OPTIMIZATION OF THE MEDIUM COMPOSITION FOR PRODUCTION OF ANTIMICROBIAL SUBSTANCES BY *Bacillus subtilis* ATCC 6633

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In the effort to overcome the increase in antimicrobial resistance of different pathogens, natural products from microbial sources appear to be the most favorable alternative to current antibiotics. Production of antimicrobial compounds is highly dependent on the nutritional conditions. Hence, in order to achieve high product yields, selection of the media constituents and optimization of their concentrations are required. In this research, the possibility of antimicrobial substances production using *Bacillus subtilis* ATCC 6633 was investigated. Also, optimization of the cultivation medium composition in terms of contents of glycerol, sodium nitrite and phosphates was done. Response surface methodology and the method of desirability function were applied for determination of optimal values of the examined factors. The developed model predicts that the maximum inhibition zone diameters for *Bacillus cereus* ATCC 10876 (33.50 mm) and *Pseudomonas aeruginosa* ATCC 27853 (12.00 mm) are achieved when the initial contents of glycerol, sodium nitrite and phosphates were 43.72 g/L, 1.93 g/L and 5.64 g/L, respectively. The results of these experiments suggest that further research should include the utilization of crude glycerol as a carbon source and optimization of composition of such media and cultivation conditions in order to improve production of antimicrobial substances using *Bacillus subtilis* ATCC 6633.

**KEY WORDS:** *Bacillus subtilis*, antimicrobial activity, cultivation medium, response surface methodology, optimization

**INTRODUCTION**

The use of antimicrobial compounds, although appropriate and conservative, contributes to the development of resistance, while a widespread unnecessary and excessive use makes it worse (1). So, the alternatives for many of the currently used antimicrobial agents are needed. In agreement with the fact that pathogenic microorganisms gradually develop resistance and increasing consumers’ awareness of the potential negative impact of synthetic preservatives, natural metabolites produced by microorganism have the potential to be used for biological control of pathogens in agriculture, food safety, and

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medicine (2). Current trend of giving value to natural, environment-friendly and renewable resources increases the potential use of these metabolites in the mentioned areas (3). Many Bacillus species, especially Bacillus subtilis strains have been identified as producers of a wide range of compounds with antimicrobial activity against different microorganisms, including pathogenic and spoilage bacteria, fungi and yeast (4). In view of the fact that Bacillus subtilis is recognized as a safe microorganism (GRAS status), it has received considerable attention as a biological control agent (5). Natural components with antimicrobial activity produced by Bacillus subtilis include cyclic lipopeptides, polypeptides, proteins (enzymes), and non-peptide products (3). To make the production of antimicrobial components feasible, it is necessary to develop the optimum production conditions. The formulation of the cultivation medium is of critical importance because its composition affects the yield and type of the synthesized components (6). In order to achieve high yields of antimicrobial agents the proper selection of carbon, nitrogen and phosphorus sources, as the most important nutrients in the cultivation medium, is required. Among these factors, carbon source has the strongest influence on the production of bioactive compounds using the bacterial strains, and the effect of this nutrient on the bioprocess efficacy has been the subject of continuous study by both industry and research groups (7). These investigations showed that Bacillus subtilis is capable to use different substrates as carbon source such as low-cost industrial by-products and waste materials (8, 9). Glycerol, a major by-product of biodiesel manufacturing process, could be used as an organic carbon substrate in a different biotechnological production process (10, 11). Its bioconversion to high value compounds through microbial cultivation is an environmental-friendly choice that also contributes to the reduction of waste treatment costs and decreases the economics of overall production of the desirable product (12). Thus, optimization of glycerol-based media seems to be a very promising and economical route to produce antimicrobial metabolites using Bacillus subtilis. When it comes to nitrogen and phosphorus sources, nitrites and phosphates appeared to be appropriate for the biosynthesis of antimicrobial substances by Bacillus subtilis (13).

Response surface methodology (RSM) is a collection of statistical and mathematical methods that are useful for the modeling and analyzing different biotechnological process. RSM defines the relationship between the controllable input parameters and the obtained responses, and also provides simultaneously information necessary for the process design and optimization (14). Successful application of RSM to enhance the antimicrobial compounds production by optimizing the culture medium has been reported (15).

