PRODUCTION OF NIGERICIN AND NIPHIMYCIN BY SOIL ISOLATE *STREPTOMYCES* SP. MS1: ANTI-*CANDIDA* BIOASSAY GUIDED RESPONSE SURFACE METHODOLOGY FOR THE OPTIMIZED CULTURE MEDIUM [†]

UDC 579.873.7 : 615.332

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Abstract. *Anti-Candida bioassay guided optimization of the culture medium was used in order to enhance the antifungal activity of the soil isolate MS1. Its morphological, physiological and biochemical characteristics, as well as 16S rDNA sequencing, assigned an MS1 isolate to genus Streptomyces. Optimization of the culture medium was achieved by experimental factorial design and response surface methodology. Maximal antifungal components production was obtained with starch, soybean meal and phosphates content of 40.52, 5.10 and 2.21 g/L, respectively. Chromatography and 1H and 13CNMR spectroscopy were employed for purification and structural characterization of antifungal antibiotics concurrently produced by this strain. These antibiotics were identified as polyether carboxylic antibiotic nigericin and guanidyl-polyol macrolide, niphimycin.*

Key words: *antifungal, nigericin, niphimycin, Streptomyces sp., media optimization*

1. INTRODUCTION

Microbial metabolites are still attractive for fungicide development because of the variability in chemical structures and the chance that a newly discovered antifungal microbial metabolite could have a new mode of action and no cross-resistance to commercial fungicides (Fruh et al., 1996; Porter, 1985; Yarbrough et al., 1993). Also, effective compounds of natural origin are expected to be more advantageous than synthetic pesticides, as they have generally a lower environmental impact and are easily biodegradable. Streptomycetes produce a wide variety of commercially important polyketide compounds, including macrolide, polyene and polyether antibiotics, which exhibit antifungal activity. Usually, one *Streptomyces* sp. strain produces several structurally different antibiotics with the same antimicrobial activity. Polyether ionophores, such as nigericin, active against Gram positive bacteria and fungi, constitute a family of over 120 structurally related natural products with the ability to selectively chelate metal ions and transport them across cell membranes (Westley, 1985). A structurally different antibiotic, polyol macrolide niphimycin is also active against Gram positive bacteria and fungi with the alkylguanidinium chain playing a major role in its fungicidal activity in cooperation with the polyol lactone ring as the enhancer (Ogita et al., 2007; Usuki et al., 2006). Interestingly, members of these two families of compounds were previously found to be synthesized by *Streptomyces* spp., a while ago (Grabley et al., 1990). The source and concentration of some components in fermentation medium have a marked effect on different metabolites production. Hence, metabolic coordination for the purpose of enhancing an activity of interest is frequently done by adjusting carbon, nitrogen and phosphorus sources (Li et al., 2007).

Herein, we further describe molecular identification of previously isolated *Streptomyces* sp. MS1 (Grahovac et al., 2014). Bioassay guided approach using *Candida albicans* as test organism was applied in order to enhance the antifungal compounds production by *Streptomyces* sp. MS1 adjusting fermentation conditions, while the effect of different levels of the medium components was examined and optimized using response surface methodology. Purification and structural characterization of antifungal antibiotics concurrently produced by this strain under optimal conditions were done using chromatography and 1H and 13C NMR spectroscopy.

2. MATERIAL AND METHODS

2.1. Isolation, identification and maintenance of MS1 soil isolate

The producing microorganism was the isolate obtained from the soil sample taken from the territory of Novi Sad, Serbia (Grahovac et al., 2014). MS1 isolate was maintained on a solid mannitol-soy flour medium (MSF) (Kieser et al., 2000), stored at 4ºC and subcultured every four weeks. MS1 soil isolate was identified on the basis of its morphological, physiological and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology (Cross, 1989; Williams et al., 1983) and sequence analysis of 16S rRNA gene. The genomic DNA from MS1 isolate was extracted as previously described (Nikodinovic et al., 2003). 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGCTACCTTGTTACGACTT-3') primers (Lane, 1991) were used for the amplification of 16S rRNA gene, while PCR was performed using a KAPA 2G Robust Hot Start PCR Kit (KapaBiosystems, Cape Town, South Africa) under the manufacturer's instructions.

