$Y = +21.35 + 0.0499\,X_1 + 0.3428\,X_2 - 0.1498\,X_3 + 0.1250\,X_1X_2 + 0.1250\,X_1X_3 + 0.1250\,X_2X_3 - 2.14\,X_1^2 - 2.14\,X_2^2 - 1.79\,X_3^2 \quad (4)$$

In this equation, *Y* represent the predicted response (BLIS activity in term of zones of inhibition) and X_1, X_2 and X_3 shows cultivation time, initial medium pH and temperature, respectively.

In order to investigate the interaction of variables, 3D response surface plots were designed. In 3D plots, the interaction between two major variables is examined by keeping other variables fixed at their center point. It is used to determine the optimal level at which the response is maximum (Tanyildizi et al., 2005). In this study, 3D response surface plots were constructed for the enhanced production of BLIS by plotting the response (BLIS activity) on Z-axis against the two independent variables (Fig. 4a–c). In Fig. 4a, gradual increase in BLIS activity was noticed by elevating both initial medium pH and incubation time at optimum point. However, further increase was resulted in the decrease of BLIS activity. Similar results were observed in Fig. 4b and c. Moreover, the circular contour lines indicated that the interaction between the variables were insignificant with a probability (*P*-value 0.7519). Result revealed that the quadratic effect of these variables (cultivation time, initial medium pH and incubation temperature) were

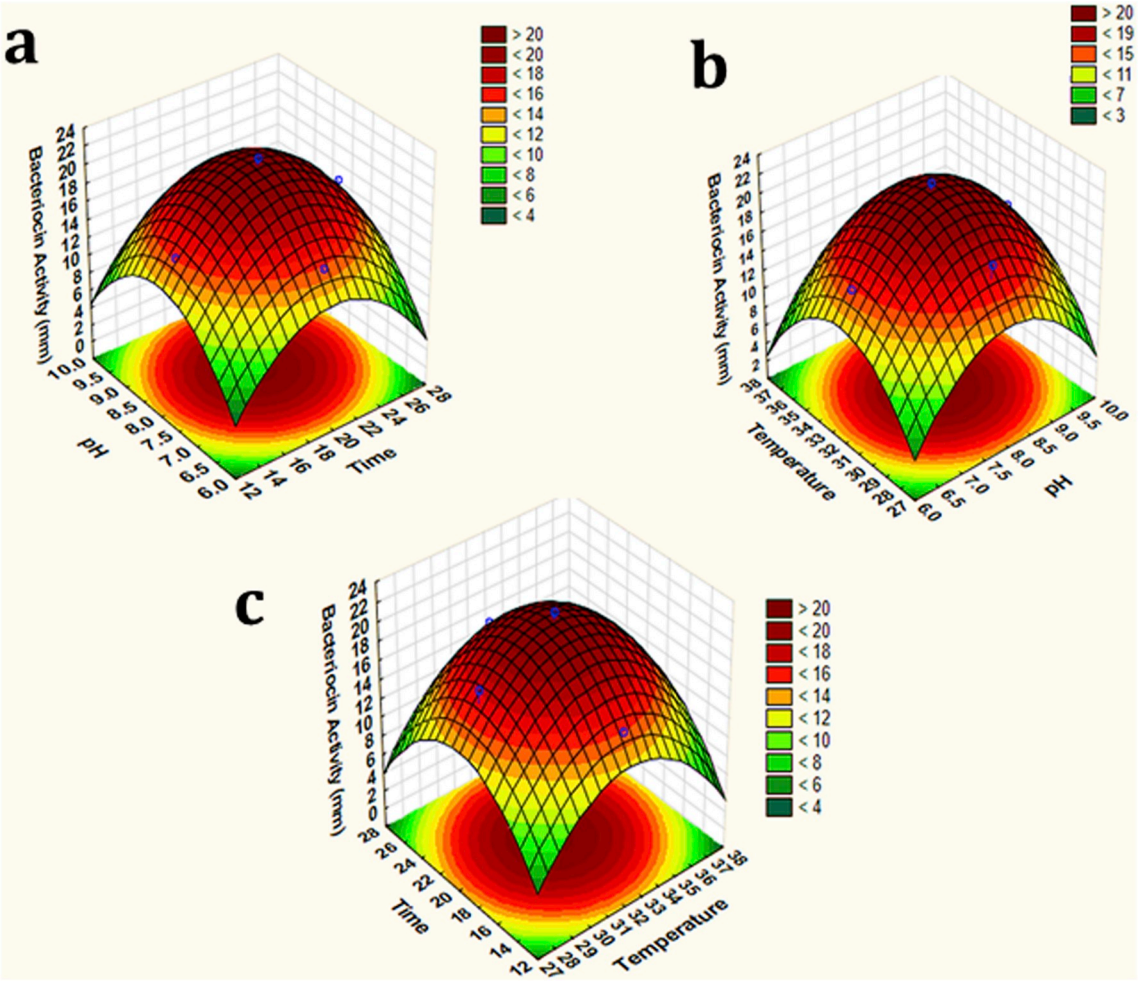

Fig. 4. 3D response surface plot presenting the effect of interactions of independent variables on BLIS production. **a**: pH *vs.* Incubation time, **b**: Temperature *vs.* pH, **c**: Incubation time *vs.* Temperature.

