# Optimization of antibiotic production against *Bacillus cereus* by a mutant strain of *Bacillus licheniformis* isolated from Egyptian Hamam Pharaon hot springs

Yasmin G. Kortam[a], Wafaa M. Abd El-Rahim[a], Olfat S. Barakat[b], Usama M. Segai[c], Mohamed Zakaria[b], Hassan Moawad[a]

[a]Department of Agricultural Microbiology, Agricultural and Biological Research Institute, National Research Centre, Departments of [b]Agricultural Microbiology, [c]Agricultural Botany, Faculty of Agriculture, Cairo University, Cairo, Egypt

Correspondence to Wafaa M. Abd El-Rahim, Prof. doctor, PhD., Department of Agricultural Microbiology, Agricultural and Biological Research Institute, National Research Centre, El-Behowth Street, PO Box 12311, Dokki, Cairo, Egypt Tel: +20 100 116 2641/20 122 213 8817; fax: +22 333 87758; e-mail: wafaa10m@hotmail.com, wafaa10m@gmail.com

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**Background**
The growing threat of multidrug-resistant bacteria necessitates the development of new and effective antibiotics for pathogen control. Bacteria are widely distributed in nature and have a wide applied biotechnological potential for cleaning up the environment. They have been demonstrated by their ability to produce thousands of novel bioactive metabolites with many potential applications in agriculture, medical, pharmaceutical industries, and bioremediation. The nature and yield of the secondary metabolites produced by the bacteria are greatly influenced by nutrition factors and fermentation conditions. So, the bioactive metabolites can be increased by several folds by applying optimization studies of the factors affecting the production process.
**Objective**
To optimize antibiotic production against *Bacillus cereus* by a potent bacterial strain isolated from hot springs and identification of the antibiotic compounds produced by such a bacterial strain.
**Materials and methods**
*Bacillus licheniformis* mutant (M15/Amo) obtained by ethyl methane sulfonate treatment was optimized for antibiotic production. A Plackett–Burman Design was employed to determine the influence of independent variables on the antibiotic production by the *B. licheniformis* mutant strain. Response surface methodology was further used by applying a Box–Behnken Design to optimize the more effective variables. Supernatants were extracted using ethyl acetate as a solvent. The crude compounds were assayed to confirm the presence of antibacterial bioactive metabolites. The analysis of the target antimicrobial metabolites was performed using liquid chromatography–electrospray ionization–tandem mass spectrometry.
**Results and conclusion**
The study is a trail to find microorganisms capable of producing antibiotics against *B. cereus* and optimize the conditions of antibiotic production by the isolated bacteria from hot springs. The highest antibiotic production was achieved in the presence of glucose and peptone as optimum carbon and nitrogen sources, respectively. The optimization of medium composition and fermentation conditions to produce antibiotics by the *B. licheniformis* mutant strain was studied. The effect of seven factors on antibiotic production was investigated. Optimization of antibiotic production by the *B. licheniformis* mutant strain using statistical modeling revealed that the mutant (M15/Amo) gave the highest antibiotic production using 3% glucose, 2.5% peptone, incubation time of 60 h at pH 7.5. The analysis of the extracts using liquid chromatography–electrospray ionization–tandem mass spectrometry showed the presence of seven compounds that have antipathogenic activity.

**Keywords:**
antibiotic production, antibiotic-resistant bacteria, *B*acillus *licheniformis*, Box–Behnken Design, liquid chromatography, response surface methodology, thermophiles

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## Introduction

Thermophiles and haloalkaliphiles have high economic value, as their products are used in various industrial biotechnological applications [1,2]. Metabolites such as extracellular enzymes, osmotically active

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compounds, exopolysaccharides, antibiotics, and specific lipids are all economically viable products [3,4]. The extreme environmental conditions, whether physical (high temperature and pH) or chemical (salinity, alkalinity, and industrial pollutants), cause deep changes in environmental biological systems [5–8]. Extremophiles have been identified as promising sources of novel bioproducts, including antimicrobials [9,10]. The search for novel microbial products under harsh environmental conditions has sparked the interest of the scientific community in recent years [11].

Phylogenetic studies of antibiotic natural resistance genes suggest the long-term prevalence of genes encoding resistance to various types of antibiotics in nature well known before the antibiotic era [12]. The phylogeny of the beta-lactamase and housekeeping genes in *Klebsiella oxytoca* is extremely consistent, showing that these genes have been evolved in this host for over a million years [13]. The phylogenetic analysis of β-lactamases in metagenomic clones obtained from 10 000-year-old 'cold-seep' sediments revealed that many of these enzymes are the consequence of ancient evolution, not current evolution [14].

Antibiotics now utilized in human medicine are mainly natural secretions of bacteria or fungi found in the environment. Streptomyces isolated from soil samples is the source of the bulk of antibiotics currently in use [15]. Microorganisms defend themselves in their natural environment by creating antimicrobial compounds and developing resistance mechanisms to other antibiotics to avoid self-toxicity [16].

Multidrug-resistant bacterial species as well as resistance genes to currently used antibiotics were discovered. Several million years have passed since the blaOXA genes that code for beta-lactamases were discovered [17]. Jardine *et al.* [18] discovered surface from soil samples in 30 000-year-old permafrost samples from Polar Regions. Cave *et al.* [19] discovered many resistance genes in the genome of a permafrost-isolated *Staphylococcus hominis*. In vitro, 65% of the 93 strains cultivated by Bhullar *et al.* from the four-million-year-old Lechuguilla Cave (New Mexico) were resistant to three or four antibiotic classes [20]. Resistance genes to lactams and glycopeptides were also discovered in the gut microbiota of the 5300-year-old mummy [21]. In the antibiotic-free Mackay Glacier region, 177 antimicrobial resistance genes belonging to 23 families (representing all modes of resistance, i.e., mutation, efflux, and antibiotic inactivation) were discovered recently [22]. Studies suggest that resistance genes can be transferred from the producers in the soil to human pathogenic organisms [23]. Under selection pressure owing to heavy human antibiotic use, horizontal gene transfer can take place from the resistome to clinical strains [24].

The aim of this study was to find microorganisms capable of producing antibiotics against *Bacillus cereus* and optimize the conditions of antibiotic production by the isolated bacteria from hot springs.

## Materials and methods

Optimization of nutritional factors for antibiotic production by mutagenic strains *Bacillus licheniformis* using the one-variable-at-a-time approach

A mutant strain of *Bacillus licheniformis* (M15/Amo) was obtained by 200 mM of ethyl methane sulfonate (EMS). The carbon and nitrogen sources were applied using a one-variable-at-a-time approach to optimize the major key factors affecting the microbial growth and active component production while keeping the other factors constant.