The aim of this study was to examine the possibility of the production of antimicrobial substances effective against different pathogenic microorganisms, using Bacillus subtilis ATCC 6633. In order to increase the antimicrobial activity and reduce the costs of substrate preparation further research included the optimization of the cultivation medium composition in terms of contents of glycerol, sodium nitrite, and phosphate.
MATERIALS AND METHODS

Producing microorganism

*Bacillus subtilis* ATCC 6633 was used in the experiments for antimicrobial substances biosynthesis. The strain of producing microorganism was stored at 4 °C on commercial nutrient agar (Torlak, Serbia) medium and subcultured at monthly intervals.

Cultivation media

The inoculum preparation was performed on a nutrient broth (Torlak, Serbia). The biosynthesis of antimicrobial substances was carried out on the nutrient broth and the semisynthetic glycerol-based media formulated according to the chosen experimental design. In agreement with the defined aim of study and the applied experimental design, contents of glycerol, sodium nitrite and phosphates (in the form of K$_2$HPO$_4$) in the medium were varied. The media also contained (g/L): yeast extract (0.5), CaCO$_3$ (17.0), MgSO$_4$·7H$_2$O (0.5) and MnSO$_4$·4H$_2$O (0.05), and their pH value was adjusted to 7.0 prior to sterilization by autoclaving at 121 °C and pressure of 2.1 bar for 20 min.

Inoculum preparation and biosynthesis conditions

The inoculum was prepared on the growth medium in two steps, first, by refreshing the culture by incubation for 48 h at 28 °C and second, by double passage of the microorganism. Second passage was inoculated with 10 % (v/v) of inoculum obtained in first, and the incubation of each passage lasted 48 h at 28 °C. The samples were spontaneously aerated and externally mixed (laboratory shaker, 150 rpm). The inoculation of the cultivation media was performed by adding 10 % (v/v) of the prepared inoculum. Production of substances with antimicrobial activity was performed in 300 mL Erlenmeyer flasks containing 100 mL of cultivation media of the appropriate composition. The biosynthesis of antimicrobial metabolites was carried out under aerobic conditions at the temperature of 28°C and agitation rate of 150 rpm on a laboratory shaker (Ika® Werke IKA® KS 4000i control, Germany) for 96 h.

Analytical methods

The supernatants obtained by centrifugation at 10,000 rpm for 15 min (Eppendorf Centrifuge 5804, Germany) were analyzed in terms of glycerol, nitrogen and phosphorus residues. The residual glycerol content was determined by high pressure liquid chromatography (HPLC). The samples were filtered through a 0.22 μm nylon membrane (Agilent Technologies Inc, Germany) and then analyzed. The HPLC instrument (Thermo Scientific Dionex UltiMate 3000 series) was equipped with a pump HPG-3200SD/RS, autosampler WPS-3000(T)SL (10 μL injection loop), column ZORBAX NH2 (250 mm x 4.6 mm, 5 μm), and detector RefractoMax520. The eluent was 70% (v/v) acetonitrile at a flow rate of 1.0 mL/min, and elution time of 10 minutes at column temperature of 30 °C.
The determination of the residual content of total nitrogen was performed by the Kjeldahl method (16), while residual content of total phosphorus was determined using spectrophotometric method (17).

**In vitro antimicrobial activity assay**

Antimicrobial activity of the obtained cultivation broths was tested in *in vitro*, by standard disk-diffusion method using sterile disks (18). The test microorganisms were grown on the following commercial media: Muller-Hinton agar, Torlak®, Serbia (*Pseudomonas aeruginosa* ATCC 27853), nutrient agar, Torlak®, Serbia (*Bacillus cereus* ATCC 10876), Sabouraud-Maltose agar, HiMedia®, India (*Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404). Agar was melted, cooled to 50±1 °C, and mixed in sterile conditions with the prepared suspension of test microorganism in a ratio of 9:1. The test microorganisms were treated with 15 μL of cultivation broth. A disk load with commercial antibiotic was prepared as a positive control, and a disk load with only distilled water was prepared as a negative control. After the incubation at 30 °C for 48 h, diameters of the zones of inhibition were measured using a zone scale from Himedia®, and expressed in millimeters (including disk diameter). Activity of each sample was tested in three replicates for each microorganism.

