

Optimization of glycerol-based medium composition for antifungal metabolites production by *Bacillus subtilis*

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Bacillus species are promising producers of various compounds that have pronounced antimicrobial, antiviral and antitumor activities. Due to its GRAS status, *Bacillus subtilis* represents an excellent candidate for the usage in plant pathogens biocontrol. In this research, evaluation of antifungal metabolites biosynthesis by *Bacillus subtilis* ATCC 6633 and optimization of glycerol-based medium composition, using response surface methodology, for the production of compounds effective against *Neurospora crassa* were investigated. The results of disc-diffusion method indicate that applied *Bacillus* strain produces compounds with antifungal activity against tested fungus. In order to find optimal cultivation medium composition, the experiments were carried out in accordance with Box-Behnken design, and optimization was performed using the concept of desirability function combined with previously defined mathematical equation, which describes examined bioprocess. The optimization model predicts that maximum inhibition zone diameter against *Neurospora crassa* of 32.24 mm is achieved when initial content of glycerol, NaNO₂ and K₂HPO₄ were 49.68 g/L, 2.90 g/L and 6.49 g/L, respectively. Additionally, the second optimization set was made to minimize the consumption of medium components and costs of medium preparation. The obtained results are the basis for further research aimed to develop medium appropriate for economically justified production of bioactive compounds at industrial scale.

Keywords: *Bacillus subtilis*. Antifungal metabolites. *Neurospora crassa*. Cultivation medium composition. Response surface methodology.

INTRODUCTION

Fungi are worldwide responsible for crops and food raw materials contamination and some human and animal diseases (Rajaofera *et al.*, 2018). Fungal growth and mycotoxins formation in agricultural products cause not only health and food safety issues, but also huge economic losses (Mwakinyali *et al.*, 2019). Hence, prevention of fungal contamination is very important for the food industry and agriculture as well as human health in general. Synthetic fungicides based on different chemicals are usually used to control the growth and

reproduction of fungi (Rajaofera *et al.*, 2018). Besides possible toxicological risks, synthetic fungicides may show various degrees of undesirable side effects that can lead to the appearance of fungicide-resistant populations (Leelasuphakul, Hemmanee, Chuenchitt, 2008). Therefore, novel studies are focused on finding alternative solutions to replace the usage of chemicals in food crops disease treatment. Application of natural products obtained from plants is limited because its chemical composition and biological activities may vary depending on the geographical region, collecting time and plant source (Meneses, Durango, García, 2009). Another disadvantage is that some plants' metabolites may show different antifungal activity against various strains from the same genera (Moro *et al.*, 2017). The use of antagonistic microorganisms that produce inhibitory

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substances for fungal pathogens has shown to be one of the most promising solutions (Radovanović *et al.*, 2018).

Production of antifungal metabolites is a feature of different kinds of bacteria and fungi and may represent a survival mechanism whereby organisms can eliminate competition and colonize a niche. Due to their ability to synthesize more than 5000 different bioactive compounds, microorganisms of genera *Bacillus*, *Penicillium*, *Streptomyces*, *Cephalosporium* and *Micromonospora* are mostly used in biological control of plant pathogens (Khan *et al.*, 2011). Members of the *Bacillus* genera are the soil bacteria that produce a high proportion of agriculturally important compounds (Rajaofera *et al.*, 2018; Leelasuphakul, Hemmanee, Chuenchitt, 2008). The genus *Bacillus* comprises 377 species (Caulier *et al.*, 2019) which produce biofungicides, enzymes that degrade fungal structural polymers and antifungal volatiles (Radovanović *et al.*, 2018; Leelasuphakul, Hemmanee, Chuenchitt, 2008). Among the *Bacillus* genus, *Bacillus subtilis* is highly promising producer of more than 70 different metabolites with antimicrobial activity (Fernandes *et al.*, 2007). It is reported that *Bacillus subtilis* have approximately 4–5 % of its entire genome devoted solely to antibiotic synthesis (Wise *et al.*, 2012). In addition, GRAS (Generally Regarded as Safe) status qualifies *Bacillus subtilis* as an excellent candidate for the usage in biocontrol of phytopathogenic microorganisms (Leelasuphakul, Hemmanee, Chuenchitt, 2008).

Antifungal compounds produced by microorganisms are secondary metabolites whose biosynthesis begins at the early stage of the stationary phase (Leelasuphakul, Hemmanee, Chuenchitt, 2008). Hence, it is important to formulate the medium composition so that favors their synthesis with limited growth of biomass. The proper selection of carbon, nitrogen and phosphorus sources, as the most important nutrients in the cultivation medium, and precise definition of their concentrations are essential to increase the yield of desired metabolites (Grahovac *et al.*, 2014).