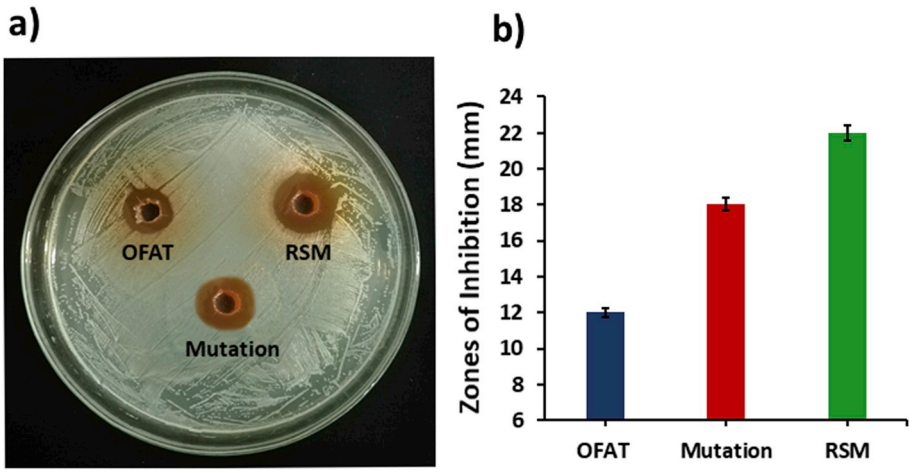

Fig. 6. Enhanced activity of BLIS after applying combinatorial strategies: **a**: Agar well diffusion assay against *Listeria monocytogenes* ATCC 7644 **b**: Graphical illustration in terms of zones of inhibition (mm).

found to be significant for the production of BLIS. Therefore, this model could be used as a powerful tool for the enhancement of BLIS/bacteriocin production.

3.5.1. *Model accuracy and validation of the optimum conditions*

The normality percentage plot is used to measure the distribution of the experimental residuals. Results showed that the observed values were aligned adjacent to the ideal line of regression demonstrating the significance and accuracy of the model. In this plot, straight line showed the normal distribution of the experimental residues and confirms the validation of the model (Fig. 5). In order to verify the results and to determine the model accuracy, experiments were conducted in triplicate with the following optimized conditions: initial pH of the medium; 9.0, temperature; 35 °C at 16 h of incubation under static conditions. Results revealed that the experimental values (22.0 mm) were in accordance with the predicted values (22.35 mm) which strongly prove the suitability of the developed model. This sequential statistical optimization resulted in the enhanced production of BLIS with approximately 2.0-fold increase in the antilisterial activity as compared to the OFAT optimization (Fig. 6).

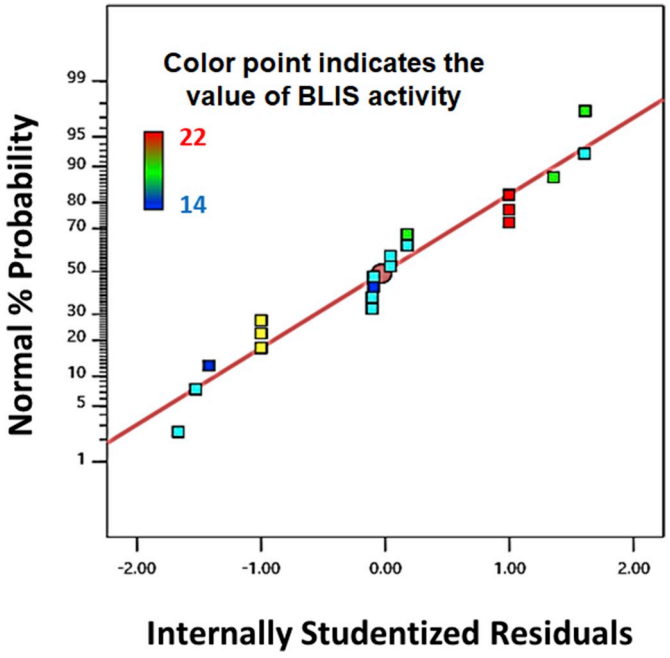

Fig. 5. The normal probability plot of residuals for the production of BLIS.

4. Conclusions

Lactic acid bacteria and their bacteriocins have emerged as a great alternative to chemical preservatives and traditional antibiotics in the fields of food science and technology. In the present study, combinatorial strategies were used for the enhanced production of BLIS from *L. plantarum* KIBGE-IB45. It can be concluded from this study that the sequential statistical approach is more effective and reliable as compared to conventional method for the enhanced production of BLIS. RSM was resulted in approximately 2.0-fold enhancement of BLIS at optimal levels of parameters (cultivation time: 16 h, temperature: 35 °C and initial medium pH: 9.0). The inhibitory potential of BLIS against *L. monocytogenes* has enforced its application to resolve the uncontrolled issues of food deterioration and preservation.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgements

The authors gratefully acknowledge KIBGE, University of Karachi, Karachi, Pakistan for providing labaratory facilities during this research project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101386.

Funding

This research work was funded by Higher Education Commission (HEC) Pakistan under National Research Program for Universities NRPU (Grant number: 20-3900/R&D/HEC/14/874).

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