### Carbon sources

Nine carbon sources were used (10 g/l): monosaccharides such as glucose, fructose, and galactose; disaccharides such as maltose, sucrose, lactose, and trehalose; and polysaccharides such as soluble starch and dextrin.

### Nitrogen sources

Seven inorganic nitrogen sources were used (10 g/l): ammonium chloride, ammonium citrate, ammonium nitrate, ammonium sulfate, ammonium phosphate, potassium nitrate and sodium nitrate, and four organic nitrogen sources (yeast extract, meat extract, peptone, and tryptone).

### Statistical optimization of antimicrobial compounds produced by the mutant *Bacillus licheniformis* strain using response surface methodology design

Carbon and nitrogen sources ranging from 1 to 3.0 % for carbon and 0.5–2.5 % for nitrogen were used to study their effects on antibiotic production for the tested strain. The effects of incubation time (24–96 h), pH (6–8), temperature (45–65°C), and agitation (100–140 rpm) were also studied.

### Screening of the most significant parameters of antibiotic production by the bacteria using Plackett–Burman design

The Plackett–Burman design was used to screen the most significant nutritional and physical factors affecting antibiotic production by a selected bacterial strain of *B. licheniformis*. The experimental design and subsequent analysis of the experimental data were carried out using Minitab 15 (Minitab Inc., State College, Pennsylvania, USA).

In this experimental design, a total of seven ($n$) variables, including two nutritional (carbon source and nitrogen source concentrations) and five physical factors (pH, incubation period, temperature, agitation speed, and Mg concentration) were studied in 12 ($n$+1) experiments, as shown in Table 1. The average observation was used as the response of the design, and each variable was represented by two levels: high (+) and low (–). Table 1 shows the variables, their symbol codes, and the actual low and high levels of the variables. Table 2 shows the details of the design. Each row represents a trial run, and each column represents an independent variable. The experiment was carried out in triplicate, and the average antimicrobial activity against *B. cereus* was taken as the response. The variables' confidence levels above 95% were considered to have a significant effect on antibiotic production and thus used for further optimization.

**Table 1 Low and high levels of each variable used in Plackett–Burman Design**

| | | Levels | |
|---|---|---|---|
| Symbols | Factors | (low) –1 | (high) +1 |
| A | Carbon source conc. (%) | 1 | 2 |
| B | Nitrogen source conc. (%) | 0.5 | 1.5 |
| C | Incubation period (h) | 24 | 48 |
| D | pH | 6 | 7 |
| E | Temperature (°C) | 45 | 55 |
| F | Agitation speed (rpm) | 100 | 140 |
| G | Mg conc. (%) | 0.015 | 0.025 |

**Table 2 Plackett–Burman Design experimental matrix for selection of the significant variables of antibiotic production by *Bacillus licheniformis* mutant**

| | Variables | | | | | | |
|---|---|---|---|---|---|---|---|
| Run no. | A | B | C | D | E | F | G |
| 1 | 1 | 1 | –1 | 1 | –1 | –1 | –1 |
| 2 | –1 | 1 | –1 | –1 | –1 | 1 | 1 |
| 3 | 1 | –1 | –1 | –1 | 1 | 1 | 1 |
| 4 | –1 | –1 | 1 | 1 | 1 | –1 | 1 |
| 5 | 1 | –1 | 1 | –1 | –1 | –1 | 1 |
| 6 | –1 | 1 | 1 | –1 | 1 | –1 | –1 |
| 7 | –1 | 1 | 1 | 1 | –1 | 1 | 1 |
| 8 | –1 | –1 | –1 | –1 | –1 | –1 | –1 |
| 9 | 1 | 1 | 1 | –1 | 1 | 1 | –1 |
| 10 | –1 | –1 | –1 | 1 | 1 | 1 | –1 |
| 11 | 1 | –1 | 1 | 1 | –1 | 1 | –1 |
| 12 | 1 | 1 | –1 | 1 | 1 | –1 | 1 |

### Maximization of antibiotic production by the *Bacillus licheniformis* mutant using response surface methodology

Response surface methodology was used with the Box–Behnken Design to optimize the selected major variables. The four selected independent variables were carbon source concentration, nitrogen source concentration, incubation period, and pH. The variables were tested at 3 different levels: –1, 0, and +1 (Table 3). The experiment was carried out in 27

**Table 3 Low, intermediate, and high levels of each variable used in Box–Behnken Design to optimize antibiotics production by *Bacillus licheniformis* mutant against *Bacillus cereus***

| | | Levels | | |
|---|---|---|---|---|
| Symbol | Factors | –1 | 0 | 1 |
| A | Carbon source conc. (%) | 2 | 2.5 | 3 |
| B | Nitrogen source conc. (%) | 1.5 | 2 | 2.5 |
| C | Incubation period | 48 | 60 | 72 |
| D | pH | 7 | 7.5 | 8 |

**Table 4 Box–Behnken Design experimental matrix for maximization of antibiotics production by the *Bacillus licheniformis* mutant against *Bacillus cereus***

| | Variables | | | |
|---|---|---|---|---|
| Run no. | A | B | C | D |
| 1 | –1 | –1 | 0 | 0 |
| 2 | 0 | 0 | –1 | 1 |
| 3 | 0 | 1 | 1 | 0 |
| 4 | 0 | 1 | –1 | 0 |
| 5 | –1 | 0 | 1 | 0 |
| 6 | 1 | 0 | 1 | 0 |
| 7 | 0 | 1 | 0 | 1 |
| 8 | –1 | 0 | 0 | 1 |
| 9 | –1 | 0 | 0 | –1 |
| 10 | 0 | 0 | 0 | 0 |
| 11 | 0 | 0 | –1 | –1 |
| 12 | 0 | 1 | 0 | –1 |
| 13 | 1 | 0 | 0 | –1 |
| 14 | 0 | –1 | 0 | –1 |
| 15 | 0 | –1 | 0 | 1 |
| 16 | 1 | 0 | 0 | 1 |
| 17 | 0 | 0 | 1 | –1 |
| 18 | –1 | 0 | –1 | 0 |
| 19 | 0 | 0 | 0 | 0 |
| 20 | 1 | 1 | 0 | 0 |
| 21 | 0 | 0 | 0 | 0 |
| 22 | –1 | 1 | 0 | 0 |
| 23 | 0 | –1 | –1 | 0 |
| 24 | 1 | –1 | 0 | 0 |
| 25 | 1 | 0 | –1 | 0 |
| 26 | 0 | –1 | 1 | 0 |
| 27 | 0 | 0 | 1 | 1 |

trials (Table 4) with three replicates at the center point. All variables were taken at a central coded value of zero. The statistical software package Design-Expert Software (Version 12; Stat-Ease Inc., Minneapolis, Minnesota, USA) was used to analyze the experimental data. The supernatant crude extract was collected to check the activity of antibiotic production by measuring the diameter of the inhibition zone against *B. cereus*.