Production of bioactive compounds using bacterial strains is greatly influenced by variation of carbon sources in cultivation media. According to literature data, glycerol is suitable substrate for the cost-effective biosynthesis of antimicrobial compounds by *Bacillus subtilis* (El-

Banna, 2005). Crude glycerol is the main by-product of biodiesel production. Considering that glycerol from biodiesel manufacturing process is an impure compound, the significant cost of purification prevents its use in the food and pharmaceutical industries (Brandão *et al.*, 2013). In addition, the growth of biodiesel production results in an increase in the amount of crude glycerol which represents the huge problem since its disposal in the environment is not allowed (de Sousa *et al.*, 2014). Glycerol bioconversion into valuable products, such as bioactive compounds, reduces production costs of desired metabolite as well as environmental problems caused by accumulation of this waste (da Silva, Mack, Contiero, 2009; Konstantinović *et al.*, 2016). Different inorganic salts, such as ammonium salts, nitrates, nitrites and phosphates are used as nitrogen and phosphorus sources in media for antifungal metabolites production (Fernandes *et al.*, 2007; El-Banna, Quddoumi, 2007). Their addition should be used in limited amount because of the negative influence on the biosynthesis. When the level of these nutrients in media is low, the rate of producing microorganism cells growth is lower and synthesis of secondary bioactive metabolites is favored (El-Banna, Quddoumi, 2007).

In order to ensure maximal biosynthesis of desired antimicrobial compounds it is necessary to identify the best medium composition and process conditions for cultivation of selected producing strain. Response surface methodology (RSM) combined with appropriate experimental design is considered as an adequate technique for bioprocess modeling and optimization (Deepak *et al.*, 2008). RSM is a well-known method, often applied in biotechnology for evaluation of the varied factors effects and for building of mathematical models that describe examined bioprocess and can be used to define optimal conditions for multi-response systems (Wang, Liu, 2008). One of the main advantages of this approach is its possibility to study many variables simultaneously with a low number of observations, saving not just time, but also costs, (El-Sersy, Ebrahim, Abou-Elela, 2010; Deepak *et al.*, 2008).

The aims of this study were the evaluation of antifungal metabolites biosynthesis by *Bacillus subtilis* ATCC 6633 and optimization of glycerol-based medium

composition, in terms of carbon, nitrogen and phosphorus sources content, using response surface methodology, for the production of desired bioactive compounds. Test microorganism used in these experiments was local wild-type *Neurospora crassa* strain.

MATERIAL AND METHODS

Microorganisms

The reference strain *Bacillus subtilis* ATCC 6633 was used in these experiments as producing microorganism. *Neurospora crassa*, used in this research as test microorganism for *in vitro* determination of antifungal activity, was the wild-type strain isolated from contaminated dried sugar beet pulp, grown on the territory of the Republic of Serbia and aimed to use as animal feed. The pure cultures of both microorganisms were stored on agar slants at 4°C and subcultured every four weeks. The commercial semi-synthetic medium, Nutrient Agar (HiMedia®, India), was used as storage medium for producing microorganism, while the medium used for storage of test microorganism was Sabouraud-Maltose Agar (HiMedia®, India).

Inoculum preparation

The inoculum was prepared in two steps. First, the producing culture was refreshed on agar slant (Nutrient Agar, HiMedia®, India) in test tube by stationary incubation at 28°C for 48 h. Second step was involved the cells multiplication by double passaging procedure. A loop of producing microorganism cells was transferred from a freshly prepared agar slant into 25 mL Erlenmeyer flasks containing 10 mL of liquid medium (Nutrient Broth, HiMedia®, India). Second passage was inoculated with broth obtained from the first and performed in 250 mL Erlenmeyer flasks containing 100 mL of cultivation medium. The incubation of each passage was carried out in aerobic conditions at 28°C and 150 rpm (laboratory shaker KS 4000i control, Ika® Werke, Germany) for 24 h. The prepared inoculum was used for inoculation of all investigated biosynthesis media.

Biosynthesis conditions

Production of antifungal metabolites was carried out in 300 mL Erlenmeyer flasks containing 100 mL of cultivation medium with appropriate composition (flask volume to medium volume ratio of 3:1 was applied). The media used for biosynthesis were Nutrient Broth (HiMedia®, India) as well as media formulated in accordance with the selected experimental design. In these media contents of glycerol (20.0-50.0 g/L), NaNO₂ (1.0-3.0 g/L) and K₂HPO₄ (5.0-15.0 g/L) were varied. Media also contained yeast extract (0.5 g/L), CaCO₃ (1.7 g/L), MgSO₄·7H₂O (0.5 g/L) and MnSO₄·4H₂O (0.05 g/L). The pH value of all media was adjusted to 7.0±0.2 prior to sterilization by autoclaving at 121°C and 2.1 bar for 20 min. The inoculation was performed in sterile conditions by adding 10% (v/v) of inoculum prepared as previously described. The biosynthesis of compounds with antifungal activity was carried out under aerobic conditions at 28°C and 150 rpm (laboratory shaker KS 4000i control, Ika® Werke, Germany) for 96 h.

