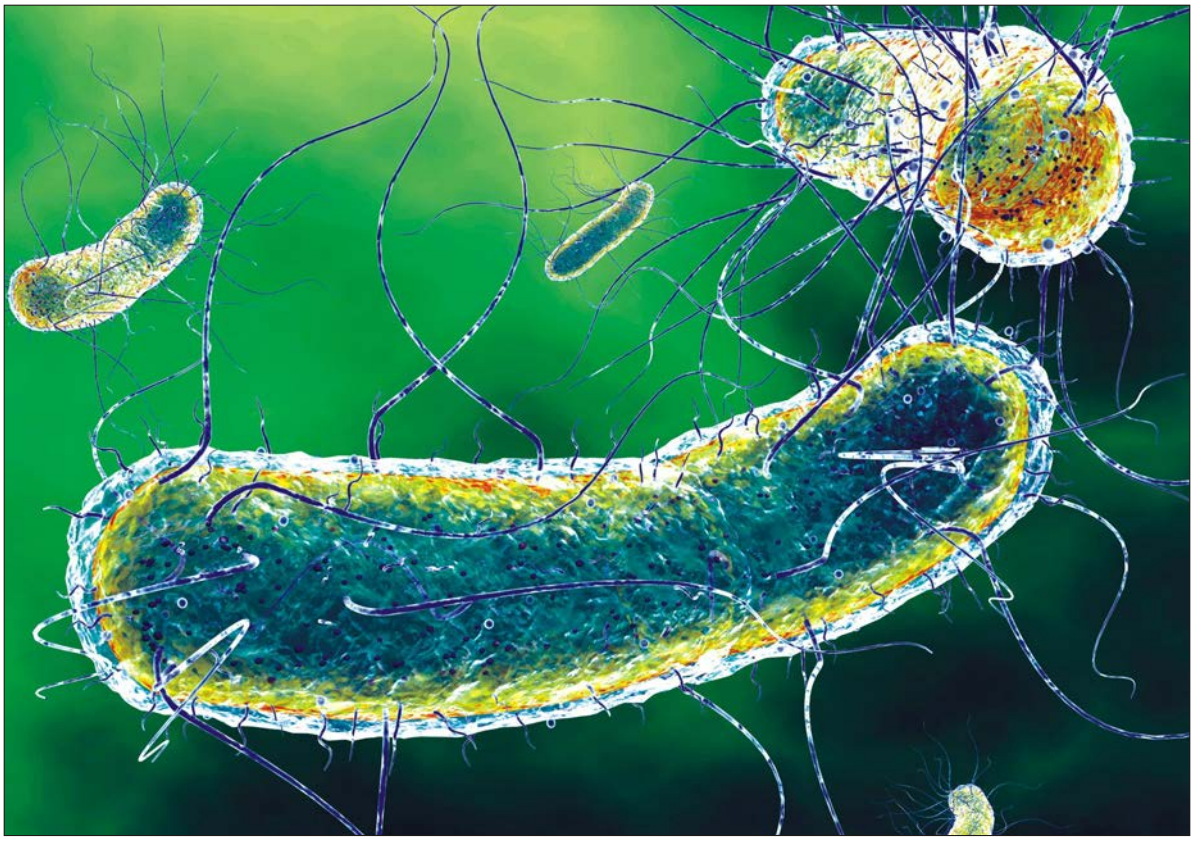

# Current Research in Bacteriology

ISSN 1994-5426

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Current Research in Bacteriology 3 (1): 1-14, 2010
ISSN 1994-5426


# Response Surface Methodology as a Tool for Optimizing the Production of Antimicrobial Agents from *Bacillus licheniformis* SN2

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**Abstract:** Response surface methodology was used for optimization of fermentation medium and conditions which were screened by Plackett Burman design for antibacterial agents production by *Bacillus licheniformis* SN2 and suppression of *Staphylococcus aureus* ATCC 6538 growth. Seven factors were chosen to be screened by Plackett Burman design, yeast extract, peptone, NaCl, pH, temperature, incubation period and culture volume. Among ingredients, high concentration of yeast extract had a positive effect on antibacterial peptide production. Alkaline pH and high temperature favored the growth of *B. licheniformis* SN2. The optimal response region of the significant factors was predicted by using a second order polynomial model fitted to the results obtained by applying the Box Behnken statistical design. In this experiment, near basal concentration of yeast extract (7.4 g $L^{-1}$), low level of peptone (2 g $L^{-1}$) and near basal concentration of NaCl (2.8 g $L^{-1}$) was highly increase the suppression % to 1.3 fold. Moreover, the optimized condition accelerated the reaction by reducing 6 h in suppression process.