The amplified fragments were sequenced using Applied Biosystems 3130 Genetic Analyzer (Foster City, CA, USA). The Seqmatch tool was used to search for similar sequences compiled by the Ribosomal Database Project-II Release 9.4 (RDP; http://rdp.cme.msu.edu) (Cole et al., 2009) in parallel with BLASTN search (Altschul et al., 1997). The alignment of the obtained 16S rDNA sequence and sequences taken from RDP, which were all trimmed to the same length, was performed with the Clustal W 2.0 algorithm (Larkin et al., 2007). A phylogenetic tree was constructed by the maximum-likelihood algorithm using Jukes-Cantor distance correction and Bootstrap resampling method, all included in MEGA6 package (Tamura et al., 2013). The tree was rooted using 16S rDNA sequence of Streptomyces hindustanus IFO 15115 strain. 16S rDNA sequence of Streptomyces sp. MS1 was deposited under GenBank Accession number KT026467.

2.2. Cultivation of *Streptomyces* sp. MS1 for maximal production of antifungal compounds

Cultivation was carried out in an Erlenmeyer shake flasks containing one third of the cultivation medium, under aerobic conditions and an agitation rate of 150 rpm at the temperature of 27ºC for 7 days. In the cultivation medium. the contents of starch (10 g/L; 35 g/L and 60 g/L), soybean meal (5 g/L; 15 g/L and 25 g/L) and phosphates (0.5 g/L; 1.5 g/L and 2.5 g/L) were varied according to the experimental plan. Phosphates were added in the form of $(NH_4)_2HPO_4$ and K_2HPO_4 (2:1 ratio). Other components of the culture medium were: $CaCO_3$, (3.0 g/L), NaCl, (3.0 g/L), $MgSO_4$, (0.5 g/L). the culture media pH values were adjusted to 7.2±0.1 (Consort C863, Turnhout, Belgium). Erlenmeyer flasks with 100 mL of the culture medium were inoculated and incubated for 7 days at 26±1°C under aerobic conditions. A rotary shaker (150 rpm) was used for cultivation medium mixing. At the end of the cultivation, the separation of the solid from liquid phase in the cultivation medium was carried out by centrifugation at 10 000×g for 10 min (Eppendorf Centrifuge 5804, Germany). Residual carbon content was estimated after *Streptomyces* sp. MS1 cultivation according to the spectrophotometric method that involves starch staining with Lugol's iodine solution and measuring absorbance at 580 nm. Total nitrogen content was determined by the Kjeldahl method (Herlich, 1990). The value of RNA content, representing the indirect parameter of bacterial growth, was determined from the solid phase of the cultivation medium by the modified method of Munro and Fleck (1966) including prior proteins precipitation and absorbance measuring in a UV area at 260 nm. The results are expressed in mg of RNA per 100 g of cultivation liquid (mg/100 g CL).

2.3. Purification and identification of antifungal compounds from *Streptomyces* sp. MS1 culture

The extraction of *Streptomyces* sp. MS1 whole culture with ethyl acetate (1:1/vol:vol) was performed by vigorous mixing at 30ºC for 14 h. The ethyl acetate extract was separated from the cell debris by centrifugation (5000 rpm for 20 min at 4°C; Eppendorf 5804R bench top centrifuge). The mycelium residue was afterwards extracted with methanol (1/10 of the original culture volume) by vigorous mixing at 30ºC for 30 min. The methanolic extract was separated from the cell debris by centrifugation (5000 rpm for 20 min at 4°C; Eppendorf 5804R bench top centrifuge). Both extracts were then separately dried with anhydrous $MgSO_4$, followed by drying under vacuum. The methanolic extract of the mycelium residue was further purified by flash chromatography using silica gel 60 (230-400 mesh) and the collected fractions were analyzed by thin layer chromatography using alumina plates with 0.25 mm silica layer

(Kieselgel 60F254, Merck) and by UV–Vis spectral analysis. The following solvent system was used for the purification of nigericin: n-hexane and ethyl acetate (7:3 ratio, 100 mL), n-hexane and ethyl acetate (3:7 ratio, 100 mL), ethyl acetate and methanol (98:2 ratio, 100 mL), ethyl acetate and methanol (80:20 ratio, 100 mL), followed by methanol (30 mL). The purification of niphimycin was performed with the following solvent system: n-hexane and ethyl acetate (7:3 ratio, 100 mL), n-hexane and ethyl acetate (3:7 ratio, 100 mL), ethyl acetate and methanol (9:1 ratio, 100 mL), followed by methanol (60 mL). The appropriate fractions were combined, dried under vacuo, and weighted. The wave length scan of the purified fractions was done from 200 to 800 nm using a UV/Visible SpectophotometerUltrospec 3300pro (Amersham Biosciences). ^{1}H and ^{13}C NMR spectra of the purified fractions were recorded on a BrukerAvance II spectrometer (Bruker, Germany) operating at 400 and 100.6 MHz, respectively. DEPT and 2D experiments (HSQC) were run on the same instrument with the usual pulse sequences. All NMR spectra were measured at 25ºC in CDCl$_3$ with TMS as an internal standard. The purified fractions were also analyzed on an Agilent MSD TOF spectrometer coupled with an Agilent 1200 HPLC. The HPLC analysis was performed with a Zorbax Eclipse XDB-C18 RRHT column - 1.8 μm, 4.6 mm × 50 mm and the mobile phase consisted of water containing 0.2% formic acid (v/v) and acetonitrile.