Determination of biomass concentration

The biomass concentration was estimated based on the turbidity of cultivation broth. Turbidity measurement was carried out spectrophotometrically, measuring the absorbance of the cultivation broth samples at a wavelength of 660 nm.

Determination of glycerol content

Glycerol content in supernatants, obtained by centrifugation of cultivation broths samples at 10000 rpm (Rotina 380 R, Hettich Lab Technology, Germany) for 15 min, was determined by high pressure liquid chromatography (HPLC). The samples were filtered through a 022 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. 70% (v/v) acetonitrile was used as

eluent with a flow rate of 1.0 mL/min and elution time of 10 minutes at column temperature of 30°C.

Determination of antifungal activity

At the end of the bioprocess, samples of cultivation broths were centrifuged at 10000 rpm (Rotina 380 R, Hettich Lab Technology, Germany) for 15 min. Obtained supernatants were concentrated by evaporation on a rotary vacuum evaporator (Ika® Werke, Germany) at 45°C to one tenth of the initial mass, filtered throughout a 0.22 µm nylon membrane filter (Agilent Technologies Inc, Germany) and then analyzed. The antifungal activity of prepared samples against selected test microorganisms was determined *in vitro* by disc-diffusion method (Bauer *et al.*, 1966). The spore suspension of test microorganism was homogenized with Sabouraud-Maltose Agar medium (HiMedia®, India) and poured into Petri dishes. Sterile 5 mm disks (HiMedia®, India) were placed on the inoculated agar plates and impregnated with 10 µL of the concentrated samples. After the incubation of test plates at 28°C for 72 h, the diameters of inhibition zones were measured and recorded in millimeters (mm). The evaluation of antifungal activity was carried out in three repetitions.

Experimental design and data analysis

The selection of experimental design is a key step in the application of the RSM. The effects of carbon, nitrogen and phosphorus sources content in cultivation media on antifungal metabolites production were investigated using an experimental design proposed by Box and Behnken with three factors on three levels and three repetitions in the central point (Ferreira *et al.*, 2007). Independent variables, i.e. examined factors and their values are glycerol content (X_1 : 20 g/L, 35 g/L and 50 g/L), NaNO_2 content (X_2 : 1 g/L, 2 g/L and 3 g/L) and K_2HPO_4 content (X_3 : 5 g/L, 10 g/L, 15 g/L).

For description of the response Y, i.e. inhibition zone diameter (mm), a second degree polynomial model was fitted to data:

$$Y_{1-10} = b_0 + b_1 \cdot X_1 + b_2 \cdot X_2 + b_3 \cdot X_3 + b_{11} \cdot X_1^2 + b_{22} \cdot X_2^2 + b_{33} \cdot X_3^2 + b_{12} \cdot X_1 \cdot X_2 + b_{13} \cdot X_1 \cdot X_3 + b_{23} \cdot X_2 \cdot X_3 \quad (1)$$

where b_0 represents intercept, b_1 , b_2 and b_3 represent linear, b_{11} , b_{22} , b_{33} represent quadratic, and b_{12} , b_{13} and b_{23} represent interaction effect of the factors.

Statistical and graphical analyses of the experimental results and mathematical modeling were performed using Statistica software v. 13.2 (Dell Inc., USA). The data were statistically processed by the analysis of variance (ANOVA) at the significance level of $\alpha=0.05$. The adequacy and significance of generated model were evaluated by determination coefficient (R^2) and p -value of the model. The individual effects of factors and their interactions were estimated based on their t -values and p -values. Defined mathematical equation and method of desirability function were applied for determination of optimal values of examined factors, which was carried out in the software package Design-Expert 8.1. (Stat-Ease, Inc., USA).

RESULTS AND DISCUSSION

Experimental results

This research was conducted to estimate the production of compounds effective against *Neurospora crassa* isolate by *Bacillus subtilis* ATCC 6633. In the environment, the members of filamentous fungus from the genus *Neurospora*, including species *Neurospora crassa* are responsible for the degradation of burned plant material (Jacobson *et al.*, 2006; Turner, Perkins, Fairfield, 2001). In addition, *Neurospora crassa* strains are widely used as model organisms for determination of antifungal activity of different biological agents (El-Mounadi *et al.*, 2016; Koch *et al.*, 2014). The results obtained in this investigation are specific and unique because they indicate the antifungal activity of compounds produced by reference *Bacillus subtilis* strain against local wild-type *Neurospora crassa* isolate.

The selected producing strain was grown in nutrient broth to test its ability for antifungal metabolites biosynthesis. Inhibition zone diameter of 10 mm obtained by diffusion-disc method indicates that selected *Bacillus* strain produces compounds with antimicrobial activity against tested fungal isolate. However, nutrient broth is not cost effective medium for the industrial production

of biological control agents. Furthermore, the obtained yield of desired compounds is not satisfactory because this medium does not fulfill all requirements in terms of nutrients necessary for growth of the cells as well as for biosynthesis of secondary metabolites (Kumar, Saini, Shrivastava, 2009). Therefore, this study was also aimed to define optimal medium in order to reduce production costs and increase product yield.

