OPTIMIZATION OF CULTIVATION MEDIUM FOR THE PRODUCTION OF ANTIBACTERIAL AGENTS

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Optimization of the cultivation medium for production of antibiotic effective against pathogenic bacteria Staphylococcus aureus using strain of Streptomyces spp. isolated from the environment represents the aim of this study. After the biosynthesis, the medium was analyzed by determining residual sugar and nitrogen, and the antibiotic activity was determined using diffusion-disc method. Experiments were carried out in accordance with the Box-Behnken design, with three factors varied on three levels (glucose: 10.0, 30.0 and 50.0 g/L; soybean meal: 5.0, 15.0 and 25.0 g/L; phosphates: 0.5, 1.0 and 1.5 g/L) and for the optimization of selected parameters Response Surface Methodology was used. The obtained model with the desirability function of 0.985 estimates that the lowest amounts of residual sugar (0.89 g/L) and nitrogen (0.24 g/L) and the largest possible inhibition zone diameter (21.88 mm) that with its antibiotic activity against S. aureus creates the medium containing 10.0 g/L glucose, 5.0 g/L soybean meal and 1.04 g/L phosphates.

KEY WORDS: antibiotic activity, cultivation medium, RSM, Streptomyces spp.

INTRODUCTION

Staphylococcus aureus is one of the most common causes of hospital- and community-acquired infections, and the main threat to human health is the emergence of resistant forms of this bacteria. The increasing resistance in staphylococci has created a need for the development of new antimicrobial agents and new targets for antibiotic therapy is currently of high priority all over the world [1].

Actinomycetes represent a group of bacteria that is especially useful to the pharmaceutical industry for their apparently unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities. Tens of thousands of such compounds have been isolated and characterized, many of which have been developed into drugs for treatment of a wide range of diseases in human, veterinary and agriculture sectors [2]. Streptomyces synthesize an amazing variety of chemically distinct substances,
many of which act as antibiotics, fungicides, cytostatics, or modulators of immune responses, and a huge diversity of ever increasing numbers of inhibitors for many different cellular processes [3].

Production of various metabolites with the same strain of *Stereotomycetes* to the greatest possible extent depends on the medium composition and process parameters. The source of carbon and nitrogen in the cultivation medium plays an important role, since microbial and products of biosynthesis are largely composed of these elements. Optimization of cultivation medium is as important as the selection of an organism to obtain antibiotic production. Optimization of medium components for the production of antibiotic effective against *S. aureus* by Response Surface Methodology (RSM) has been reported in case of different strains of *Streptomyces* [4, 5].

Optimization through factorial design and response surface analysis is a common practice in biotechnology [6]. Employing RSM in the optimization enables the study of interaction effect among different variables. The main objective of RSM is to determine the optimum operational conditions for the system or to determine a region that satisfies the operating specifications [7]. Also, it can be used to evaluate the relative significance of several affecting factors, even in the presence of complex interactions [8]. In this research, RSM was used to optimize the cultivation medium components, contents of glucose, soybean meal and phosphates, for the production of antibiotic effective against opportunistic pathogen *S. aureus*, where a strain of *Streptomyces* sp. isolated from the environment was used as the production microorganism.

EXPERIMENTAL

Production microorganism

The producing microorganism was the isolate of *Streptomyces* sp. obtained from the soil sample taken from the territory of Novi Sad, Serbia. The isolate was identified as *Stereotomycetes hygroscopicus* on the basis of its morphological and physiological characteristics according to Bergey’s Manual of Systematic Bacteriology [9, 10]. The strain of producing microorganism was stored at 4°C and subcultured every four weeks.

Cultivation medium

The medium for the growth of production microorganisms had the following characteristics (g/L): glucose (15.0), soybean meal (10.0), CaCO₃, (3.0), NaCl, (3.0), MgSO₄, (0.5), (NH₄)₂HPO₄, (0.5), K₂HPO₄, (1.0) [11]. In mediums for antibiotic production, contents of glucose (10.0 g/L; 30.0 g/L and 50.0 g/L), soybean meal (5.0 g/L; 15.0 g/L and 25.0 g/L) and phosphates (0.5 g/L; 1.5 g/L and 2.5 g/L) were varied based on the experimental plan and defined aim of the study. Phosphates were added in the form of (NH₄)₂HPO₄ and K₂HPO₄ in a 2:1 ratio. The mediums were also enriched with the following ingredients (g/L): CaCO₃ (3.0), NaCl (3.0) and MgSO₄ (0.5). Before sterilization (121°C, 20 minutes), the pH value of the mediums was adjusted to 7.2 ± 0.1.
Cultivation