2.4. Antimicrobial assays

To monitor the antifungal activity during culture medium optimization experiments, *Streptomyces* sp. MS1 culture supernatants against *Candida albicans* ATCC 10231 were tested. The volume of each sample was 10 μL of the supernatant evaporated to the one tenth of its initial mass. 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 suspension was diluted until the final cell concentration of 10^6cfu/mL was achieved. A Muller-Hinton agar (Torlak®) was used for growth of the test microorganism, after it was melted, cooled to 50±1°C and mixed in sterile conditions with prepared suspension of test microorganism in a 9:1 ratio. After the incubation at 35ºC for 48 h, the inhibition zones were measured by a HiAntibioticZoneScale ruler (Himedia®).

Standard disc diffusion assay with *C. albicans* ATCC 10231, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* NCTC1001 as test organisms was performed with the ethyl acetate extract of *Streptomyces* sp. MS1 culture and the methanolic extract of *Streptomyces* sp. MS1 mycelium. Briefly, the late stationary phase cells of the test microorganism were spread on Sabouraud dextrose agar plates (containing glucose, 40 g/L; peptone, 10 g/L; agar, 15 g/L; pH 5.8) for *C. albicans* and LB agar plates for bacteria (Sambrook et al., 1989). Sterile cellulose discs (6 mm diameter, HiMedia, Mumbai, India) were then applied to the surface of the plates containing test microorganisms. The extracts and purified compounds were dissolved in dimethyl sulfoxide (DMSO, 50 mg/mL) and applied on the discs (1-10 μL) and the same volume of the DMSO solvent as a control. The plates were incubated at 30°C and the zones of inhibition were measured after 24 h. As a positive control (0.15 mg/disc), nystatin was used for *C. albicans,* while gentamycin was used for *S. aureus* and *E. coli.*

Minimum inhibitory concentration (MIC) of the extracts was studied by using a previously reported 96-well microtiter plate assay (Brady et al., 2003). The microtiter plate-based assay allowed bacterial growth at 30°C and its inhibition to be assessed over time. A dilution series of the purified fractions were prepared in DMSO. Controls containing the solvent were carried out in each assay. MIC was determined to be the lowest concentration of the compound at which no evidence of growth was observed.

2.5. Statistical analysis

All experiments in this study were carried out in triplicate and the average values of the experimental results were calculated. The reproducibility of these measurements was good, and the standard deviations between parallel experiments were in the range of ± 4.7%. The experimental results were statistically analysed using the analysis of variance at the significance level of α = 0.05. A polynomial equation of the second-degree (1) was established to evaluate and quantify the influence of the factors variation on selected responses (inhibition zone radius, residual carbon content, residual nitrogen content and RNA content):

$$Y = b_o + \sum b_i X_i + \sum b_{ii}^2 X_{ii}^2 + \sum b_{ij} X_i X_j \qquad (1)$$

where b_0 is the intercept, b_i is the linear, b_{ii} the quadratic and b_{ij} the interaction coefficients. The factor variables are the contents of starch (X_1), soybean meal (X_2) and phosphates (X_3). Combinations of the experimental factors for each experiment were obtained using the Box-Behnken experimental design. The adequacy of the model was evaluated using the coefficient of determination (R^2) value. The significance of regression coefficients was assessed by their *p*-value at the 0.05 significance level. Statistica 12.0 Software (StatSoft Inc., USA) was used for a statistical and graphical analysis of the experimental data. The obtained response surface plots present the effect of two varied factors on a selected response, while the third factor was set to its medium value. The method of desirability function was applied for determination of examined factors optimal values, according to the assigned optimization results (Design-Expert 7.01, trial version, StatEase, Inc., USA).