**Key words:** Response surface methodology, *Bacillus licheniformis*, suppression, *S. aureus*

## INTRODUCTION

Production of antimicrobial compounds seems to be a general phenomenon for most bacteria. Admirable arrays of microbial defense systems are produced, including broad-spectrum classical antibiotics, metabolic by-products such as organic acids and lytic agents such as lysozyme. In addition, several types of proteins, exotoxins and bacteriocins, which are biologically active peptide moieties with bactericidal mode of action, were described (Riley and Wertz, 2002; Yeaman and Yount, 2003). This biological arsenal is remarkable in its diversity and natural abundance, since some substances are restricted to some bacterial groups while others are widespread produced (Riley and Wertz, 2002).

*Bacillus* strains is of major interest in bacteriocin research since this genus produces a diverse array of antimicrobial peptides with several different basic chemical structures (Stein, 2005; Pakpitcharoena *et al*., 2008). Antimicrobial agents produced by Gram-positive bacteria have attracted much attention because of their potential use as food preservatives (Gould, 1996) some representatives of *Bacillus* sp., such as *B. subtilis* and *B. licheniformis*, are Generally Recognized as Safe (GRAS) bacteria (Sharp *et al*., 1989). Choopan *et al*. (2008) studied the anti-methicillin resistant *S. aureus* activity of *Brevibacillus laterosporus* strain SA14.

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*Staphylococcus aureus* has extensive genomic variability and easily acquires tools for resisting against antimicrobials, in particular against B-Lactam Antibiotics (BLAs). Therefore, it is one of the most successful and adaptable human pathogens (Daini and Akano, 2009). Staphylococcal antibiotic resistance has been associated with resistant plasmids that have the ability to mediate the production of drug inactivating enzymes such as β-lactamases (Adeleke and Odelola, 1997) and other functions (King *et al*., 2006; Diep *et al*., 2008). Methicillin-resistant *S. aureus* (MRSA) has remained a major cause of nosocomial disease world-wide (Larsen *et al*., 2008).

Resistance of several pathogenic bacteria to antimicrobial agents is an emerging problem. Twenty years ago, bacteria that were resistant to antimicrobial agents were easy to detect in the laboratory because the concentration of the drug required to inhibit their growth was usually quiet high and distinctly different from that of susceptible strains. Newer mechanisms of resistance, however, result in much more subtle shifts in bacterial population distributions, for example, one of the most difficult phenotypes to detect, is decreased susceptibility to β-lactams. There is certainly an urgent need for new antimicrobial agents, given the increase in drug resistance in many common bacterial pathogens, together with the emergence of new diseases (Davis and Webb, 1998). *Bacillus licheniformis* inhibited the growth of several Gram positive and negative bacteria (Pakpitcharoena *et al*., 2008).

The nutritional and environmental conditions have a great influence on production of antimicrobial agents. To achieve maximum production, knowledge regarding the environmental factors affecting this process needs to be well identified. Experimental designs are excellent techniques for optimization of culture conditions to achieve optimal production (Hassan, 2008).

In the present study, we have designed an optimization strategy to study the effect of the different fermentation variables and their interactions on the antimicrobial agents production from *Bacillus licheniformis* SN2 a*gainst Staphylococcus aureus* ATCC 6538.

## MATERIALS AND METHODS

### Bacterial Strains

The experimental bacterium used throughout the present investigation was *Bacillus licheniformes* SN2 which was kindly provide by Dr. Hassan A.H. Ibrahim (NIOF). The bacterial indicators were *Staphylococcus aureus* ATCC 6538, *Micrococcus luteus* ATCC 10240, *Pseudomonas aeruginosa* ATCC 8739, *Candida albicans* ATCC 14053, *Vibrio damsela*, *Streptococcus faecalis* and *Escherichia coli.* This study was conducted during 2009.

### Media for Antagonistic Activity

Luria Bertani broth medium (Barrow and Feltham, 1993), consisting of the followings (g $L^{-1}$): yeast extract, 5; peptone, 10 and sodium chloride, 5.