#### Experimental validation

*The B. licheniformis* mutant strain was cultured in nonoptimized and optimized production media, which yielded the maximum response for experimental validation. The clear supernatants of the fermented broths were assayed for antibacterial activity against test pathogenic bacteria.

#### Identification of antibiotic compounds

##### *Extraction of bioactive compound (antibiotics)*

An aliquot of 100 µl of *B. licheniformis* culture was inoculated into three flasks containing 300 ml of nutrient broth. The culture was incubated at 55°C with shaking at 100 rpm for 3 days. After 3 days, the supernatants were separated by centrifugation at 8000 rpm at 4°C for 15 min. Supernatants were stored for antibiotic extraction. Supernatants were extracted by each of the following organic solvents: ethyl acetate, acetone, methanol, and chloroform (1 : 1 v/v). The mixture of supernatant and each solvent was shaken for one hour and kept stationary for 15 min to separate the organic phase from the water phase. The organic phases of solvent extracts were evaporated in a vacuum and stored at −20°C until use. The crude compounds were processed by the agar disc diffusion method to confirm the presence of antibacterial bioactive metabolites [25,26].

##### *Identification of bioactive crude antibiotic compounds using liquid chromatography–electrospray ionization–tandem mass spectrometry*

The analysis of the target antimicrobial agent was performed using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) with an ExionLC AC system for separation and SCIEX Triple Quad 5500+ MS/MS system equipped with an ESI for detection in negative ion mode. The separation was performed with Ascentis C18 Column (4.6×150 mm, 3 µm). The mobile phases consisted of two eluents: (A) 5 mM ammonium formate (pH 8, adjusted by NaOH), and (B) acetonitrile (LC grade). The mobile phase gradient was programmed as follows: 10% B at 0–1 min, 10–90% B from 1 to 33 min, 90% B from 33 to 37 min, 10% at 37.1, and 10% from 37.1 to 40 min. The flow rate was 0.7 ml/min, and the injection volume was 10 µl. For MS/MS analysis, negative ionization mode was applied with a scan (EMS-IDA-EPI) from 100 to 1000 D for MS1 with the following parameters: curtain gas: 25 psi; ion spray voltage: −4500; source temperature: 500°C; ion source gases 1 and 2 were 45 psi and from 50 to 800 D for MS2 with a declustering potential: −80; collision energy: −35; and collision energy spread: 15. Compounds' identification was performed using MS-DIAL Software Version 4.70. The detailed procedure is described by Cherfia *et al.* [27] and Radwan *et al.* [28].

## Results and discussions

#### Optimization of nutritional factors for production of antibiotics by the *Bacillus licheniformis* mutant strain against *Bacillus cereus* using one-variable-at-a-time approach

The effects of carbon and nitrogen sources on antibiotic production by mutant strains were studied using the one-variable-at-a-time technique, which involves changing one independent variable while keeping the other factors constant.

#### Effect of carbon sources on antibiotic production

Nine carbon sources were assessed in relation to antibiotic production by the potent mutant strain of *B. licheniformis*. Among the nine tested carbon sources, the highest antibiotic production was achieved in the presence of glucose, where the inhibition zone against *B. cereus* reached 34 mm, followed by fructose with a diameter zone of 26 mm, whereas the lowest antimicrobial activity was recorded in the presence of lactose, dextrin, and maltose as carbon sources in the media, where the inhibition zone did not exceed 10 mm (Table 5). Similar effects of the same sugars on antibiotic production were reported by other authors [29–31].

#### Effect of nitrogen sources on antibiotic production

Eleven nitrogen sources were tested in relation to antibiotic production by the mutant strain. The inhibition zones against the test pathogenic bacteria (*B. cereus*) were greater in the presence of organic nitrogen rather than inorganic sources in the media. The highest antibiotic production was obtained when peptone was used in the media, followed by tryptone, meat extract, and glutamic acid. The other nitrogen sources did not enhance the antibiotic production by the *B. licheniformis* mutant, as the inhibition zones were much smaller and ranged between 7 and 12 mm (Table 5). The enhancement of antibiotic production by organic nitrogen sources was reported by Popov *et al.*

**Table 5 Effect of different carbon and nitrogen sources on antibiotic production by *Bacillus licheniformis* mutant against *Bacillus cereus***

| C and N sources | Inhibition zone diameter (mm) |
|---|---|
| Carbon sources | – |
| Glucose | 34 |
| Fructose | 26 |
| Soluble starch | 21 |
| Galactose | 16 |
| Sucrose | 16 |
| Trehalose | 13 |
| Maltose | 8 |
| Dextrin | 7 |
| Lactose | 7 |
| Nitrogen sources | – |
| Peptone | 37 |
| Tryptone | 31 |
| Meat extract | 25 |
| Glutamic acid | 21 |
| Ammonium phosphate | 12 |
| Ammonium chloride | 10 |
| Ammonium nitrate | 10 |
| Ammonium sulfate | 9 |
| Yeast extract | 9 |
| Ammonium citrate | 8 |
| Sodium nitrate | 8 |
| Potassium nitrate | 7 |

[30] and Goswami *et al*. [31]. The high production of antibiotics in media containing organic nitrogen sources could be due to the organic sources not only acting as nitrogen sources but also as an additional source for growth factors enhancing antibiotic secretion against the test pathogenic bacteria [32–34].