**Experimental design and optimization by RSM**

The response surface methodology based on the Box-Behnken experimental design was used for the optimization of media composition for antimicrobial substances production. The independent variables and their varied contents (g/L) were: \(X_1\) - glycerol (20, 35, 50), \(X_2\) - NaNO₂ (1, 2, 3) and \(X_3\) - K₂HPO₄ (5, 10, 15). In this regard, according to the Box-Behnken experimental design with three factors at three levels and three repetitions in the central point (15) a set of 15 experiments was carried out.

The relations between the independent variables and the responses \(Y_i\) (inhibition zone diameter (mm), residual glycerol (g/L), residual nitrogen (g/L) and residual phosphorus (g/L) content) were determined by the second-order polynomial equation:

\[
Y_i = b_0 + \sum b_iX_i + \sum b_{ii}X_i^2 + \sum b_{ij}X_iX_j
\]  

[1]

where \(Y_i\) is the selected response, \(b_0\) is the intercept, \(b_i, b_{ii}\) and \(b_{ij}\) are the linear, quadratic and interaction regression coefficient, respectively, while \(X_i\) and \(X_j\) are the varied factors.

The adequacy of the model was evaluated by coefficient of determination (R²) and model p-value. The significance of regression coefficients was assessed by p-values at the 0.05 significance level. The statistical software package Statistica v. 13.0 was used for the regression analysis of the experimental data, and also to generate the response surface graphs. The method of desirability function was applied for the determination of optimal values of examined variables (Design-Expert 8.1).
RESULTS AND DISCUSSION

In order to examine the possibility of antimicrobial substances production using *Bacillus subtilis* ATCC 6633 a preliminary experiment was carried out, which included cultivation of producing microorganism on commercial nutrient broth. Based on the inhibition zone diameters of 10.0 mm and 6.0 mm for *Bacillus cereus* ATCC 10876 (Gram-positive bacteria) and *Pseudomonas aeruginosa* ATCC 27853 (Gram-negative bacteria) obtained by the disk-diffusion method, respectively, it can be concluded that the applied cultivation broth had an antibacterial effect against tested bacteria, while no antifungal effect against *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404 was detected. Gram-positive and Gram-negative bacteria have differences in their cell wall structure, the most important being the presence of a lipid-rich outer membrane in the cell wall of Gram-negative bacteria (19). Compared with Gram-positive bacteria, Gram-negative bacteria are more resistant against different extreme environmental conditions and antimicrobial agents because of the complex structure of their cell wall (20). So, the reason for the lower antimicrobial activity obtained against *Pseudomonas aeruginosa* ATCC 27853 may be the difference in the cell wall structure between tested bacteria. On the other hand, the fungal cell wall consists of glycoproteins and polysaccharides, mainly glucan and chitin, extensively cross-linked, to form a complex network (21). The obtained results indicate that the antimicrobial substances produced in applied experimental conditions do not decompose complex structure of the fungal cell wall, which may be the reason for the high resistance of *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404.

In a large-scale production of antimicrobial components, commercial semisynthetic broths are not economically feasible medium, and in order to reduce the costs of raw material, some cheaper substrates are to be sought. Also, from the economical aspect of the production process, production of antimicrobial compounds with high yields is essential, and can be achieved primarily by media manipulation. Therefore, the other aim of this study was to optimize the medium composition in order to increase the product yield, reduce costs of substrate preparation and content of unused nutrients, which consequently cause reduction of the organic load of waste streams that will be generated after the separation of the bioactive components. In this research, the media for the production of antimicrobial substances by *Bacillus subtilis* ATCC 6633 was formulated by varying content of glycerol, sodium nitrite and phosphates. The values of the examined factors were selected based on the available literature data (13, 22).

**Statistical analyses and definition of the mathematical models**

Based on the results of experiments formulated by the Box-Behnken design and regression analysis, quadratic polynomial equations were established to identify the relation between the selected responses and examined factors. The selected responses were: the inhibition zone diameter obtained against *Bacillus cereus* ATCC 10876 (mm, $Y_1$), the inhibition zone diameter obtained against *Pseudomonas aeruginosa* ATCC 27853 (mm, $Y_2$), residual glycerol content (g/L, $Y_3$), residual nitrogen content (g/L, $Y_4$)
and the residual phosphorus content (g/L, Y_5). The results of the statistical analyses are presented in Table 1.