### Antagonistic Action Against Indicator Microorganisms

The well-cut diffusion technique was used to test the ability of the bacterial isolates to inhibit the growth of indicator bacteria and yeast. Fifty milliliter of nutrient agar medium inoculated with indicator microorganisms were poured into all plates. After solidifying, wells were punched out using 0.5 cm cork borer and each of their bottoms was then sealed with two drops of sterile water agar. One hundred microliter of tested bacterial filtrates were transferred into each well after sterilizing by ultra-filtration using 0.22 μL sterilized filters. All plates were incubated at appropriate temperature for 24-48 h. After incubation period, the radius of clear zone around each well (Y) and the radius of the well (X) were linearly

measured in mm, where dividing $Y^2$ over $X^2$ determines an Absolute Unit (AU) for the clear zone. The absolute unit of each antagonistic isolate, which indicates a positive result in the antagonistic action, was calculated according to the following equation (El-Masry *et al*., 2002):

$$AU= Y^2/X^2$$

**Effect of Enzymes and Heat on Antimicrobial Activity**

This step aimed to categorize the antimicrobial agent as an antibiotic, a siderophore or a bacteriocin depending on its nature. After treating with proteolytic enzymes (proteinase k and trypsin) and heating to 100°C for 15 and 30 min. The cell free culture of *B. licheniformis* SN2 was exposed to proteolytic enzymes represented by (1) Proteinase K (20 mg $mL^{-1}$) dissolved in $T_{10}$ $E_1$ buffer (10 mM Tris, 1 mM EDTA, pH 8), (2) Trypsin (5 mg $mL^{-1}$) dissolved in 0.1 M Tris-HCl buffer, pH 7. Each inhibitory substance was mixed with an equal volume of the proteolytic enzyme solution and incubated at 37°C for 1 h with agitation at 100 rpm $min^{-1}$. Samples of untreated culture supernatant and protease (in buffer only) served as control. After the incubation time, enzyme reaction in all treatments was inactivated by boiling for 30 min, where then inhibitory effect of the enzyme treated antimicrobial agent was assayed (Deraz *et al*., 2005) by boiling for different intervals (15, 30, 45 and 60 min) and autoclaving for 15 and 30 min (Lavermicocca *et al*., 1999).

The treated and untreated supernatants in all previous steps were assessed for antimicrobial activity using the method described in the bioassay and then the suppression percentage was calculated.

**Experimental Designs Applied for Optimizing the Production of Antimicrobial Agents**

Optimization was performed by a two-phase experimental design. The first step was to evaluate the relative importance of various constituents within a complex cultural medium by applying a fractional factorial design. In the second phase, levels of the variables, which had significant influences on the production process, were further studied using an appropriate optimization design.

**Plackett-Burman Design**

Application of a complete factorial design would require $2^n$ experiments if n factors have to be investigated. In the present case, seven variables would lead to 128 trials, which is a very large number. Using a fraction of the factorial design without losing information about the main effects of variables can reduce the number of experiments (Oijkaas *et al*., 1998). The Plackett-Burman experimental design, a fractional factorial design (Plackett and Burman, 1946; Yu *et al*., 1997) was applied in this research to reflect the relative importance of various environmental factors on the production of antimicrobial agent(s) in liquid cultures.

Seven independent variables were screened in nine combinations organized according to the Plackett-Burman design matrix described in the Results section. For each variable, a high (+) and low (-) level was tested. All trials were performed in duplicates and the averages of observation results were treated as the responses. The main effect of each variable was determined using the following equation:

$$E_{xi} = (\Sigma M_{i+}-\Sigma_{i-})/N$$

where, $E_{xi}$ is the variable main effect, $M_{i+}$ and $M_{i-}$ are suppression percentages of *S. aureus* 6538 growth in trials where the independent variable (xi) was present in high and low levels,

respectively and N is the number of trials divided by 2. A main effect with a positive sign indicates that the high level of this variable is nearer to optimum. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for determination of variable significance.

**Box-Behnken Design**

In the second phase of medium formulation for optimum production of antimicrobial agents, the Box-Behnken experimental design, which is a central composite design (Box and Behnken, 1960), was applied. In this model, the most significant independent variables, namely ($X_1$), ($X_2$) and ($X_3$) are included and each factor can be examined at three different levels, low (-), high (+) and central or basal (0).

Here, concentration of yeast extract ($X_1$) peptone ($X_2$) and sodium chloride ($X_3$) were treated as independent variables. Thirteen combinations were examined and their observations (results section) were fitted to the following second order polynomial model:

$$Y= b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+ b_{22}X^2_2+ b_{33}X_3^2$$

where, Y is the dependent variable (suppression %); $X_1$, $X_2$ and $X_3$ are the independent variables; $b_0$ is the regression coefficient at center point; $b_1$, $b_2$ and $b_3$ are linear coefficients; $b_{12}$, $b_{13}$ and $b_{23}$ are second-order interaction coefficients; and $b_{11}$, $b_{22}$ and $b_{33}$ are quadratic coefficients.

The values of the coefficients were calculated using Origin 6.1 software and the optimum concentrations were predicted using Microsoft Excel 2007. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination, $R^2$. Three-dimensional graphical representations were also constructed using Statistica software to reflect the effects as well as the interactions of independent variables on the objective.