The inoculum was prepared on the growth medium in two steps, first, by refreshing the culture by incubation for 48 h at 27°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 27°C. The samples were spontaneously aerated and externally mixed (laboratory shaker, 150 rpm). The inoculation of the cultivation mediums was performed by adding 10% (v/v) of prepared inoculums. Cultivation on antibiotic production mediums was carried out in an Erlenmeyer shake flasks containing one third of the cultivation medium, under aerobic conditions and agitation rate of 150 rpm at the temperature of 27°C for 7 days. At the end of the cultivation, the separation of the solid from the liquid phase in the cultivation medium was carried out by centrifugation at 10,000·G for 10 minutes (Eppendorf Centrifuge 5804, Germany).

Analytical methods

Supernatant of cultivation mediums was used for all analytical methods. The content of reducing sugars was determined using the method with dinitrosalicylic acid (DNS) [12]. Total nitrogen content was determined by the Kjeldahl method [13]. The antibiotic activity of the supernatants of obtained cultivation medium against *S. aureus* ATCC 11632 was tested by the diffusion-disc method using sterile discs (HiMedia, India) and the volume of analyzed samples was 10 μL of the supernatant evaporated to the one tenth of its initial mass [14]. A few colonies of the test microorganism were picked with a wire loop from the original culture plate and introduced into a test tube containing 10 mL of saline solution. This solution was diluted until the final cell concentration of 10^6 cfu/mL was achieved. For the growth of test the microorganism, Muller-Hinton agar (Torlak®) was used. Agar was melted, cooled to 50±1°C and mixed in sterile conditions with the prepared suspension of test microorganism in a ratio 9:1. After the incubation at 30°C for 48 h [15], the inhibition zones were measured by a HiAntibioticZoneScale ruler (HiMedia®).

Data processing

The RSM is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from the properly designed experiments to simultaneously solve multivariable equations. In the application of the RSM the most important step represents the selection of a corresponding experimental plan. The Box-Behnken design for three independent variables on three levels and three repetitions in the central point was used to obtain the combination of the values that optimizes the response within the region of three-dimensional observation space, which allows one to minimize the number of experiments required [16, 17]. The results were statistically processed by the analysis of variance at the significance level of α=0.05. The adequacy of the model was evaluated by the coefficient of determination (R^2) and p-value of the model. For the description of the responses Y1-Y3 (inhibition zone diameter (mm), residual sugar (g/L) and residual nitrogen (g/L)) the obtained data were fitted to a second-degree poly-
nominal model. The independent variables and their levels are $X_1$: content of glucose (10.0-50.0 g/L, interval value 20.0 g/L), $X_2$: content of soybean meal (5.0–25.0 g/L, interval value 10.0 g/L) and $X_3$: content of phosphates (0.5–2.5 g/L, interval value 1.0 g/L).

Experimental data were subjected to the statistical analysis using the software STATISTICA 9.0 (StatSoft, USA). The response surface curves were plotted with a constant value of one of the parameters, while the remaining two parameters were varied. The obtained data were analyzed using the software package DESIGN EXPERT 8.1 (StatEase, Inc., USA) and the method of desired function was applied for the determination of the optimal values of the examined parameters.

RESULTS AND DISCUSSION

Statistical data analysis

The response surface procedure consists of a series of steps that include conducting experiments and fitting of the measured values using the method of response surface function, the definition of desired function to each of the responses and optimization of the total desired function with respect to the parameters of the experiment [18]. The results of fitting the selected responses by a second-degree polynomial are shown in Tables 1 and 2. For the selected responses, the results of the statistical analyses are presented in Table 1 and the ANOVA results are reported in Table 2. The regression coefficients, with a significance of 95%, are significant if the value of their significance coefficients ($p$-value) is less than 0.05 and these values are bolded in the table.