Optimization of medium composition was performed using response surface methodology combined with Box-Behnken design and desirability function approach. Media

for antifungal metabolites production by selected *Bacillus* strain were formulated by varying content of glycerol, NaNO_2 and K_2HPO_4 . Values of examined factors were selected based on available literature data (El-Banna, Quddoumi 2007; de Faria *et al.*, 2011; El-Banna, Quddoumi, 2016;), and along with the experimentally obtained values of selected response are given in Table I. The inhibition zone diameter against *Neurospora crassa* was considered as response because it is a direct indicator of the amount of produced antifungal metabolites in applied conditions (Cladera-Olivera, Caron, Brandelli, 2004).

TABLE I - Combinations of varied factors and values of selected response

Varied factors			Selected response
Glycerol content (X_1 , g/L)	NaNO_2 content (X_2 , g/L)	K_2HPO_4 content (X_3 , g/L)	Inhibition zone diameter (Y, mm)
20	1	10	29
50	1	10	29
20	3	10	28
50	3	10	27
20	2	5	30
50	2	5	31
20	2	15	29
50	2	15	25
35	1	5	23
35	3	5	30
35	1	15	22
35	3	15	14
35	2	10	24
35	2	10	23
35	2	10	22

The results presented in Table I show the effect of all tested samples on *Neurospora crassa* growth, i.e., the variable antifungal activity of cultivation broths with different initial nutrient content is evident. The measured

values of inhibition zone diameter were in the range from 14 mm to 31 mm. By comparing these results and values of inhibition zone diameter obtained using nutrient broth (10 mm) and nutrient yeast dextrose broth (14-21 mm)

(Kim *et al.*, 2003), it can be noticed that cultivation of *Bacillus subtilis* ATCC 6633 on glycerol-based medium resulted in better production of desired metabolites. The highest antifungal activity was achieved when cultivation medium initially contained 50 g/L glycerol, 2 g/L NaNO₂ and 5 g/L K₂HPO₄. The value of inhibition zone diameter in this case was 31 mm. The antifungal activity was also highly pronounced when cultivation medium contained 20 g/L glycerol, 2 g/L NaNO₂ and 5 g/L K₂HPO₄ as well as 35 g/L glycerol, 3 g/L NaNO₂ and 5 g/L K₂HPO₄. The obtained results indicate that the highest production of antifungal compounds by selected *Bacillus* strain was obtained when concentration of K₂HPO₄ in cultivation medium was minimal (5 g/L). This is probably due to the fact that phosphorus is limiting nutrient for the biosynthesis of secondary metabolites (Lang, Glassey, Archibald, 1982). In addition, it can be noticed that concentration of carbon and nitrogen sources has significant effect on antifungal activity of broths obtained by cultivation of applied producing microorganism. This observation is in accordance with literature data which suggest that production of antimicrobial substances depends on nutrient (C and N) limitation (El-Banna, 2005; El-Banna, Quddoumi 2007).

From the results given in Table I, it is clear that increase in NaNO₂ and K₂HPO₄ content in medium leads to decrease in the inhibition zone diameter. This can be explained by the fact that higher concentration of nitrogen and phosphorous sources stimulates bacterial growth so their consumption is more directed to biomass accumulation than to biosynthesis of secondary metabolites (Demoling, Figueroa, Bååth, 2007).

Statistical analysis of modeled response

The statistical analysis of the modeled response was performed in the form of analysis of variance (ANOVA), which is important tool for determining the adequacy and significance of quadratic models. The analysis was done by means of Fisher's F-test. In general, the F-value with a low *p*-value indicates high significance of the regression model. According to the ANOVA summary results given in Table II, defined mathematical model was found to be statistically significant at the 99% confidence level, which is confirmed by extremely low *p*-value (*p*=0.000002).

TABLE II - Analysis of variance (ANOVA) of modeled response

	Residual			Model					
	SS	DF	MS	SS	DF	MS	F-value	<i>p</i> -value	R ²
Y	15.500	5	3.100	10204.500	10	1020.450	329.177	0.000002	0.946

The coefficient of determination (R²) was used to check the adequacy of generated model. The high value of this parameter (0.946), shown in Table 2, suggests the very good fit of experimental results with the second-order polynomial model. This means that only 5.4% of the total variations in the analyzed data could not be

explained to a satisfactory degree by defined mathematical model. Additionally, comparison between experimental and predicted values of inhibition zone diameter against *Neurospora crassa* is graphically presented in Figure 1. The points clustered around the diagonal line indicate the significant correlation between mentioned data groups.