**Bioassay of Antibacterial Activity**

The bioassay used for detecting the suppression of *S. aureus* ATCC 6538 by selected isolate *B. licheniformis* SN2 was modified according to Nissen-Meyer *et al.* (1992). Seeding culture of *S. aureus* ATCC 6538 was freshly prepared in LB with optical density 0.7 at Absorbance 550 nm using Spectionic 21D Milton Roy spectrophotometer. On the other hand, cultures of producers were prepared according to the trials conditions of experimental designs then centrifuged at 5000 rpm for 20 min. The supernatants were then followed by filtration using 0.45 μ membrane filters (Gelman Laboratory, USA).

In test tubes containing 2 mL sterilized LB, 20 μL $mL^{-1}$ of *S. aureus* ATCC 6538 were added to obtain control, while blank was applied as LB without any treatments. Treatments included *S. aureus* ATCC 6538 (20 μL $mL^{-1}$) with different concentrations of filtrated supernatant (25, 50, 75 and 100 μL $mL^{-1}$).

All tubes were incubated in a shaker (New Brunswick Scientific, Edison, N.J., USA) at 37°C. As soon as, the optical density of *S. aureus* ATCC 6538 as a control reached to 0.3 at $A_{550}$ nm, the optical densities of all treatments were recorded and then suppression percentages were calculated according to the following equation (Al-Ajlani and Hasmain, 2006):

$$\text{Suppression \%} = \frac{A_{550}\ \text{control} - A_{550}\ \text{treatment}}{A_{550}\ \text{control}} \times 100$$

## RESULTS

### Effect of Enzymes and Heat on Antimicrobial Activity

The protentious nature of the antimicrobial agent secreted by *B. lichenformis* SN2 was tested by subjection to proteolytic enzymes (proteinase k and trypsin) and heating to 100°C for 15 and 30 min. Results from Table 1 clarify that, suppression % was decreased from 76% to 11.1 by using proteinase k and to 11.0% by using trypsin, which may confirm its protentious nature. Table 1 also clarify a direct relationship between boiling time and suppression % decrease.

### Influence of Fermentation Parameters on the Production of *B. lichenformis* SN2 Antimicrobial Agent

For elucidation of medium components affecting the production of anti- *S. aureus* ATCC 6538 protein by *B. lichenformis* SN2, the independent variables examined in the Plackett Burman experiment and their settings are shown in Table 2. The main effect and t-value of each variable was calculated according to the dry weight and suppression % results (Table 3). The data (Table 4) indicated that, the presence of high levels of yeast extract (7 g $L^{-1}$) in the growth medium, with culture volume of 65 mL and incubation period of

Table 1: Influence of enzymes and heat on antimicrobial activity of *B. licheniforms* SN2 culture filtrate

| Treatment | Suppression (%) |
|---|---|
| **Control** | 76.0 |
| Proteinase K | 11.1 |
| Trypsin | 11.0 |
| **Boiling** | |
| 15 min | 15.1 |
| 30 min | 8.3 |

Table 2: Factors examined as independent variables affecting the production of anti-*S. aureus* ATCC *6538* protein by *B. lichenformis* SN2 and their levels in the Plackett-Burman experiment

| | | Level | | |
|---|---|---|---|---|
| Factors | Symbol | -1 | 0 | +1 |
| Yeast extract (g $L^{-1}$) | YE | 3 | 5 | 7 |
| Peptone (g $L^{-1}$) | Pep. | 8 | 10 | 12 |
| NaCl (g $L^{-1}$) | Na | 3 | 5 | 7 |
| pH | pH | 5 | 7 | 9 |
| Temperature (°C) | Temp. | 30 | 35 | 40 |
| Incubation period (h) | IP | 12 | 24 | 36 |
| Culture volume (mL) | CV | 35 | 50 | 65 |

Table 3: Experimental results of the applied Plackett-Burman experimental design

| | Response | |
|---|---|---|
| | Growth of *B. licheniforms* SN2 | |
| Trial | Dry wt. (mg $mL^{-1}$) | Suppression % of *S. aureus* |
| 1 | 1.840 | 43.24 |
| 2 | 1.570 | 86.82 |
| 3 | 0.401 | 45.61 |
| 4 | 0.412 | 33.78 |
| 5 | 0.281 | 38.81 |
| 6 | 1.040 | 40.54 |
| 7 | 1.950 | 38.18 |
| 8 | 1.312 | 49.32 |
| 9 | 1.964 | 77.10 |