Table 1. Regression coefficients and their significance of models for the inhibition zone diameter against applied test microorganism, residual sugar and residual nitrogen

<table>
<thead>
<tr>
<th>Responses</th>
<th>$Y_1$</th>
<th>$Y_2$</th>
<th>$Y_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$b_0$</td>
<td>33.2396</td>
<td>6.4287</td>
<td>0.0536</td>
</tr>
<tr>
<td>Linear</td>
<td>-0.6688</td>
<td>-0.1902</td>
<td>0.0123</td>
</tr>
<tr>
<td>$b_1$</td>
<td>-4.0625</td>
<td>0.3469</td>
<td>-0.0050</td>
</tr>
<tr>
<td>$b_2$</td>
<td>-3.1250</td>
<td>-11.4653</td>
<td>0.1292</td>
</tr>
<tr>
<td>Quadratic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$b_{11}$</td>
<td>0.0068</td>
<td>0.0181</td>
<td>-0.0019</td>
</tr>
<tr>
<td>$b_{22}$</td>
<td>0.4271</td>
<td>0.9165</td>
<td>0.00099</td>
</tr>
<tr>
<td>$b_{33}$</td>
<td>-0.0417</td>
<td>3.3102</td>
<td>-0.03457</td>
</tr>
<tr>
<td>Interaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$b_{12}$</td>
<td>0.0063</td>
<td>-0.0223</td>
<td>-0.00001</td>
</tr>
<tr>
<td>$b_{13}$</td>
<td>0.1500</td>
<td>0.1700</td>
<td>-0.00092</td>
</tr>
<tr>
<td>$b_{23}$</td>
<td>-0.2500</td>
<td>0.5094</td>
<td>-0.00079</td>
</tr>
</tbody>
</table>

Relatively high values of the coefficient of determination ($R^2$) obtained for all responses indicate good fit of experimental data to the second-degree polynomial. All polynomial models tested for the selected responses were significant at the 95% confidence level. The model F-values of 171.1303, 66.9874 and 309.9173 for the inhibition zone dia-
meter toward \textit{S. aureus}, residual sugar and residual nitrogen, respectively, imply that the models for selected responses are significant.

\textbf{Table 2.} Analysis of variance (ANOVA) of the modeled responses

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
 Response                  & Residual & Model & F  & p-value & R^2  \\
                        & DF & SS   & MS & DF & SS   & MS &       &       \\
\hline
 Inhibition zone diameter & 5  & 9.917 & 1.983 & 10  & 3394.083 & 339.408 & 171.1303 & <0.0001 & 0.944  \\
 Residual sugar           & 5  & 39.995 & 7.999 & 10  & 5358.292 & 535.829 & 66.9874 & <0.0001 & 0.985  \\
 Residual nitrogen        & 5  & 0.005 & 0.001 & 10  & 3.434 & 0.344 & 309.9173 & <0.0001 & 0.989  \\
\hline
\end{tabular}
\end{table}

DF – degrees of freedom; SS – sum of squares; MS – mean square

The type and amount of product obtained in the biotechnological process are significantly affected by the cultivation medium. Since antibiotics are mostly products of secondary metabolism, the optimization of the content of the medium presents a challenge. Firstly, the microorganism needs to be provided with a sufficient amount of nutrients for the growth phase and also stimulated to enter the stationary phase where the desired component is produced. The intensity of the antibacterial effect of the cultivation media is a consequence of different amounts of the synthesized bioactive molecule (significant effect of the linear coefficient for glucose, \( b_1 \)), which is very likely a consequence of higher amount of activity of enzymes of the corresponding metabolic pathway (this is indicated by the significance of the linear coefficients for the starting amount of glucose and soybean meal, \( b_1 \) and \( b_2 \); as well as the coefficient of the interaction of the initial contents of carbon and phosphates, \( b_{13} \)).

The three-dimensional response surfaces, plotted in Figures 1-3, correspond to the combined effects of the contents of glucose-soybean meal, glucose-phosphates, and soybean meal-phosphates, respectively, while the content of the third variable was kept at a constant level.