Table 1. Coefficients of regression equations and their significance for the inhibition zone diameters, residual glycerol, nitrogen and phosphorus content

<table>
<thead>
<tr>
<th>Responses</th>
<th>Y_1</th>
<th></th>
<th>Y_2</th>
<th></th>
<th>Y_3</th>
<th></th>
<th>Y_4</th>
<th></th>
<th>Y_5</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Intercept</td>
<td></td>
<td>b_0</td>
<td></td>
<td>-38.751</td>
<td>0.068</td>
<td>-9.725</td>
<td>0.117</td>
<td>23.588</td>
<td>0.2295</td>
<td>-0.038</td>
</tr>
<tr>
<td>Linear</td>
<td></td>
<td>b_1</td>
<td></td>
<td>3.445</td>
<td>0.002</td>
<td>0.409</td>
<td>0.064</td>
<td>0.428</td>
<td>0.4927</td>
<td>-0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b_2</td>
<td></td>
<td>-2.961</td>
<td>0.718</td>
<td>14.167</td>
<td>0.002</td>
<td>-20.78</td>
<td>0.0486</td>
<td>0.181</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b_3</td>
<td></td>
<td>-0.454</td>
<td>0.781</td>
<td>-0.183</td>
<td>0.716</td>
<td>-4.032</td>
<td>0.0534</td>
<td>0.029</td>
</tr>
<tr>
<td>Quadratic</td>
<td></td>
<td>b_{11}</td>
<td></td>
<td>-0.036</td>
<td>0.004</td>
<td>-0.006</td>
<td>0.003</td>
<td>0.008</td>
<td>0.3262</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b_{22}</td>
<td></td>
<td>-2.854</td>
<td>0.124</td>
<td>-2.896</td>
<td>0.002</td>
<td>3.705</td>
<td>0.0679</td>
<td>0.003</td>
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<td></td>
<td></td>
<td>b_{33}</td>
<td></td>
<td>-0.049</td>
<td>0.463</td>
<td>0.004</td>
<td>0.835</td>
<td>0.124</td>
<td>0.1097</td>
<td>-0.000</td>
</tr>
<tr>
<td>Interaction</td>
<td></td>
<td>b_{12}</td>
<td></td>
<td>0.000</td>
<td>0.999</td>
<td>0.008</td>
<td>0.795</td>
<td>-0.108</td>
<td>0.3392</td>
<td>-0.000</td>
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<tr>
<td></td>
<td></td>
<td>b_{13}</td>
<td></td>
<td>-0.015</td>
<td>0.483</td>
<td>0.005</td>
<td>0.449</td>
<td>0.005</td>
<td>0.8306</td>
<td>0.000</td>
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<tr>
<td></td>
<td></td>
<td>b_{23}</td>
<td></td>
<td>1.250</td>
<td>0.008</td>
<td>-0.175</td>
<td>0.113</td>
<td>1.030</td>
<td>0.0202</td>
<td>-0.005</td>
</tr>
</tbody>
</table>

Bolded values represent the significant coefficients at a confidence level of 95%.

The mode of interactions between the examined factors is indicated by the regression equations coefficients. A positive sign for the values of coefficients of interaction indicates a synergistic effect, while a negative sign represents an antagonistic effect of the factors on the selected response. The _p_-values serve as a tool for checking the significance of each of the coefficients of the regression equations. The factors with _p_-values less than 0.05 are significant at a confidence level of 95%. These coefficients are bolded in Tables 1.

The adequacy and significance of the quadratic model was checked using the analysis of variance (ANOVA) which was tested using Fisher’s statistical analysis and the results are shown in Table 2. All polynomial models were significant at the 95% confidence level. The model F-values of 124.593, 151.900, 40.602, 331.611 and 32.847 for the inhibition zone diameter obtained against _Bacillus cereus_ ATCC 10876 (mm, Y_1) and _Pseudomonas aeruginosa_ ATCC 27853 (mm, Y_2), residual content of glycerol (Y_3), nitrogen (Y_4) and phosphorus (Y_5), respectively, imply that the models for the selected responses are significant. The fitness of the model was checked and confirmed by the coefficient of determination (R^2). The R^2 value closer to 1 denotes better correlation between the observed and predicted values. The relatively high values of the determination coefficient obtained for all responses indicate a good fit of the experimental data to Eq. (1).
Table 2. Analysis of variance of the modeled responses