Table 4: Statistical analysis of the Plackett-Burman experiment

| | Suppression % of *S. aureus* ATCC 6538 | | Dry wt. | |
|---|---|---|---|---|
| Variable | Main effect | t-value[1] | Main effect | t-value |
| Yeast extract | **11.32** | 0.944018 | -0.0037 | -0.071420 |
| Peptone | -12.67 | -1.092620 | -0.0162 | -0.316840 |
| Sodium chloride | -10.81 | -0.896090 | 0.0036 | 0.174798 |
| pH | -11.66 | -0.977310 | 0.0039 | 0.180672 |
| Temperature | -7.35 | -0.579790 | 0.1130 | 0.180672 |
| Incubation period | **14.86** | 1.313087 | 0.1138 | 5.099805 |
| Culture volume | **16.04** | 1.452817 | -0.4190 | -0.8636 |

[1]t-value significant at the 1% level = 3.70, bold t-value significant at the 5% level = 2.446, bold t-value significant at the 10% level = 1.94, bold t-value significant at the 20% level =1.372, standard t-values are obtained from statistical methods (Snedecor and Cochran, 1989)

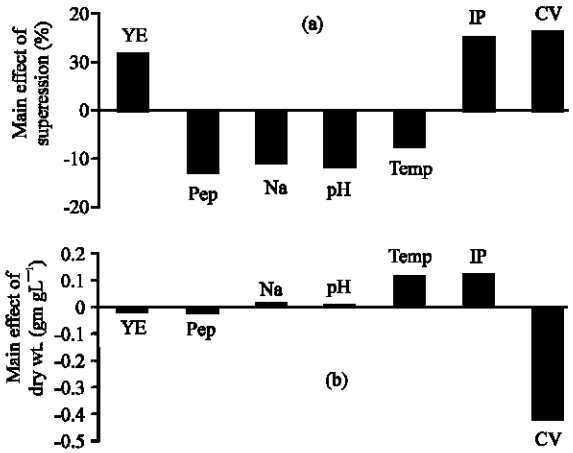


Fig. 1: Elucidation of fermentation factors affecting (a) suppression % and (b) dry wt.

36 h affect the suppression % positively. While, low level of peptone (8 g $L^{-1}$) and NaCl (3 g $L^{-1}$) with low pH (5) and incubation at low temperature (30°C) will also enhance the suppression % (Fig. 1). On the other hand, the growth was promoted with the incubation at high temperature (40°C) and incubation period of 36 h, while low culture volume (35 mL) will promote the growth (Fig. 2). According to this results it can be predicted that the near optimum medium for high suppression % by *B. lichenformis SN2* (g $L^{-1}$): Peptone, 8; yeast extract, 7; NaCl, 3, while pH value is 5 incubated at 30°C with incubation period of 36 h and culture volume of 65 mL medium.

In order to evaluate the accuracy of the applied Plackett-Burman screening test, a verification experiment was carried out in triplicate. The predicted near optimum levels of independent variables and the far from optimum were examined and compared to the basal condition setting. The average of biomass production and suppression % was recorded (Table 5). Suppression % reached about 86.13% which is approximately 1.1 times higher than that obtained from basal medium (78.39%). On the basis of the calculated t-values (Table 4), the incubation period is considered to be highly significant for the suppression % and growth enhancement, while culture volume is significant for suppression % only.

**Optimization of the Production of *B. lichenformis* SN2 Antimicrobial Agent by Box-Behnken Design**

In this second optimization step the levels of the three independent variables Yeast extract ($X_1$); Peptone ($X_2$); and NaCl ($X_3$) were further investigated each at three different

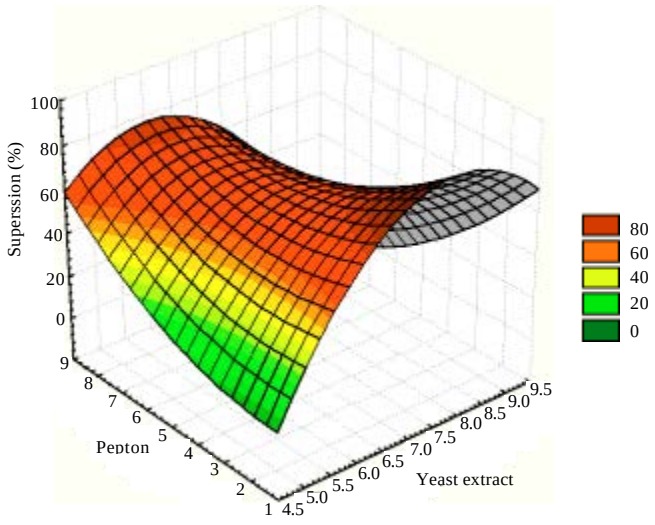


Fig. 2: Surface plot showing the interaction effect between peptone and yeast extract concentrations (g $L^{-1}$) on the suppression % of *S. aureus*