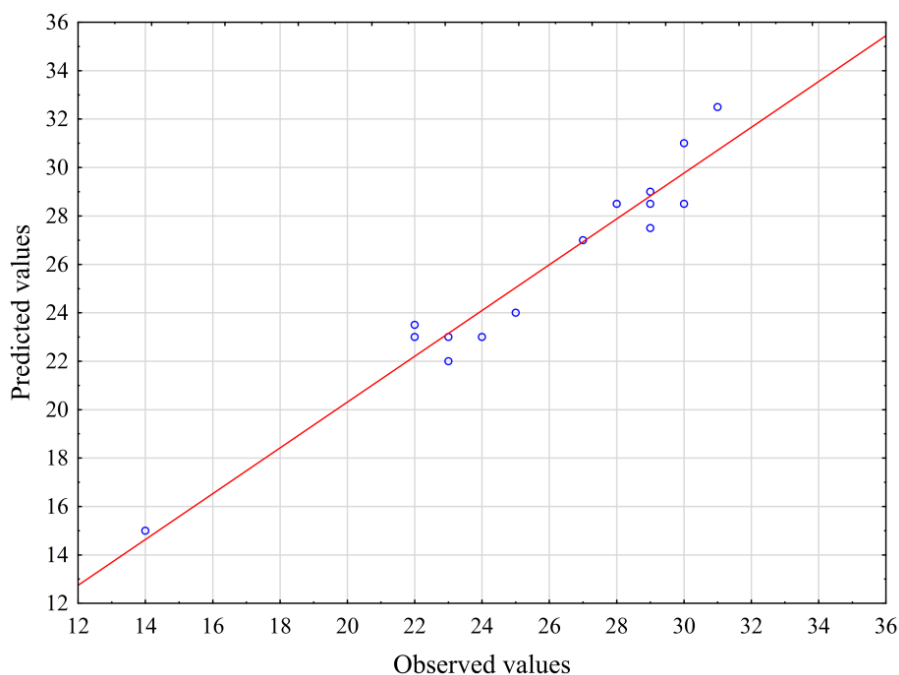


FIGURE 1 - The correlation between observed and predicted values of inhibition zone diameter against *Neurospora crassa*.

Mathematical model of the inhibition zone diameter against *Neurospora crassa*

Applying response surface methodology, the results of the Box-Behnken design (Table I) were fitted with a second-degree polynomial model, which resulted in generation of mathematical relationship between response and factor variables:

$$Y_1 = 38.153 - 1.661 \cdot X_1 + 10.083 \cdot X_2 + 1.583 \cdot X_3 + 0.026 \cdot X_1^2 - 0.625 \cdot X_2^2 - 0.005 \cdot X_3^2 - 0.017 \cdot X_1 X_2 - 0.017 \cdot X_1 X_3 - 0.075 \cdot X_2 X_3 \quad (2)$$

where Y was the predicted value of inhibition zone diameter against *Neurospora crassa*, and X_1 , X_2 and X_3 were the coded values of glycerol content, NaNO_2 content and K_2HPO_4 content, respectively.

A positive sign of the linear and quadratic coefficients in obtained regression equation points to a

direct relationship between the variables, while negative sign of these coefficients indicates an inverse relationship. On the other hand, a positive sign of the interaction coefficients refers to a synergistic effect of certain factors, but if these coefficients are negative, the antagonistic effect on the analyzed response is evident.

The individual effects of varied medium components and their interactions can be discussed from the Pareto charts presented in Figure 2. The length of each bar is proportional to the absolute value of its associated regression coefficient or estimated effect. The effects of all parameters, interactions as well as quadratic effects, are standardized (each effect is divided by its standard error). The order in which the bars are displayed corresponds to the order of the effect size. The charts also include a vertical line that corresponds to the 95% limit indicating statistical significance. An effect is, therefore, significant ($p < 0.05$) if its corresponding bar crosses this vertical line.

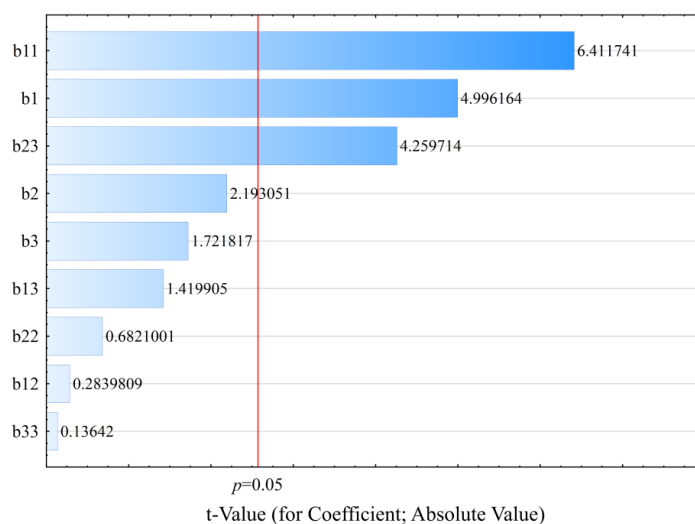


FIGURE 2 - Pareto chart of standardized effects for inhibition zone diameter against *Neurospora crassa*.