<table>
<thead>
<tr>
<th>Response</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F-value</th>
<th>p-value</th>
<th>R²</th>
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<tbody>
<tr>
<td></td>
<td>⁵r</td>
<td>⁰m</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Y₁</td>
<td>5</td>
<td>10</td>
<td>44.10⁰</td>
<td>8.82¹</td>
<td>124.59</td>
<td>0.975</td>
</tr>
<tr>
<td></td>
<td>10m</td>
<td>10m</td>
<td>10990.15⁰</td>
<td>1099.01⁰</td>
<td>0.000023</td>
<td></td>
</tr>
<tr>
<td>Y₂</td>
<td>5</td>
<td>10</td>
<td>4.16²</td>
<td>0.83⁴</td>
<td>151.90</td>
<td>0.943</td>
</tr>
<tr>
<td></td>
<td>10m</td>
<td>10m</td>
<td>1265.83⁴</td>
<td>126.58³</td>
<td>0.00014</td>
<td></td>
</tr>
<tr>
<td>Y₃</td>
<td>5</td>
<td>10</td>
<td>47.03⁶</td>
<td>9.40⁷</td>
<td>40.60²</td>
<td>0.968</td>
</tr>
<tr>
<td></td>
<td>10m</td>
<td>10m</td>
<td>3819.56²</td>
<td>381.96³</td>
<td>0.00370</td>
<td></td>
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<tr>
<td>Y₄</td>
<td>5</td>
<td>10</td>
<td>0.003</td>
<td>0.00¹</td>
<td>331.61</td>
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<tr>
<td></td>
<td>10m</td>
<td>10m</td>
<td>2.04³</td>
<td>0.20⁴</td>
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<tr>
<td>Y₅</td>
<td>5</td>
<td>10</td>
<td>0.019</td>
<td>0.00⁴</td>
<td>32.84²</td>
<td>0.956</td>
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<tr>
<td></td>
<td>10m</td>
<td>10m</td>
<td>1.27⁴</td>
<td>0.12⁷</td>
<td>0.00619</td>
<td></td>
</tr>
</tbody>
</table>

DF - degree of freedom; SS - sum of squares; MS - mean squares. r - residual; m - model.

Three-dimensional response surfaces were plotted on the basis of the model equation, to investigate the interaction among the examined factors and to determine the range of concentration of each factor for maximum antimicrobial compounds biosynthesis by *Bacillus subtilis* ATCC 6633. In the response surface plots, two factors varied when the third factor was kept at a fixed level (zero level).

Figure 1A shows the effects of the initial content of glycerol and sodium nitrite at a constant concentration of phosphates (10.0 g/L) on the production of compounds with antimicrobial activity against *Bacillus cereus* ATCC 10876. As it can be seen from the response surface plot, the increase of the initial glycerol concentration in the medium results in the increase of inhibition zone in whole investigated range of sodium nitrite content. Also, the results in this figure indicate that over the glycerol concentration of about 40.0 g/L in the medium there is no significant increase of antimicrobial activity with further increase of nitrogen content. According to the predictions of the model, the inhibition zone is maximum (about 33.0 mm) at maximum values of initial glycerol content (40-50 g/L), regardless of the sodium nitrite concentration.

The response surface plot presented in Figure 1B illustrates the effects of the initial content of glycerol and phosphates on the inhibition zone diameter at a constant sodium nitrite concentration (2.0 g/L). From this plot is evident that the antimicrobial activity increased with the increase of the initial glycerol concentration in the whole range of the initial phosphates content. Therefore, a maximum inhibition zone diameter of about 35.0 mm model predicts at the maximum values of the initial glycerol concentration (40-50 g/L), regardless of the initial phosphates content.