Table 5: Verification of the Plackett-Burman experimental results

| Response | Basal medium | Near optimum medium[1] | Far from optimum medium[2] |
|---|---|---|---|
| Suppression (%) | 78.39 | 86.13 | 14.84 |
| Biomass (mg $mL^{-1}$) | 1.89 | 1.53 | 0.06 |

[1]A near optimum medium formula was predicted according to the results obtained from the Plackett-Burman experiment.
[2]A far from optimum medium formula was predicted as the contrary of the near optimum medium formula

Table 6: The results of the Box-Behnken experiment applied on the production of anti-*S. aureus* ATCC 6538 by *B. licheniforms* SN2

| Concentration (g $L^{-1}$) | | | Observed response |
|---|---|---|---|
| $X_1$ (Yeast extract) | $X_2$ (Peptone) | $X_3$ (NaCl) | Suppression % of *S. aureus* |
| 9 | 8 | 3 | 30.00 |
| 9 | 2 | 3 | 85.71 |
| 5 | 8 | 3 | 53.77 |
| 5 | 2 | 3 | 31.95 |
| 9 | 5 | 5 | 21.61 |
| 9 | 5 | 1 | 11.75 |
| 5 | 5 | 5 | 38.46 |
| 5 | 5 | 1 | 45.32 |
| 7 | 8 | 5 | 74.03 |
| 7 | 8 | 1 | 49.61 |
| 7 | 2 | 5 | 66.23 |
| 7 | 2 | 1 | 74.29 |
| 7 | 5 | 3 | 93.99 |

levels (Table 6). Near optimum levels of the other factors, suggested by the Plackett- Burman experimental results were used in all trials. All cultures were performed in duplicate and the averages of the observations (Suppression % of *S. aureus*) were used. Presenting experimental results in the form of surface plots (Fig. 2-4) showed that gradual increase in peptone concentration together with high level of yeast extract (8 g $L^{-1}$) will highly promote the suppression %, while more increasing in yeast extract concentration than 8 g $L^{-1}$ will decrease suppression % (Fig. 2). The interaction between NaCl and yeast extract concentration (g $L^{-1}$) was shown in Fig. 3. where a maximum peak of suppression was

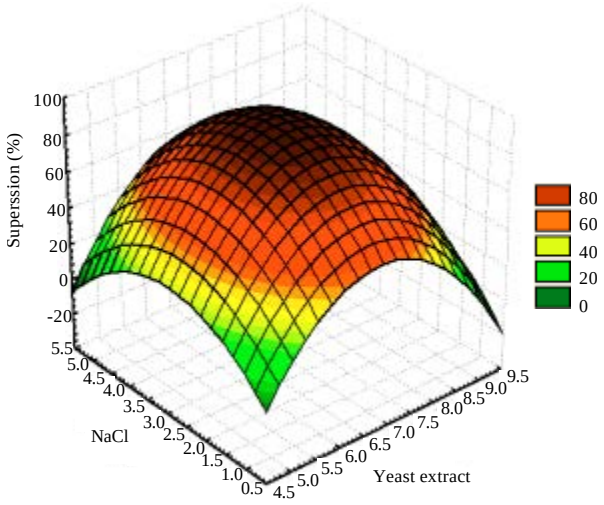


Fig. 3: Surface plot showing the interaction effect between NaCl and yeast extract concentrations (g $L^{-1}$) on the suppression % of *S. aureus*

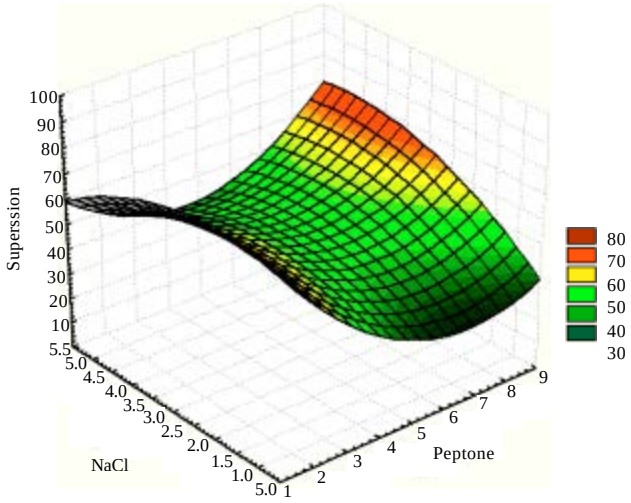


Fig. 4: Surface plot showing, the interaction effect between NaCl and peptone concentrations (g $L^{-1}$) on *S. aureus* suppression %

appeared at NaCl concentration of 3.5 g $L^{-1}$ and yeast extract concentration of 7.5 g $L^{-1}$ while the suppression % was decreased at any other concentrations of both NaCl or Yeast extract. Moreover, Fig. 4 clarifies the interaction between NaCl and peptone concentration (g $L^{-1}$), where the gradual increase in their level the highest suppression % was achieved.