### Screening of the most significant antibiotic production parameters using Plackett–Burman design

A Plackett–Burman screening experimental design was employed to determine the influence of independent variables on the antibiotic production by the potent *B. licheniformis* mutant strain. A set of 12 experiments were conducted to study the effects of seven factors, that is, glucose concentrations, peptone concentrations, temperature, pH, incubation period, agitation speed, and magnesium concentration, on antibiotic production against *B. cereus*. Table 6 shows the response of antibiotic production to seven values. The results show wide variations in antibiotic production by the *B. licheniformis* mutant, depending on growth media composition. The maximum antibiotic production was achieved in run number 12 (41 mm diameter), where all factors were at a high level (+1) except the incubation period and agitation speed, which were at a low level (-1). Run number 8 was the lowest (6 mm diameter) in antibiotic production by the

**Table 6 Plackett–Burman Design using seven independent factors-two levels (high and low) factorial showing actual values for antibiotic production by *Bacillus licheniformis* mutant against *Bacillus cereus***

| | Variables | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Run no. | A | B | C | D | E | F | G | Inhibition zones (mm) |
| 1 | 1 | 1 | –1 | 1 | –1 | –1 | –1 | 24 |
| 2 | –1 | 1 | –1 | –1 | –1 | 1 | 1 | 7 |
| 3 | 1 | –1 | –1 | –1 | 1 | 1 | 1 | 18 |
| 4 | –1 | –1 | 1 | 1 | 1 | –1 | 1 | 17 |
| 5 | 1 | –1 | 1 | –1 | –1 | –1 | 1 | 11 |
| 6 | –1 | 1 | 1 | –1 | 1 | –1 | –1 | 11 |
| 7 | –1 | 1 | 1 | 1 | –1 | 1 | 1 | 15 |
| 8 | –1 | –1 | –1 | –1 | –1 | –1 | –1 | 6 |
| 9 | 1 | 1 | 1 | –1 | 1 | 1 | –1 | 21 |
| 10 | –1 | –1 | –1 | 1 | 1 | 1 | –1 | 17 |
| 11 | 1 | –1 | 1 | 1 | –1 | 1 | –1 | 21 |
| 12 | 1 | 1 | –1 | 1 | 1 | –1 | 1 | 41 |

A, carbon source conc. (%); B, nitrogen source conc. (%); c, incubation period (h); D, pH; E, temperature (°C); F, agitation speed (rpm); G, Mg conc.

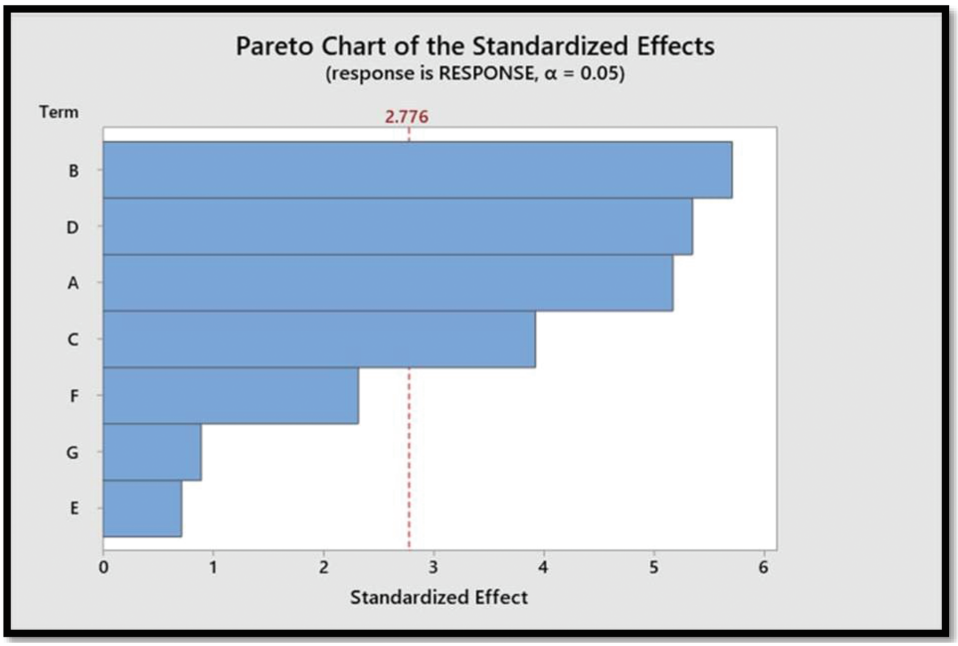
**Table 7 Statistical analysis of variance of Plackett–Burman Design for different variables affecting production of antibiotic by mutant strain of *Bacillus licheniformis* against *Bacillus cereus***

| Variables | *F* value | *P* value |
|---|---|---|
| Model | 15.69 | 0.009 |
| A-Carbon source conc. (%) | 26.70 | 0.007 |
| B-Nitrogen source conc. (%) | 32.51 | 0.005 |
| C-Incubation period (h) | 15.37 | 0.017 |
| D-pH | 28.57 | 0.006 |
| E-Temperature (oC) | 0.51 | 0.515 |
| F-Agitation speed (rpm) | 5.37 | 0.081 |
| G-Mg conc. (%) | 0.79 | 0.423 |
| $R^2$=96.49%, $R^2$ (pred)=97.33% | | |

*B. licheniformis* mutant. This run had the lowest level of all the tested media variables. Table 7 shows that carbon source, nitrogen source, incubation period, and pH are the main factors significantly affecting the antibiotic production by *B. licheniformis* against *B. cereus*.

According to the analysis of variance analysis of the Plackett–Burman Design presented in Table 7, the significance of each factor, *P* values, *F* values, standard division, and $R^2$=determination coefficient of the main effect of each variable were determined. The high *F* value and low *P* value ($P<0.05$) and variables having a *P* value less than 0.05 were considered significant. The results show that four variables [carbon source conc. (A), nitrogen source conc. (B), incubation period (C), and pH (D)] had an apparent effect on antibiotic production by the potent *B. licheniformis* mutant. As shown in Fig. 1, confidence levels of these four

**Figure 1**




Pareto chart of the most significant factors affecting the production of antibiotics by *Bacillus licheniformis* mutant. (a) Carbon source conc. (%), (b) nitrogen source conc. (%), (c) incubation period (h), (d) pH, (e) temperature (°C), (f) agitation speed (rpm), and (g) Mg concentration (%).

variables were higher than 95%, which indicates their significant effect compared with those of other variables. The same was confirmed from the Pareto graph (Fig. 1), in which higher effects were presented in the upper portion and then progressed down to the lower effect. It shows that the most important factors influencing antibiotic production by *B. licheniformis* mutant were carbon source concentration, nitrogen source concentration, incubation period, and pH. The determination coefficient ($R^2$), which had values ranging from 0.98 to 1.00, indicates that all factors, levels, responses, and correlations between the experimental and predicted values are strongly correlated to each other (Tables 9 and 10). Statistical optimization of antibiotic production by the Plackett–Burman Design was reported by several authors [35–37].