According to the results shown in Figure 2, it is evident that the linear and quadratic coefficients of initial glycerol content as well as coefficient that describes the interaction between NaNO_2 and K_2HPO_4 content in cultivation medium have statistically significant effect on the antifungal metabolites production in applied experimental conditions. Among them, the interaction coefficient has the lowest influence on the observed response. This can be explained by the fact that carbon is essential nutrient for both, cell growth and secondary metabolites biosynthesis (Thompson *et al.*, 2006).

The mathematical model of the inhibition zone diameter against *Neurospora crassa* can be presented graphically by the response surface plots, which provide a visual interpretation of the interaction between two factors and facilitate the location of their optimal values. In order to understand the effects of varied medium constituents on the antifungal metabolites production by *Bacillus subtilis* ATCC 6633 in applied conditions, these dimensional plots were generated (Figure 3, 4 and 5). Each figure represents the influence of two factors on selected response, whereas the third factor had central value from the experimental design.

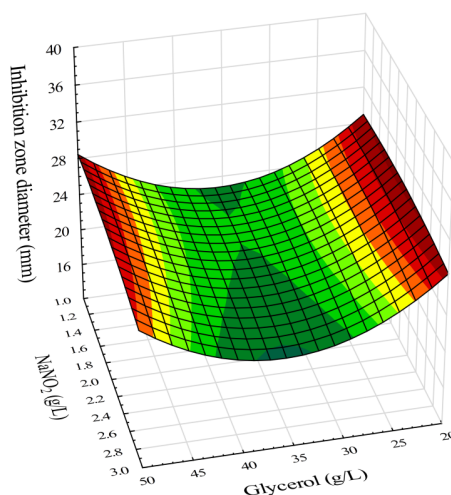


FIGURE 3 - The effects of glycerol and NaNO_2 contents on the inhibition zone diameter against *Neurospora crassa* at K_2HPO_4 content of 10 g/L.

The effects of initial glycerol and NaNO_2 content on the inhibition zone diameter against *Neurospora crassa* formed by the medium with constant content of K_2HPO_4 (10 g/L) are presented in Figure 3. From this response surface plot it is evident that, in applied experimental conditions, antifungal compounds production does not depend on the initial content of used inorganic nitrogen source. On the other hand, initial glycerol content in cultivation medium greatly affects the value of selected response. According to the predictions of the mathematical model, maximal inhibition zone diameter of about 29 mm is noted at initial glycerol content of 20 g/L for all investigated concentrations of NaNO_2 . In addition, it can be observed that increase in the initial glycerol content to about 35 g/L leads to decrease in the value of inhibition zone diameter, while further increase in this carbon source concentration results in an increase in the value of selected response. Hence, it is clear that there is no linear correlation between glycerol

content and metabolic activity of applied producing strain. This situation can be explained by the facts from previously published literature. Decrease in the values of observed response with increasing substrate concentration indicates that higher concentration of carbon source in cultivation medium can negatively affect the biosynthesis of secondary metabolites, such as desired antimicrobial compounds (Veličković *et al.*, 2018). On the other hand, literature data indicate that metabolic activity of microorganisms is inhibited at high substrate concentration (Liu, 2013). Consequently, it can be stated that values of inhibition zone diameter obtained for media with initial glycerol content higher than 35 g/L are increased due to high residual glycerol content that has an inhibitory effect on growth of test microorganism. High concentrations of residual glycerol in analyzed samples are the results of limited metabolic activity of producing microorganism in media with high carbon source concentration.

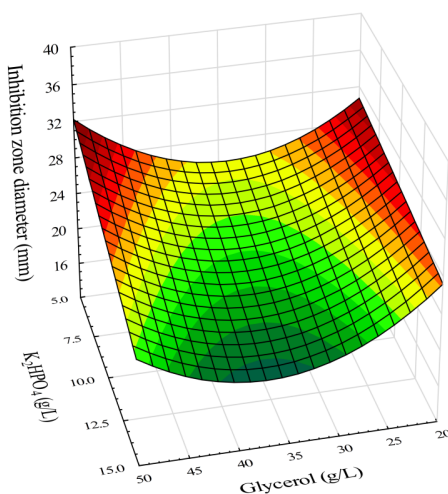


FIGURE 4 - The effects of glycerol and K_2HPO_4 contents on the inhibition zone diameter against *Neurospora crassa* at NaNO_2 content of 2 g/L.

Figure 4 illustrates the effects of initial glycerol and K_2HPO_4 content on the inhibition zone diameter against *Neurospora crassa* formed by the medium with constant content of NaNO_2 (2 g/L). Graphically presented results suggest an identical dependence of response value on the initial carbon source concentration as in the previous case.

Additionally, it can be seen that production of antifungal metabolites increases with a decrease in the initial K_2HPO_4 content in cultivation medium. The results shown in this figure indicate that the highest value of the inhibition zone diameter of about 30 mm is noted at initial glycerol content of 20 g/L or 50 g/L and initial K_2HPO_4 content of 5 g/L.