For predicting the optimal point, a second order polynomial function was fitted to the experimental results of suppression % of *S. aureus* (Y):

$$Y=-518.02+152.4X_1-20.258X_2-23.89X_3-3.23X_1X_2-1.045\ X_1X_3+1.35\ X_2X_3-10.05X_1^2-0.38X_2^2-6.128X_3^2$$

The fit of the model is expressed by coefficient of determination, $R^2$, which was calculated to be 0.89. The closer the $R^2$ value to 1.00 the stronger the model is and the better it predicts response. Accordingly, our calculated $R^2$ value indicates that the model could explain 89.0% of the variability in the response. The predicted optimal concentrations of the three components as obtained from the solver function of Microsoft Excel tool were calculated to be (g $L^{-1}$): yeast extract (7.4), peptone (2) and NaCl (2.8) with a predicted suppression % of 100.

**Verification of the Model**

In order to determine the accuracy of the model and to verify the optimization results, an experiment was performed under basal and predicted optimal conditions where suppression% was monitored. pH value was adjusted at 5, culture was incubated at 35°C with incubation period of 36 h and culture volume of 65 mL medium. Under the optimized condition, 98.2% suppression was reached after 30 h, while the basal control medium reached 78.4% after 36 h. These results indicate that the optimized condition accelerated the reaction rate with suppression % of 1.3 fold increase.

Matching the predicted suppression (100%) and the observed suppression (98.2%) under optimal condition also proves the accuracy and validity of the model. According to this results it can be predicted that the optimum medium for *S. aureus* suppression% by *B. lichenformis* SN2 is (g $L^{-1}$): yeast extract,7.4; Peptone, 2; NaCl, 2.8; pH value was adjusted at 5, culture was incubated at 30°C with incubation period of 30 h and culture volume of 65 mL medium.

## DISCUSSION

Although, marine microorganisms have been increasingly of interest as a source of new bioactive molecules, a great percentage of them have not been described (Pomponi, 1999). To discover novel by-products from marine environments, maintenance of not simply abundant but diverse microorganisms is necessary (Park *et al*., 2002).

*Bacillus* species are widely distributed in nature and have remarkable ability to survive strong environmental stresses (Sneath *et al*., 1986). Moreover, members of the genus *Bacillus* can be easily isolated from aquatic habitats and marine ecosystems (Borsodi *et al*., 2007). Borsodi *et al*. (2007) isolated 40 *Bacillus* and related strains from aquatic habitats.

Bacteria belonging to the genus *Bacillus* have a long and distinguished history in the field of biotechnology (Kambourova *et al*., 2001). Since, members of this genus are used for the synthesis of a very wide range of important medical, agriculture, pharmaceutical and other industrial products (Parry *et al*., 1983). These include a variety of antibiotics, bacteriocins, enzymes, amino acids, sugars, surfactants and flavor enhancers (Ying *et al*., 2005; Bhaskar *et al*., 2007).

*Bacillus* species produce many kinds of antibiotics which share a full range of antimicrobial activity such as bacitracin, pumulin and gramicidin (Todar, 2005). Bacitracin is produced by *Bacillus licheniformis* which is a mixture of at least 5 polypeptides. This antibiotic consists of 3 separate compounds, bacitracin A, B and C.

Bacitracin A is the chief constituent. It is active against many Gram positive organisms, such as *Staphylococci*, *Streptococci*, *anaerobic cocci*, *Corynebacter* and *Clostridia*, but not against most other Gram negative organisms (Hussein and AL-Janabi, 2006).

The results showed that the antimicrobial substance was sensitive to proteinase K and trypsin. The substance showed no thermal resistance, since a 60.9% loss of the activity was observed after incubation at 100°C for 15 min.

Bacitracin is one of the most important polypeptide antibiotics; it is produced by some strains of *B. licheniformis* and *B. subtilis* and functions as an inhibitor of the cell wall biosynthesis. It is a potent antibiotic used clinically in combination with other microbial drugs (Cao and Helmann, 2002).

Haddar *et al.* (2007) studied the optimal production of bacitracin by *Bacillus licheniformis* B5 which gave 192 units $mL^{-1}$ when using 7.5% polyacrylamide gel as an immobilization material.