### Enhancing of antibiotic production by the *Bacillus licheniformis* mutant strain using response surface methodology

Based on the results of the Plackett–Burman Design, the four significant variables influencing antibiotic production by the mutant strain were optimized using Box–Behnken Design, and the obtained results were analyzed by ANOVA (Tables 8 and 9). The Box–Behnken matrix of the *B. licheniformis* mutant comprised a total of 27 experiments with combinations of glucose concentrations (A), peptone concentrations (B), incubation period (C), and pH (D). Previous factors were at three levels and coded as −1, 0, and +1 (0 as normal level of factors, −1 as low level of factors, and +1 as high level of factors) and were demonstrated along with actual responses in Tables 8 and 9.

The minimum antibiotic production for all response values was in run 9, where the carbon source concentration and pH were at the low level (Table 8).

The model *F* values of 1171.5 and 2036.78 for antibiotic production by *B. licheniformis*, shown in Tables 8 and 9 and Fig. 2, implied that the model was strongly significant. The *P* value was also less than 0.0001, revealing that the model was highly significant.

The results show that the modification of media composition in run 20, where the glucose and nitrogen sources were at a high level, developed the highest amount of antibiotic production as compared with the basic media (Table 8). Enhancing microbial antibiotic production using response surface methodology was successfully applied in previous studies [38–41].

The response surface curves of antibiotic production by mutant strain *B. licheniformis* are plotted to explore the effects of changing the factor levels on the response and to determine the optimum level of each variable to reach the maximum amount of antibiotic production

**Table 8 The Box–Behnken Design experimental matrix for four independent factors of different concentrations effect for antimicrobial production by *Bacillus licheniformis* mutant against *Bacillus cereus***

| | Variables | | | | |
|---|---|---|---|---|---|
| Run no. | A | B | C | D | Inhibition zone (mm) |
| 1 | –1 | –1 | 0 | 0 | 20 |
| 2 | 0 | 0 | –1 | 1 | 20 |
| 3 | 0 | 1 | 1 | 0 | 34 |
| 4 | 0 | 1 | –1 | 0 | 24 |
| 5 | –1 | 0 | 1 | 0 | 19 |
| 6 | 1 | 0 | 1 | 0 | 32 |
| 7 | 0 | 1 | 0 | 1 | 32 |
| 8 | –1 | 0 | 0 | 1 | 20 |
| 9 | –1 | 0 | 0 | –1 | 14 |
| 10 | 0 | 0 | 0 | 0 | 24 |
| 11 | 0 | 0 | –1 | –1 | 18 |
| 12 | 0 | 1 | 0 | –1 | 26 |
| 13 | 1 | 0 | 0 | –1 | 27 |
| 14 | 0 | –1 | 0 | –1 | 18 |
| 15 | 0 | –1 | 0 | 1 | 21 |
| 16 | 1 | 0 | 0 | 1 | 30 |
| 17 | 0 | 0 | 1 | –1 | 21 |
| 18 | –1 | 0 | –1 | 0 | 15 |
| 19* | 0 | 0 | 0 | 0 | 24 |
| 20 | 1 | 1 | 0 | 0 | 47 |
| 21 | 0 | 0 | 0 | 0 | 24 |
| 22 | –1 | 1 | 0 | 0 | 19 |
| 23 | 0 | –1 | –1 | 0 | 19 |
| 24 | 1 | –1 | 0 | 0 | 21 |
| 25 | 1 | 0 | –1 | 0 | 25 |
| 26 | 0 | –1 | 1 | 0 | 21 |
| 27 | 0 | 0 | 1 | 1 | 28 |

| | | Levels | | |
|---|---|---|---|---|
| Symbol | Factors | –1 | 0 | 1 |
| A | Carbon source conc. (%) | 2 | 2.5 | 3 |
| B | Nitrogen source conc. (%) | 1.5 | 2 | 2.5 |
| C | Incubation period (h) | 48 | 60 | 72 |
| D | pH | 7 | 7.5 | 8 |

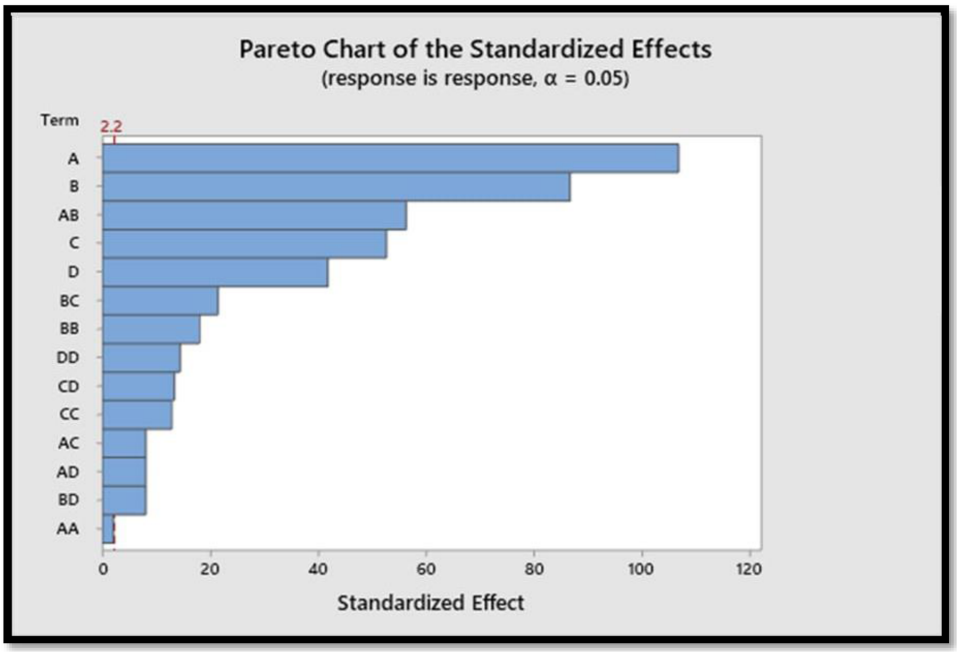
*Basic medium.