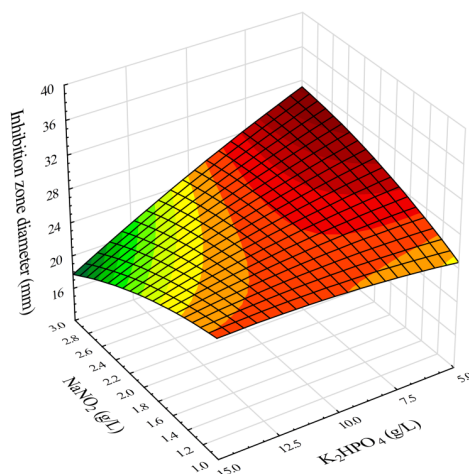


FIGURE 5 - The effects of NaNO_2 and K_2HPO_4 contents on the inhibition zone diameter against *Neurospora crassa* at glycerol content of 35 g/L.

The influence of initial NaNO_2 and K_2HPO_4 content on the inhibition zone diameter against *Neurospora crassa* formed by the medium with constant glycerol content (35 g/L) is given in Figure 5. From the graphically presented results it can be seen that increase in the initial NaNO_2 content at maximal investigated initial content of K_2HPO_4 (15 g/L) results in formation of minimal inhibition zone diameter. On the other hand, opposite results are obtained at minimal examined initial content of phosphorus source (5 g/L). This is in accordance with the negative value of interaction coefficient b_{23} (equation 2), which also points to an antagonistic effect of NaNO_2 and K_2HPO_4 content in medium on antifungal compounds production. However, maximal inhibition zone diameter against tested fungal isolate of about 30 mm is formed

by cultivation broth that initially contained 3 g/L NaNO_2 and 5 g/L K_2HPO_4 .

Optimization of medium composition

The final step in this research was definition of optimal content of carbon, nitrogen and phosphorus sources in glycerol-based medium to achieve maximal production of desired antifungal compounds. For this purpose, the concept of desirability function was applied since it is one of the most popular optimization procedures in biotechnology. Depending on the selected goals, two sets of optimization were made and the obtained results are presented in Table III.

TABLE III - Optimal values of varied factors and predicted values of selected response

Factors and response	First set		Second set	
	Goal	Predicted value	Goal	Predicted value
Glycerol (g/L)	in range	49.68	minimize	20.00
NaNO_2 (g/L)	in range	2.90	minimize	1.40
K_2HPO_4 (g/L)	in range	6.49	minimize	5.00
Inhibition zone diameter (mm)	maximize	32.24	maximize	28.69
Overall desirability function		1.00		0.91

The only goal in the first optimization set was to achieve maximal inhibition zone diameter against *Neurospora crassa*. The results presented in Table III indicate that overall desirability function has maximal value (1.00) when cultivation medium is contained of 49.69 g/L glycerol, 2.90 g/L NaNO₂ and 6.49 g/L K₂HPO₄. By applying the medium with that composition, developed model predicts formation of inhibition zone diameter of 32.24 mm.

In order to minimize the consumption of nutrients and therefore the cost of cultivation medium preparation, second optimization set was made. Thus, the maximal value of inhibition zone diameter and minimal initial content of varied medium constituents were selected as goals. According to the model prediction, the highest value of overall desirability function (0.91) is achieved when the initial content of glycerol, NaNO₂ and K₂HPO₄ are 20.00 g/L, 1.40 g/L and 5.00 g/L, respectively, while the expected inhibition zone diameter against *Neurospora crassa* is 28.69 mm.

By comparing the results of both optimization sets, reduction of consumption of glycerol by 59.74%, NaNO₂ by 51.72%, and K₂HPO₄ by 22.96% and a decrease of the inhibition zone diameter by 11.01% can be noticed. These results represent great basis for further techno-economic analysis of the bioprocess to select optimal medium composition for the production of antifungal compounds at industrial scale.

Antifungal metabolites production on optimal medium

In order to validate developed optimization model (second set in Table III), the production of desired antifungal metabolites was carried out in applied experimental conditions on glycerol-based medium with optimal nutrients content using *Bacillus subtilis* ATCC 6633. The biomass growth, the consumption of glycerol, and the production of compounds effective against local wild-type *Neurospora crassa* strain were examined during the 96 h of cultivation. The obtained results are graphically presented in Figure 6.