The effects of the initial contents of glucose and soybean meal at a constant concentration of phosphates (1.5 g/L) on the diameter of inhibition zone toward \textit{S. aureus} are presented in Figure 1. Figure 1 shows that, in the applied experimental conditions, the glucose content affects the zone diameter to some extent, while the use of the lowest amount of soybean meal maximizes the diameter of inhibition zone. The initial content of soybean meal had an almost constant effect on the tested response for all initial glucose concentrations. For all the amounts of glucose the increase in the starting amount of soybean meal results in an almost linear decrease of the inhibition zone diameter from about 20 mm to about 13 mm. This can be explained by the fact that antibiotics are products of secondary metabolism, so that the reduction of the starting amount of nitrogen in the medium limits the biomass growth and stimulates the synthesis of the desired component. For the same initial values of soybean meal the smallest inhibition zone diameters are obtained when the content of glucose was between 25.0 g/L and 35.0 g/L, while the lowest and highest values for glucose result in an almost uniform diameter of the inhibition zone of about 20 mm.
Figure 1. Response surface curve of the inhibition zone diameter toward \textit{S. aureus} as a function of the contents of glucose and soybean meal for the content of phosphates of 1.5 g/L.

Figure 2. Response surface curve of the inhibition zone diameter toward \textit{S. aureus} as a function of the contents of glucose and phosphates for the content of soybean meal of 15.0 g/L.

The combined influence of glucose and phosphates on the inhibition zone diameter against \textit{S. aureus}, with a constant content of soybean meal (15.0 g/L) is shown in Figure 2. As it can be seen from the figure, the increase in the amount of glucose at the minimal
The initial amount of phosphates results in the minimum inhibition zone diameter of about 10 mm, while at the maximum initial amount of phosphates gives the reverse result, the maximum inhibition zone diameter of about 19 mm. When the medium values of glucose were used, no significant effect of phosphates on the responses was observed. It can be noticed that for the highest production of antibiotic effective against applied test microorganism, in the applied experimental condition, one must use the lowest contents of glucose and phosphates (10.0 g/l and 0.5 g/L, respectively) or the highest content of both nutrients (50.0 g/L of glucose and 2.5 g/L of phosphates).

Figure 3. Response surface curve of the inhibition zone diameter toward *S. aureus* as a function of the contents of soybean meal and phosphates for the glucose content of 30.0 g/L

The response surface curve of the inhibition zone diameter of cultivation medium against *S. aureus* as a function of the contents of soybean meal and phosphates at a constant content of glucose (30.0 g/L) is presented in Figure 3. Based on the shape of the three-dimensional plot it can be seen that the diameter of the inhibition zone for all concentrations of soybean meal does not depend of the initial content of phosphates. For all used initial concentrations of phosphates the increase in the amount of soybean meal produced a decrease in the diameter of inhibition zone of antibiotic effective against *S. aureus* from about 20 mm to about 12 mm.

**Optimization of the antibiotic cultivation medium**

The final goal of the application of the response surface procedure is the optimization of the observed process, so that the developed models can be used for simulations and optimizations. The observed responses are the residual sugar, residual nitrogen, and the
inhibition zone diameter for the applied test microorganism. To optimize the composition of culture medium for the production of antibacterial agents a number of factors must be taken into consideration, and it is necessary that the process is the most economical and environmentally friendly. The effluents from the process should not contain large amounts of nutrients that, on the one hand require additional processing prior to their release into the environment, and represent an economically unjustified use of substrates on the other, since increase the cost of the entire process. In accordance with the aforementioned, the residual sugar and residual nitrogen were minimized, while the inhibition zone diameter against \textit{S. aureus} is maximized. The obtained model (Table 3) with a desirability function \((D)\) of 0.985, predicts that the maximum inhibition zone diameter (21.88 mm) against microorganism \textit{S.aureus}, minimum amount of residual sugar (0.89 g/L) and the minimum amount of residual nitrogen (0.24 g/L), in applied experimental conditions, is achieved when the contents of nutrients in the cultivation medium are: glucose 10.0 g/L, soybean meal 5.0 g/L and phosphates 1.04 g/L. Looking at it in absolute terms, the predicted minimum values of responses for the residual sugar and residual nitrogen corresponds to the values at which fermentation is usually terminated in the industrial conditions.