**Table 9 Statistical analysis of variance of Box–Behnken Design for different variables affecting the production of antibiotics using *Bacillus licheniformis* mutant**

| Variables | *F* value | *P* value |
|---|---|---|
| Model | 2036.78 | 0.000 |
| A-Carbon source conc. (%) | 11426.4 | 0.000 |
| B-Nitrogen source conc. (%) | 7526.4 | 0.000 |
| C-Incubation period (h) | 2774.4 | 0.000 |
| D-pH | 1749.6 | 0.000 |
| A×A | 4.27 | 0.061 |
| B×B | 326.67 | 0.000 |
| C×C | 166.67 | 0.000 |
| D×D | 209.07 | 0.000 |
| A×B | 3175.2 | 0.000 |
| A×C | 64.8 | 0.000 |
| A×D | 64.8 | 0.000 |
| B×C | 460.8 | 0.000 |
| B×D | 64.8 | 0.000 |
| C×D | 180 | 0.000 |
| $R^2$=99.96%, $R^2$ (pred)=99.76% | | |

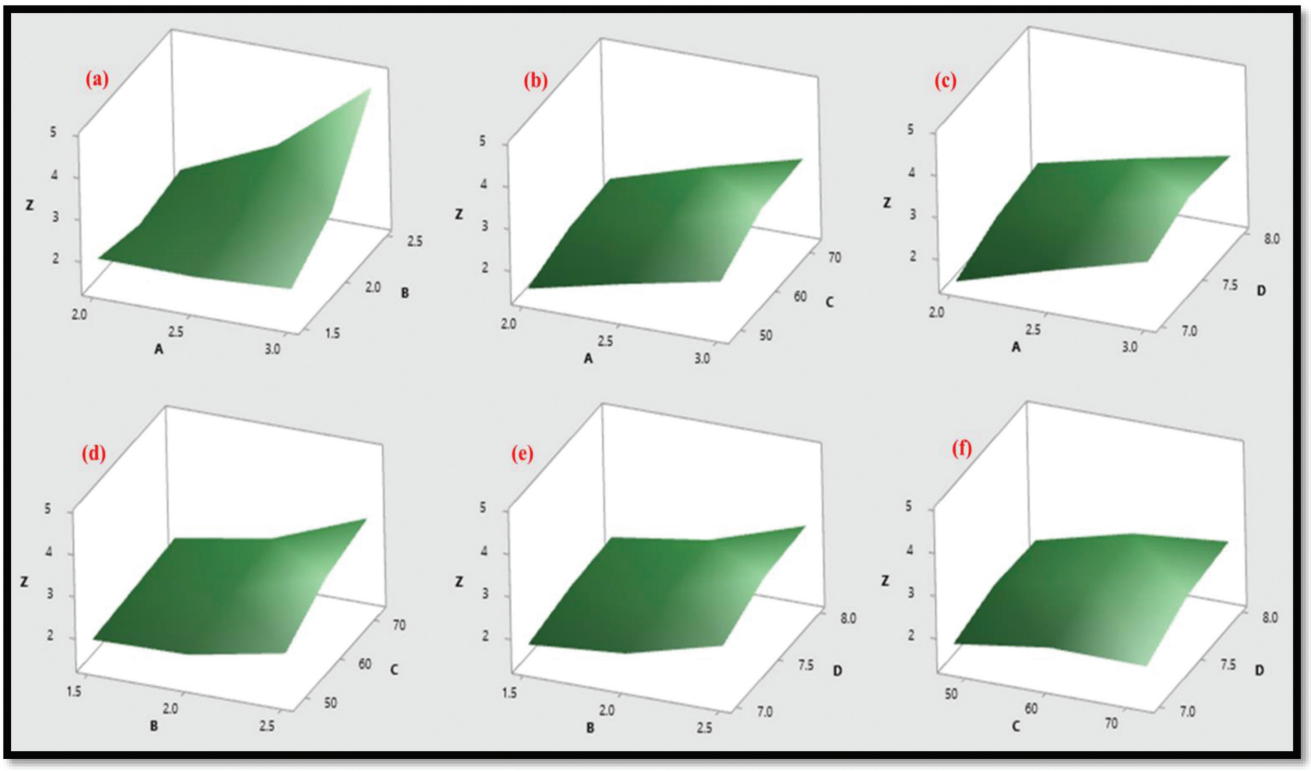
against *B. cereus*. As shown in Fig. 3, three-dimensional surface plots and contour plots were constructed. The three-dimensional surface plots show the effects and interaction of two independent variables on the response while the other independent variable remained unchanged. As shown in Fig. 3a, increasing the concentration of carbon sources increased antibiotic production. The increase in the concentration of both carbon and nitrogen sources leads to a significant increase in the production of antibiotics. In addition, the increase in the concentration of the carbon source with an increase in both the incubation period and the pH produced almost the same increase in antibiotic production (Fig. 3b and c). This may be due to the importance of increasing the concentration of carbon and nitrogen sources in the bacterial growth media to stimulate the production of antibiotics. Besides that, increasing the concentration of carbon sources, the length of the incubation period, and pH enhances the production of antibiotics. Figure 3d shows that the level of antibiotic production was increased in the presence of higher nitrogen sources with longer incubation periods. The increase of nitrogen sources with increasing pH also resulted in more antibiotic production (Fig. 3e). Meanwhile, increasing the incubation period with increasing pH values led to a slight increase and then a slight decrease in the production of antibiotics (Fig. 3f). The data in Fig. 3 show that increasing the level of both carbon and nitrogen sources increased the production of the antibiotics at a slower rate at the beginning and at a rapidly increasing rate toward the end. As the incubation period and pH increased, the antibiotic production obviously increased, especially at the optimum levels. An extended increase in the incubation period and pH negatively affects the production of antibiotics. This may be due to the higher growth rates of the bacterial cells and the accumulation of the secondary metabolite in the culture media, which leads to subsequent inhibition of antibiotic production. Figure 3 clearly shows that higher antibiotic production levels were observed after induction at higher values of both carbon and nitrogen sources. The higher values of both the incubation period and pH at the induction point slightly

**Figure 2**




Pareto chart of significant factors and their interaction after maximizing antibiotics production by *Bacillus licheniformis* mutant: (a) carbon source conc. (%), (b) nitrogen source conc. (%), (c) incubation period (h), and (d) pH.

**Figure 3**




Three-dimensional response surface plots showing the effect of four significant variables and their interacted effects on antibiotic production by *Bacillus licheniformis* mutant: (a) carbon source conc. (%), (b) nitrogen source conc. (%), (c) incubation periods (h), (d) pH, and Z-Diameter of inhibition zone (cm).

decreased antibiotic production. Considering all the responses, it is evident that the carbon and nitrogen sources had a significant effect on antibiotic production. The results agree well with the data presented in Table 9. The same was confirmed from the Pareto graph (Fig. 2) in which higher effects were presented in the upper portion and then progressed down to the lower effect. These results are in line with the results obtained by other researchers [42–44].