The effects of the initial contents of sodium nitrite and phosphates on the biosynthesis of compounds effective against *Bacillus cereus* ATCC 10876 at a constant glycerol concentration (35.0 g/L) are presented in Figure 1C. According to the predictions of the model, the inhibition zone diameter is maximum (about 35.0 mm) if the initial nitrogen and phosphorus content are 3.0 and 15.0 g/L (maximal values of both factors), respectively.
Figure 1. Effects of the cultivation medium composition on inhibition zone diameter for *Bacillus cereus* ATCC 10876 (A, B, C) and *Pseudomonas aeruginosa* ATCC 27853 (D, E, F)
Also, when medium for production of antimicrobial compounds contains minimal initial concentrations of sodium nitrite (1.0-1.4 g/L) and phosphates (5.0-9.0 g/L) the model predicts inhibition zone diameter for *Bacillus cereus* ATCC 10876 of about 30.0 mm. A positive interaction between the two independent variables (coefficient of interaction, \( b_{23} \), Table 1) points to a synergistic effect between the contents of sodium nitrite and phosphates on antimicrobial component production by *Bacillus subtilis* ATCC 6633 effective against *Bacillus cereus* ATCC 10876. The minimum response predicted by the model was around 13.0 mm at the maximal content of sodium nitrite and minimal content of phosphates, which indicates that high concentration of nitrogen source in the medium with insufficient phosphorus content have a negative effect on metabolic activity of the producing microorganism.

From the analysis of the response surfaces it can be seen that the increase of glycerol content in the medium affects positively the production of desired antimicrobial substances, which makes glycerol a limiting nutrient for their biosynthesis. Furthermore, the results presented in Figure 1A-C indicate that the nitrogen and phosphorus ratio is very important for the production of antimicrobial components effective against *Bacillus cereus* ATCC 10876. By maintaining the appropriate ratio of these nutrients in the cultivation medium a maximal production of bioactive components with antimicrobial activity is provided.

The effects of the initial content of glycerol and sodium nitrite on the inhibition zone diameter obtained for *Pseudomonas aeruginosa* ATCC 27853 at a constant content of phosphates (10 g/L) are presented in Figure 1D. The obtained plot shows that the antimicrobial substances production increased with an increase in the initial glycerol concentration up to the range of 30.0 g/L to 45.0 g/L, beyond which there is a decrease. Also, the increase of the initial sodium nitrite content up to about 1.8-2.4 g/L, has a positive effect on antimicrobial activity. With higher content of nitrogen source in the cultivation medium, the antimicrobial activity shows a significant decrease. Consequently, in accordance with the model predictions, a maximum inhibition zone diameter of approximately 12.0 mm was obtained at the initial glycerol concentration ranging from 30.0 g/L to 45.0 g/L and the initial sodium nitrite concentration ranging from 1.8 g/L to 2.4 g/L.

The effects of the initial contents of glycerol and phosphates on the production of bioactive substances in cultivation medium at a constant nitrogen concentration (2 g/L) are illustrated in Figure 1E. The presented results indicate that the decrease of phosphates content in the medium leads to a maximum of antimicrobial activity. A negative influence of the phosphates content in the medium could be explained by the fact that in the most cases antimicrobial compounds are products of the secondary metabolism, thus earlier beginning of stationary phase, during which the secondary metabolites are synthesized, is often achieved by limiting the phosphorus source content in the production medium (23). A maximum inhibition zone diameter of about 12.0 mm, according to the model predictions, is achieved at the initial glycerol content of 25.0-50.0 g/L and minimal phosphates content (5.0-8.0 g/L).

Figure 1F illustrates the effects of the initial contents of sodium nitrite and phosphates on the biosynthesis of compounds effective against *Pseudomonas aeruginosa* ATCC
27853 at a constant glycerol concentration (35.0 g/L). From this response surface plot it can be seen that increase of the initial sodium nitrite content up to about 2.2-2.6 g/L, has a positive effect on the bioactive component concentration in cultivation media, regardless of the initial phosphates content. This positive effect of sodium nitrite values in the examined range on the production of the desired components indicates that the proposed nitrogen concentrations are sufficient for the initial biomass multiplication and production of antimicrobial substances. Further increase of nitrogen content in the cultivation medium will probably cause the prolongation of the exponential phase and significant nutrients depletion of the medium before the beginning of the stationary phase, which would result in a reduced production of desired components. Also, it is evident that, in the applied experimental conditions, the production of antimicrobial compounds decreases with the increase in the phosphates content in the medium. As can be seen from the response surface plot, the inhibition zone diameter is maximum (about 12.0 mm) at the minimal content of phosphates (5.0-8.0 g/L) and sodium nitrite content of about 2.0-2.8 g/L.