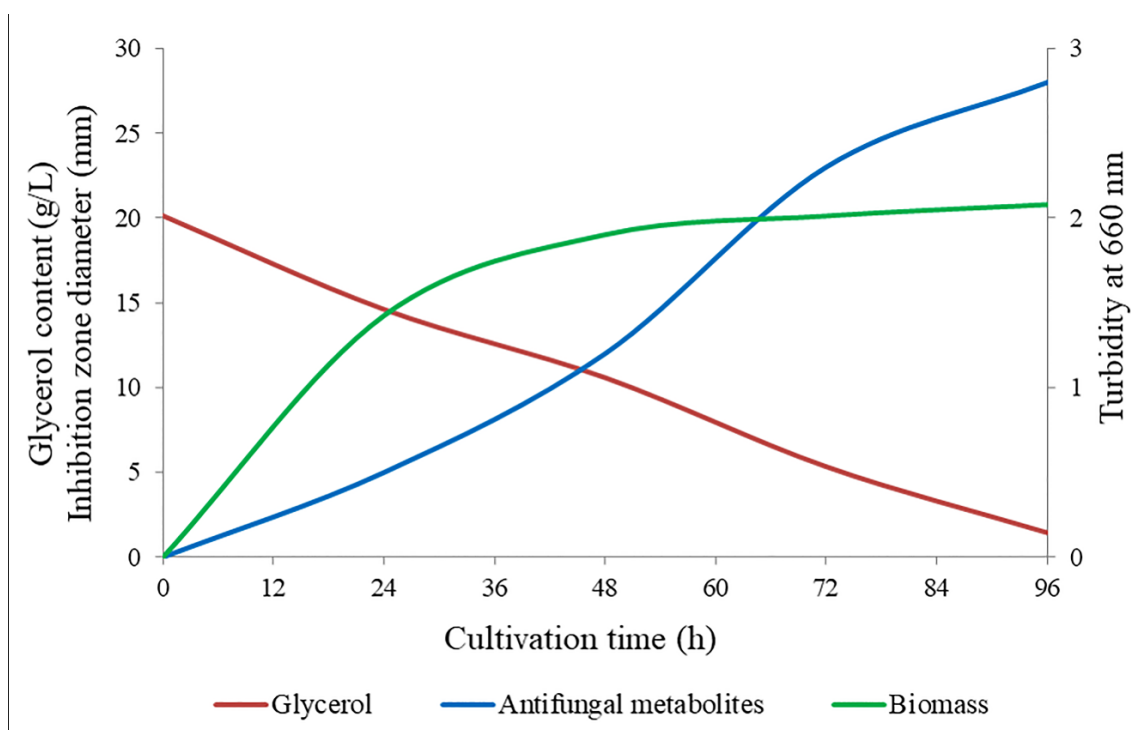


FIGURE 6 - Biomass growth, glycerol consumption and production of antifungal metabolites effective against *Neurospora crassa* during the cultivation of *Bacillus subtilis* ATCC 6633 on optimal medium.

The *Bacillus subtilis* cells growth during the cultivation was monitored by measuring the turbidity of the broth samples (green line in Figure 6). This measurement did not give an accurate number of bacterial cells in the cultivation broth, but the changes of biomass concentration could be monitored during the bioprocess. The results shown in Figure 6 indicate that producing microorganism cells growth started immediately after inoculation of the medium. It can be seen that during the first 48 h of cultivation biomass concentration increased almost in linear manner. This intensive producing microorganism cells growth corresponds to the exponential phase. With further prolongation of cultivation time, the increase of biomass concentration, i.e. changes in the turbidity of medium was insignificant. It is obvious that from this moment and till the end of cultivation the producing microorganism goes into the stationary phase.

The results shown in Figure 6 (red line) indicate that glycerol content significantly decreased during the cultivation, coinciding with an increase in biomass concentration and antifungal metabolites production. The residual content of carbon sources must be controlled at a very low level which is of particular importance for the bioprocess efficiency. After 96 h of cultivation the remaining glycerol content in culture medium was negligible (lower than 2 g/L), which is justified from economic and environmental viewpoint of biotechnological production.

Production of antifungal metabolites effective against selected *Neurospora crassa* isolate during the cultivation was monitored by determination of the inhibition zone diameter against test microorganism. According to the results presented in Figure 6 (blue line), the biosynthesis of antifungal compounds in applied experimental conditions started at exponential growth phase and continued during the stationary phase. Therefore, it cannot be stated that bioactive compounds production by *Bacillus subtilis* ATCC 6633 is growth-associated, because approximately 50% of antifungal metabolites was produced when producing microorganism cells growth reached stationary phase. It can be seen that maximum inhibition zone diameters of 28 mm was achieved after 96 h of cultivation. These

results are in excellent agreement with the predicted values (second set in Table III).

CONCLUSION

The research conducted within this study indicates that *Bacillus subtilis* ATCC 6633 has a great potential for the production of antifungal compounds effective against local wild-type *Neurospora crassa* strain. Response surface methodology combined with Box-Behnken design and desirability function approach proved to be appropriate bioprocess modeling and optimizing tool. The obtained results suggest that the highest inhibition zone diameter (32.24 mm) can be achieved if the cultivation medium is composed of 49.68 g/L glycerol, 2.90 g/L NaNO₂ and 6.49 g/L K₂HPO₄. These data are validated in laboratory conditions and represent reliable information for further investigation and consideration in order to develop economically acceptable medium for sustainable production of bioactive compounds at industrial scale.

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