\textbf{Table 3. Desirability function of the optimized model}

<table>
<thead>
<tr>
<th>Variable/Responses</th>
<th>Conditions</th>
<th>Optimum values</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (g/L) in the range</td>
<td>10.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean meal (g/L) in the range</td>
<td>5.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphates (g/L) in the range</td>
<td>1.04</td>
<td></td>
<td>0.985</td>
</tr>
<tr>
<td>Inhibition zone diameter (mm)</td>
<td>max</td>
<td>21.88</td>
<td></td>
</tr>
<tr>
<td>Residual glucose (g/L) min</td>
<td>0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual nitrogen (g/L) min</td>
<td>0.24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The effects of the concentrations of glucose and soybean meal on the value of desirability function are displayed in the contour diagram for the constant value of phosphates (1.00 g/L), as shown in Figure 4.

The highest values of desirability function, as can be seen from Figure 4, are obtained in the region of low initial concentrations of both glucose and soybean meal. From the presented figure it can be seen that the range in which the desirability function has values higher than 0.900 is very limited, and includes the initial glucose concentrations lower than 15 g/L, while the initial concentration of soybean meal is somewhat lower than 10 g/L, when the phosphates content was 1.00 g/L.

To validate the developed mathematical model, additional experiments were conducted and the results are presented in Table 4. It can be seen that the experimental values for all three modeled responses are in good agreement with the predicted values. In average, the experimentally obtained inhibition zone diameters of 21.98 mm with the standard deviation was 0.45. For the residual sugar and residual nitrogen, the average values of additional experiments were 0.86 g/L and 0.22 g/L, with the matching standard deviations 0.05 and 0.03, respectively.
Table 4. Model responses for three independent experiments to validate the adequacy of the obtained model

<table>
<thead>
<tr>
<th>Run</th>
<th>Glucose (g/L)</th>
<th>Soybean meal (g/L)</th>
<th>Phosphates (g/L)</th>
<th>Inhibition zone diameter (mm)</th>
<th>Residual glucose (g/L)</th>
<th>Residual nitrogen (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.0</td>
<td>5.00</td>
<td>1.04</td>
<td>22.12</td>
<td>0.81</td>
<td>0.22</td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
<td>5.00</td>
<td>1.04</td>
<td>21.48</td>
<td>0.91</td>
<td>0.19</td>
</tr>
<tr>
<td>3</td>
<td>10.0</td>
<td>5.00</td>
<td>1.04</td>
<td>22.35</td>
<td>0.86</td>
<td>0.25</td>
</tr>
</tbody>
</table>

CONCLUSION

The results of this study showed that the Box-Behnken experimental design and response surface methodology can be successfully used to determine the optimal concentration of glucose, soybean meal and phosphates in the culture medium for the production of antibiotics effective against the pathogenic bacteria *S. aureus*. Based on the results, it can be concluded that the model with a desirability function of 0.985 was obtained for the lowest tested contents of glucose and soybean meal, while the content of phosphates had its medium value. The obtained model included the limitations of the minimum amount of residual sugar and nitrogen and a maximum diameter of inhibition zone toward tested microorganism. The chosen method of optimization of medium composition was efficient, relatively simple, and time and material saving.
Acknowledgement

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REFERENCES


**ОПТИМИЗАЦИЈА ХРАЊИВЕ ПОДЛОГЕ ЗА ПРОИЗВОДЊУ АНТИБИОТСКИХ АГЕНЕСА**

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Циљ овог истраживања представља оптимизација састава хранљиве подлоге за производњу антибиотика који делује на патогену бактерију Staphylococcus aureus када је као производни микроорганизам примењен Streptomyces spp. изолован из природног окружења. Након биосинтезе, култивационе течности су анализирани у погледу садржаја резидуалног шећера и азота док је антибиотска активност култивационе течности одређивана диск-дифузионом методом. Сви експерименти су изведени у складу са Box-Behnken-овим експерименталним планом варирањем три фактора на три нивоа (глукоза: 10,0; 30,0 и 50,0 g/L; сојино брашно: 5,0; 15,0 и 25,0 g/L; фосфати: 0,5; 1,0 и 1,5 g/L) док је оптимизација одабраних параметара изведена применом методе одзивне површине. Добијени модел са вредношћу је могућ пречник зоне просветљења (21,88 mm) формира култивациона течност која садржи 10,0 g/L глукозе, 5,0 g/L сојиног брашна и 1,04 g/L фосфата.

Кључне речи: антибиотска активност, хранљива подлога, RSM, Streptomyces spp.