### Experimental validation

The inhibition zone formed by the *B. licheniformis* parent strain against *B. cereus* was 23 mm. The mutation of these strain by EMS technique improved the antagonism and the inhibition zone was increased by 56.5% over the parent strain. The statistical optimization by response surface methodology further increased antagonism by 104% of the parent strain (Table 10 and Fig. 4). Similar approach was applied to enhance production of bioactive compounds [45,46].

### Identification of antibiotic compounds

#### *Antimicrobial activity of Bacillus licheniformis mutant extracts*

Four different polar and nonpolar solvents (ethyl acetate, methanol, acetone, and chloroform) were used for the extraction of antibiotic compounds from bacterial supernatant. Only the ethyl acetate extract showed antibiotic activity against eight tested pathogens, whereas acetone, methanol and chloroform extracts did not show any activity. The antimicrobial activity of extracts is presented in Table 11. Table 11 shows that the highest antimicrobial activity was against *B. cereus*. In addition, the data show that the ethyl acetate crude extract inhibited all of the pathogenic bacteria tested, as measured by the diameter of the inhibition zone, which ranged from 29 to 41 mm. In the negative control (Table 11), absolute ethanol (98%), used to dilute the extract, did not exhibit any antimicrobial activity against all test microorganisms. Ethanol is a volatile organic alcohol (flash point, 13°C), so it rapidly evaporates after being dropped on a paper disc. Therefore, it is clear that the inhibition of test microorganisms was caused by the bioactive compounds contained in the extract, not the solvent. In positive control, chloramphenicol inhibited the growth of test organisms at different degrees. Similar effects were reported in previous publications [47–49].

**Table 10 Enhancing antibiotic production by *Bacillus licheniformis* against *Bacillus cereus* using ethyl methane sulfonate mutagenesis followed by statistical optimization**

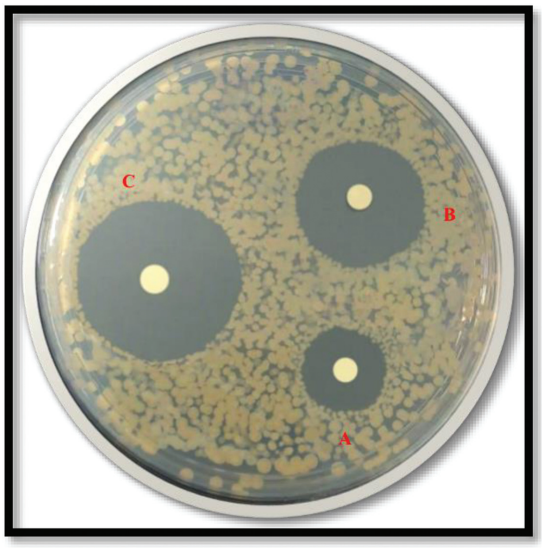
| *Bacillus Licheniformis* | Inhibition zone (mm) | Increase over inhibition by parent strain (%) |
|---|---|---|
| Parent strain | 23 | Base line |
| After EMS mutagenesis | 36 | 56.5 |
| After optimization | 47 | 104 |

EMS, ethyl methane sulfonate.

#### *Profiling of antimicrobial compound produced by Bacillus licheniformis mutant using liquid chromatography–tandem mass spectrometry*

One of the common methods for natural product analysis entails the extraction of the natural products from the culture broth, and then the extract is subjected to LC-MS/MS analysis. The LC-ESI-MS/MS

**Figure 4**




Inhibition zones of antibiotic production by *Bacillus licheniformis* against *Bacillus cereus* using EMS mutagenesis followed by statistical optimization. (a) Parent strain, (b) mutant obtained by EMS mutagenesis, (c) optimized mutant strain using response surface methodology. EMS, ethyl methane sulfonate.

**Table 11 Antimicrobial activity (inhibition zone in mm) of ethyl acetate extracts of *Bacillus licheniformis* mutant**

| Test pathogenic bacteria | Absolute ethanol | Chloramphenicol | Ethyl acetate extract |
|---|---|---|---|
| *Bacillus cereus* | – | 40 | 41 |
| *Bacillus subtilis* | – | 45 | 33 |
| *Escherichia coli* | – | 36 | 30 |
| *Salmonella typhi* | – | 47 | 30 |
| *Klebsiella sp.* | – | 35 | 38 |
| *Staphylococcus aureus* | – | 30 | 35 |
| *Micrococcus lateues* | – | 21 | 29 |
| *Pseudomonas aeruginosa* | – | 37 | 35 |

**Table 12 Tentative identification of secondary metabolites in the ethyl acetate extract of mutant strain *Bacillus lichniformis* via negative liquid chromatography–electrospray ionization–tandem mass spectrometry**

| No. | $R_t$ (min) | *m/z** [M-H]$^-$ | M. wt. | M. F. | Tentatively identified antimicrobial compounds | Chemical class |
|---|---|---|---|---|---|---|
| 1 | 8.54 | 144.95 | 146.19 gmol$^{-1}$ | $C_6H_{14}N_2O_2$ | L-lysine | α-amino acid |
| 2 | 12.36 | 133 | 134.08 gmol$^{-1}$ | $C_4H_6O_5$ | Malic acid | Betahydroxy acids and derivatives |
| 3 | 21.54 | 311.4 | 304.47 gmol$^{-1}$ | $C_{20}H_{32}O_2$ | Arachidic acid | Saturated fatty acid |
| 4 | 24.70 | 177 | 178.14 gmol$^{-1}$ | $C_9H_6O_4$ | 6,7-Dihydroxycoumarin | Organic compounds |
| 5 | 26.53 | 340.3 | 341.42 gmol$^{-1}$ | $C_{15}H_{23}N_3O_4S$ | Levosulpiride | Benzamide |
| 6 | 31.41 | 217.95 | 219.62 gmol$^{-1}$ | $C_7H_{10}ClN_3O_3$ | Ornidazole | Nitroimidazoles |
| 7 | 33.90 | 228 | 229.62 gmol$^{-1}$ | $C_6H_8ClN_7O$ | Amiloride | Pyrazines |

*m/z: mass to charge.

**Table 13 Mode of action of chemical compounds performing antibiotic compounds function**

| Antimicrobial compounds* | Mode of action | Antibiotic activity | References |
|---|---|---|---|
| Amiloride | Broad anti-motility activity | Antibacterial | [51,52] |
| Levosulpiride | Inhibiting the initiation of bacterial protein synthesis | Antibacterial | [53,54] |
| L-lysine | Increase proton motive force via transmembrane chemical gradient, resulting in aminoglycoside acumination | Antibacterial | [55,56] |
| 6,7-Dihydroxycoumarin | Damaging the cell membrane | Antibacterial, antifungal, anticoagulant, antioxidant, anticancer, and anti-inflammatory properties | [57,58] |
| Arachidic acid | Lysis of bacterial cell membranes Modification of some macromolecules, eliciting toxicity | Antibacterial | [59,60] |
| Malic acid | Lowering the pH of the bacterial cell | Inhibitory effect on yeasts, molds and bacteria | [61,62] |
| Ornidazole | Production of toxic derivatives and radicals Reduction of the nitro group | Antibacterial Antiprotozoal | [63,64] |

*Identified by liquid chromatography–tandem mass spectrometry.