Lisboa *et al.* (2006) studied the characterization of a bacteriocin like substance produced by *Bacillus amyloliqueniformis* isolated from the Barazilian Atlantic forest where the antimicrobial substances was inhibitory to pathogenic and food spoilage bacteria such as *Listeria monocytogenes*, *Bacillus cereus*, *Serratia marcescens* and *Pasteurella haemolytica.*

Antony *et al.* (2009) reported that antibacterial peptides are gaining more attention as an alternative therapeutics and food and other products from spoilage and deterioration. Antibacterial peptide producing strains were isolated from sediments of slaughterhouse sewage wastes. One among them, identified as *Bacillus licheniformis* inhibited the growth of several Gram positive bacteria.

Martirani *et al.* (2002) studied the purification and partial characterization of bacillocin 490, a novel bacteriocin produced by a thermophilic strain of *Bacillus licheniformis.*

From the previous author's studies, together with the present experimental results, possibly we can categorize the nature of the antimicrobial substance produced by *B. licheniformis* as bacteriocin-like substance (Nissen-Meyer *et al.*, 1992).

Statistical experimental designs are powerful tools for searching the key factors rapidly from a multivariable system and minimizing the error in determining the effect of parameters and the results are achieved in an economical manner (Nermeen El-Sersy, 2007; Abou-Elela *et al.*, 2009).

*Bacillus licheniformis* SN2 antimicrobial production were elaborated in a two phase of optimization approach. The first was to screen for important fermentation factors that affect the production by using (Plackett-Burman design) and the second was to optimize the medium components through a three-level multi-factorial design (Box-Behnken design).

By applying The Plackett-Burman design, the expected medium was with the following composition (g $L^{-1}$): Peptone, 8; yeast extract, 7; NaCl, 3 while pH value is 5 incubated at 30°C with incubation period of 36 h and culture volume of 65 mL medium.

Antony *et al.* (2009) studied the influence of medium components and fermentation conditions on the production of bacteriocins by *Bacillus licheniformis* AnBa9.

Torkar and Matijasic (2003) reported that bacteriocins were detected in stationary-phase of bacterial cultures. Moreover, Galvez *et al.* (1993) recorded maximal antimicrobial activity of *B. licheniformis* A12 after 72 h of incubation when grown at 28°C (Pattnaik *et al.*, 2005). In our study it was noticed that high concentration of yeast extract, positively affects the suppression % (high production of bacteriocin), while incubation period of 36 h positively affect both suppression % and the growth of *B. licheniforms* SN2.

Moreover, the high level of culture volume (65 mL), will highly affects the suppression % and negatively affects the growth of the producing strain. This is can be explained as the less aeration introduced to the culture, the less gain of the dry weight. Also, present results clarify that high temperature (40°C), will promote the growth and in contrast lowering the suppression %. This is may be due to antimicrobial agents inhibition at high temperature (Tassou *et al.*, 2000). In addition, (Egorov *et al.*, 1983) also studied the effect of temperatures ranging from 30 to 55°C on the synthesis of exoprotease and bacitracin, as well as on sporification in *Bacillus licheniformis.* The synthesis of bacitracin is substantially sensitive

to the temperature variation. The maximum synthesis of the antibiotic was observed at 50°C. Temperature is also an important regulator of the growth rate of microorganisms. A shift in temperature can alter the utilization rate of one component as compared to another, thus unbalancing the medium with respect to growth (Hunt and Stieber, 1986).

A verification experiment confirmed the data obtained and predicted near-optimum condition resulted in a 1.1 fold increase in antimicrobial agent production.

Since, Plackett-Burman experimental design is based on the first order model which does not describe interactions among factors and is used to screen and evaluate the important factors that influence the response, the Box-Behnken design was applied as a complimentary step (Banik and Pandey, 2009).

Since, the Plackett-Burman design is a preliminary optimization technique, which tests only two levels of each medium component, it cannot provide the optimal quantity of each factor that affects the response. The optimal response region of the significant factors was predicted by using a second order polynomial model fitted to the results obtained by applying the Box-Behnken statistical design. In this experiment, near basal concentration of yeast extract (7.4 g $L^{-1}$), low level of peptone (2 g $L^{-1}$) and near basal concentration NaCl (2.8 g $L^{-1}$) was highly increase the suppression % to 1.3 fold.

The levels of examined independent variables predicted to attain 100% suppression % were calculated and applied in a verification experiment. The great similarity between the predicted (100% suppression) and the observed results (98.2%) proves the accuracy of the model and its application validity as previously revealed by other workers (Antony *et al*., 2009). Moreover, the optimized condition accelerated the reaction under a relatively high yeast extract concentration. The time consumed for 100% suppression was 30 h in case of optimized medium, i.e., reducing 6 h in this reaction than basal medium. This will proof that the use of this optimization strategy was powerful for achieving our goal.

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