Optimization of the cultivation medium composition

The optimization of the medium composition for the antimicrobial compounds production was accomplished using a multi-response method called desirability function (24). This method involves the transformation of each response variable (Y_i) to an individual function of desirability (d_i). In this study, the concept of desirability function was used to optimize the contents of glycerol, sodium nitrite and phosphates in the medium for production of substances effective against Bacillus cereus ATCC 10876 and Pseudomonas aeruginosa ATCC 27853 using Bacillus subtilis ATCC 6633. Two sets of optimization were made. The obtained results are presented in Table 3.

The demonstrated antimicrobial activity of the substances produced by Bacillus subtilis ATCC 6633 against the tested Gram-positive (Bacillus cereus ATCC 10876) and Gram-negative (Pseudomonas aeruginosa ATCC 27853) bacteria indicated the potential broad-spectrum antimicrobial activity of the obtained cultivation broth. So, if the only goal of optimization is to achieve a maximum inhibition zone diameter against Bacillus cereus ATCC 10876 and Pseudomonas aeruginosa ATCC 27853 (first set, Table 3), the desirability function is of the maximum value (0.96) at the initial contents of glycerol, sodium nitrite and phosphates of 43.71 g/L, 1.92 g/L and 5.63 g/L, respectively. By applying media with such nutrients contents, the model predicts the inhibition zone diameters of 33.50 mm (Bacillus cereus ATCC 10876) and 12.00 mm (Pseudomonas aeruginosa ATCC 27853). The predicted residual content of glycerol, total nitrogen and phosphorus are 15.20 g/L, 0.32 g/L and 0.16 g/L, respectively.

When defining the media composition it must be taken into account that the efficiency of the antimicrobial substances production are improved when the residual nutrients content is minimal, because the unused nutrients represent losses from an economic viewpoint (25). One of the common concerns regarding human and environmental health is the presence of organic components in wastewater, whose inappropriate disposal is an
environmental problem, and therefore requires additional processing prior to their release into the environment (26).

**Table 3.** The optimal values of factors and predicted values of selected responses

<table>
<thead>
<tr>
<th>Factors and responses</th>
<th>Goal</th>
<th>Optimal value</th>
<th>Goal</th>
<th>Optimal value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>First set</td>
<td>Second set</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol (g/L)</td>
<td>in the range</td>
<td>43.71</td>
<td>in the range</td>
<td>34.98</td>
</tr>
<tr>
<td>NaNO₂ (g/L)</td>
<td>in the range</td>
<td>1.92</td>
<td>in the range</td>
<td>1.78</td>
</tr>
<tr>
<td>K₂HPO₄ (g/L)</td>
<td>in the range</td>
<td>5.63</td>
<td>in the range</td>
<td>5.20</td>
</tr>
<tr>
<td>Predicted values</td>
<td></td>
<td></td>
<td>Predicted values</td>
<td></td>
</tr>
<tr>
<td>Inhibition zone diameter A (mm)</td>
<td>maximize</td>
<td>33.50</td>
<td>maximize</td>
<td>29.22</td>
</tr>
<tr>
<td>Inhibition zone diameter B (mm)</td>
<td>maximize</td>
<td>12.00</td>
<td>maximize</td>
<td>12.00</td>
</tr>
<tr>
<td>Residual glycerol (g/L)</td>
<td>in the range</td>
<td>15.20</td>
<td>minimize</td>
<td>8.83</td>
</tr>
<tr>
<td>Residual nitrogen (g/L)</td>
<td>in the range</td>
<td>0.32</td>
<td>minimize</td>
<td>0.30</td>
</tr>
<tr>
<td>Residual phosphorus (g/L)</td>
<td>in the range</td>
<td>0.16</td>
<td>minimize</td>
<td>0.20</td>
</tr>
<tr>
<td>Desirability function</td>
<td>0.96</td>
<td></td>
<td>0.80</td>
<td></td>
</tr>
</tbody>
</table>

A - Bacillus cereus ATCC 10879; B - Pseudomonas aeruginosa ATCC 27853

Therefore, in the second optimization set (Table 3), in addition to achieving the maximum antimicrobial activity, the minimum residual contents of the nutrients were selected as responses. According to the model predictions for the highest value of desirability function (0.80) the optimal values of the initial contents of glycerol, sodium nitrite and phosphate are 34.98 g/L, 1.78 g/L and 5.20 g/L, respectively. The predicted inhibition zone diameter obtained against Bacillus cereus ATCC 10876 is 29.22 mm and against Pseudomonas aeruginosa ATCC 27853 is 12.00 mm, while the residual nutrients content are 8.83 g/L, 0.30 g/L and 0.20 g/L for glycerol, total nitrogen and phosphorus, respectively. Comparing the results of the first and second optimization sets, a reduced concentration of residual glycerol by 41.91% and a decrease of the inhibition zone diameter by 12.78% for Bacillus cereus ATCC 10876 were noticed, while the inhibition zone diameter for Pseudomonas aeruginosa 27853 remained unchanged. The changes in the concentrations of residual nitrogen and phosphorus were not significant.