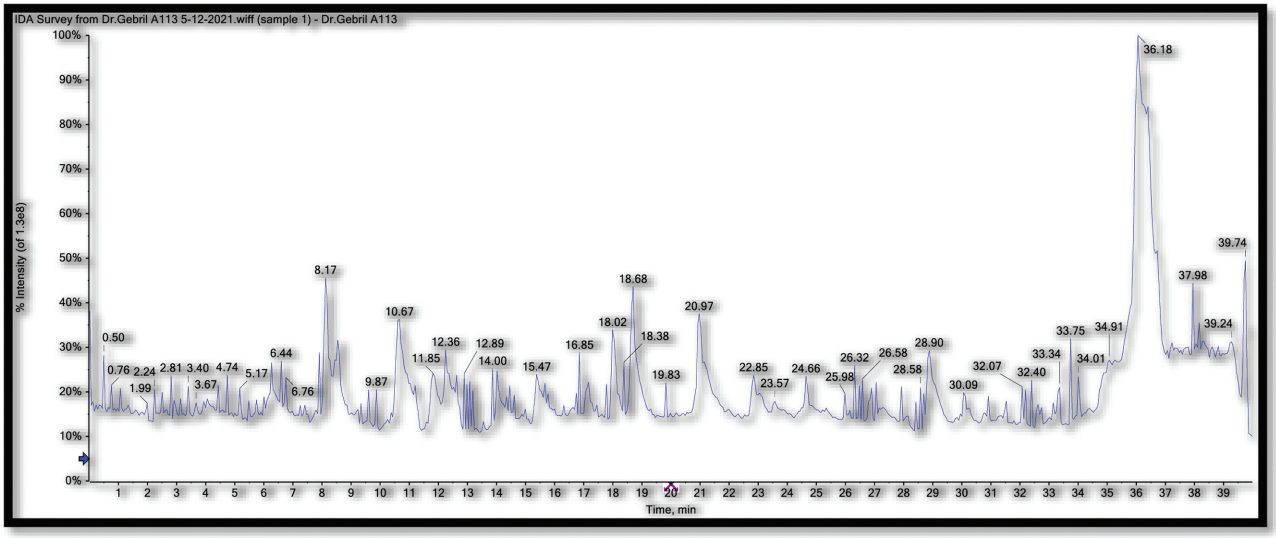
technique in negative ionization mode was used to identify the antibiotic compounds in the microbial extract of *B. licheniformis*. Tentative identification of secondary metabolites was based on mass fragmentation patterns in comparison with previously reported data. Metabolic profiling analysis is a good tool for studying metabolites and their intermediates that reflect the dynamic response to genetic modification and physiological and/or developmental stimuli [50]. Therefore, in this study, it became essential to study the metabolomics profile of the ethyl acetate extract of *B. licheniformis* mutant cultivated in nutrient broth media. The chemical profiles of the extracted *B. licheniformis* mutant are presented in Tables 12 and 13. As shown in Tables 12 and 13 and Fig. 5, some dominating peaks are visible in the LC-MS chromatogram.

Seven secondary metabolites were identified in the ethyl acetate extract of the *B. licheniformis* mutant (Tables 12 and 13 and Fig. 5). The identified compounds were categorized as α-amino acids, beta-hydroxy acids, saturated fatty acids, organic compounds, benzamide, nitroimidazoles, pyrazine derivatives, and fatty acids. The fragmentation patterns of some selected compounds are presented in Fig. 5. The modes of action of compounds identified by LC-ESI-MS/MS analysis in the extract of *B. licheniformis* mutant as described by several previous studies are presented in Table 13. Other reports stated the ability of bacteria *B. licheniformis* to produce other antibiotic compounds such as bacitracin, surfactin, biosurfactants, licheniformin, AMP, macrolactins, amicoumacins, exopolysaccharides, probiotics, lantibiotics, lichenicidin, and phenylacetic acid [51,63–65].

## Conclusion

Statistical modeling was used to identify the optimum conditions for the growth and antibiotic production by the *B. licheniformis* mutant developed from a native strain isolated from hot water springs. Plackett–Burman Design and response surface methodology have been used to achieve optimal conditions instead of studying one variable at a time, in which it is difficult to achieve the required optimization. The most significant antibiotic

**Figure 5**




Negative LC-ESI-MS/MS profile of antibiotic compounds from ethyl acetate extract of *B. lichniformis* mutant. Numbers at peaks refer to Table 12. LC-ESI-MS/MS, liquid chromatography–electrospray ionization–tandem mass spectrometry.

production *for the B. licheniformis* mutant (M15/Amo) was present in the presence of glucose, where the antagonism area reached 34 mm, with a significant increase over other carbon sources. Peptone nitrogen source significantly enhanced the antibiotic production by the mutant strain (37 mm). After determining the best carbon and nitrogen sources, experiments were conducted to determine the best concentration of these sources, in addition to a number of other five variables (incubation period, pH, temperature, agitation, and magnesium concentration). The study revealed that four variables, namely, carbon source, nitrogen source, incubation period, and pH, had a significant effect on the production of antibiotics by the *B. licheniformis* mutant (M15/Amo). The Box–Behnken method revealed that the treatment with the greatest significant effect on antibiotic production by the *B. licheniformis* mutant (M15/Amo) contained 3% glucose and 2.5% peptone after 60 h of incubation at 7.5 pH. The results show that the highest antagonistic activity of *B. licheniformis* strain (M15/Amo) was when extracting the active substances from the supernatant using ethyl acetate, where the diameter of the inhibition zones against *B. cereus* reached 41 mm. The LC analysis of the supernatant showed the presence of seven chemical compounds having antagonistic activity to pathogenic test bacteria. The study clearly shows the success of enhancing antibiotic production through the selection and mutation of bacterial strains adapted to hot spring environments. This could be achieved through optimization of the proper conditions for the growth of bacteria and optimal conditions for antibiotic production.

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**Conflicts of interest**

There are no conflicts of interest.

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