To validate the model developed in the second optimization set, a new series of experiments were carried out with the optimum values of examined factors. On average, the experimentally obtained values of inhibition zone diameters for Bacillus cereus ATCC 10879 and Pseudomonas aeruginosa ATCC 27853 were 28.67±1.53 mm and 11.50±1.32 mm, respectively. For the residual sugar, total nitrogen, and phosphorus the average values in the additional experiments were 8.29±0.92 g/L, 0.28±0.09 g/L and
0.17±0.07 g/L, respectively. These results show that the experimentally determined values are in good agreement with the statistically predicted values for all modeled responses, which confirmed the adequacy of the model.

The results obtained in this study indicate a considerable difference in the antimicrobial activity of cultivation broths against tested microorganisms, probably because they belong to the different bacterial species with characteristic differences in the structure of cell wall. In comparing the inhibition zone diameter obtained for two tested bacterial pathogens, it can be assumed that the antimicrobial components produced on semisynthetic glycerol-based media also show the different antibacterial effect against Gram-positive and Gram-negative bacteria. Therefore, a detailed study is required to explore the relationship between the antimicrobial activity of the obtained cultivation broth and its concentration. Generally, concentrated bioactive products will provide a strengthened antimicrobial effect. Hence, these results represent a basis for further research in order to produce and formulate an antimicrobial agent with increased activity against Gram-negative bacteria.

CONCLUSION

The results obtained in this study demonstrated that Bacillus subtilis ATCC 6633, in applied experimental conditions produce substances with antibacterial effect against Bacillus cereus ATCC 10876 and Pseudomonas aeruginosa ATCC 27853 on both examined cultivation media (nutrient broth and semisynthetic glycerol-based media). Statistical methodology was employed to define the composition of alternative medium with glycerol for production of antimicrobial compounds. The RSM based on the Box-Behnken design, as well as desirability function approach, were applied to find out the optimal concentration of glycerol, sodium nitrite and phosphates. The applied statistical techniques proved to be a valuable tool for improving medium composition that leads to a higher degree of antimicrobial compound production. In addition, the results of our study represent the basis for further improvements of the production of antimicrobial substances using Bacillus subtilis ATCC 6633.

REFERENCES


ге у погледу садржаја глицерола, натријум-нитрита и фосфата. Биосинтеза анти-
микробних компоненти изведена је на различitim подлогама чији је састав форму-
лисан у складу са Box-Behnken-овим експерименталним планом. За одређивање
оптималног почетног садржаја нутријената применена је метода жељене функције
у комбинацији са полиномским зависностима посматраних одзива добијених ко-
ришћењем поступка одзивне површине. Најефикасије деловање култивационе
tечности против Bacillus cereus ATCC 10876 (33,50 mm) и Pseudomonas aeruginosa
ATCC 27853 (12,00 mm), модели предвиђа при почетним садржајима глицерола, натријум-
нитрита и фосфата од 43,72 g/l, 1,93 g/l и 5,64 g/l, редоследом. Поред тога,
урађен је још један сет оптимизације са циљем минимизације резидуалних садржаја
нутријената, како би се утицало на смањене организко углередење отпадних токо-
ва, али и на укупне трошкове припреме хранљиве подлоге. Добијени резултати
представљају основу за даља истраживања којима би се унапредила производња
жељених антимикробних супстанци, а која би подразумевала испитивање могућ-
ности употребе сировог глицерола као извора углередника у подлози за биосинтезу,
оптимизацију процесних услова, као и дефинисање корака у биосепаративном низу
како би се формулисао готов биолошки препарат.

Кључне речи: Bacillus subtilis, антимикробна активност, хранљива подлога, RSM,
oптимизација

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