3. RESULTS

3.1. Identification of the *Streptomyces* sp. MS1 isolate

MS1 bacterial strain was previously isolated from the soil sample collected in Novi Sad, Serbia (Grahovac et al., 2014). MS1 isolate sporulated on the MSF solid medium after 5 days of incubation and had white to grey spores (Fig. 1A). The isolate was identified as *Streptomyces hygroscopicus* on the basis of its morphological, physiological and biochemical characteristics (Cross, 1989; Williams et al., 1983). In order to confirm this result, molecular identification by 16S rDNA sequencing was carried out.
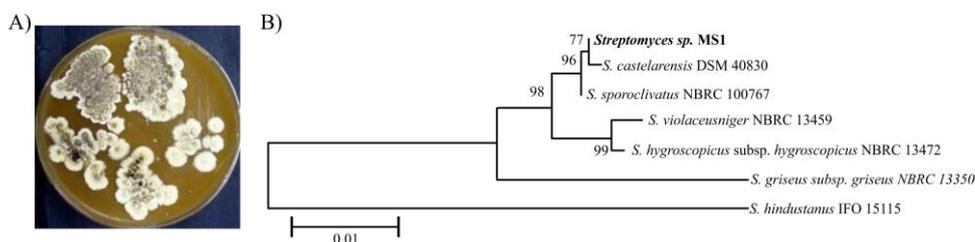
Fig. 1 Soil isolate MS1. A) Isolate growing and sporulating on MSF plates B) Phylogenetic tree showing the relationship between *Streptomyces* sp. MS1 strain and closely related members of the genus *Streptomyces* based on 16S rRNA gene sequences (constructed by the maximum-likelihood algorithm using Jukes-Cantor distance correction and Bootstrap resampling method).

A part of the 16S rDNA sequence of 1410 nucleotides was retrieved by PCR amplification from the total genomic DNA. Using 16S rDNA sequence analysis, strain *Streptomyces* sp. MS1 was confirmed to belong to genus *Streptomyces* (the sequence has been deposited under GenBank Accession number KT026467). A phylogenetic tree of 16S rDNA sequences was generated using sequences available in Ribosomal Database Project (Fig. 1B). The closest matches in Ribosomal Database Project were *Streptomyces castelarensis* DSM 40830, *Streptomyces sporoclivatus* NBRC 100767, *Streptomyces violaceusniger* NBRC 13459 and *Streptomyces hygroscopicus* subsp. *hygroscopicus* NBRC 13472. This was in agreement with the NCBI database analysis, where, for example, *Streptomyces* sp. MS1 16S rDNA sequence of 1410 nucleotides had 100% query coverage and 100% or 99% identity with the 16S rDNA sequences of *Streptomyces castelarensis* (GenBank KR063210) and *Streptomyces hygroscopicus* subsp. *hygroscopicus* (GenBankDQ445789) (data not shown).

3.2. Antifungal compound production and statistical analyses of modelled responses

In order to determine the optimal values of the cultivation medium components for antifungal compound production, Box-Behnken experimental design was performed. The experimental plan and the values of modelled responses (inhibition zone radius, residual carbon, nitrogen and phosphate content) are presented in Table 1.

Table 1 Combinations of experimental factors and values of modelled responses

Experimental factors			Responses			
Starch (g/L)	Soybean meal (g/L)	Phosphates (g/L)	Inhibition radius (mm)	Residual C (g/L)	Residual N (g/L)	RNA (mg/100g CL)
10	5	1.5	22	2.05	0.178	20.77
60	5	1.5	29	21.20	0.221	20.26
10	25	1.5	7	4.70	1.175	16.80
60	25	1.5	11	34.90	1.09	17.28
10	15	0.5	14	2.41	0.294	23.74
60	15	0.5	22	29.50	0.302	24.86
10	15	2.5	20	2.47	0.598	21.34
60	15	2.5	17	27.50	0.312	24.99
35	5	0.5	29	11.65	0.097	21.82
35	25	0.5	25	16.20	0.963	18.98
35	5	2.5	33	10.90	0.302	21.12
35	25	2.5	9	15.70	1.203	16.58
35	15	1.5	23	12.60	0.367	23.36
35	15	1.5	20	12.90	0.284	24.83
35	15	1.5	21	13.10	0.391	23.33

All examined responses values were analysed via ANOVA statistical test and the results are reported in Table 2.

Table 2 Analysis of variance (ANOVA) of the modelled response

Response	Source								
	Residual			Model					
	DF	SS	MS	DF	SS	MS	F-value	p-value	R^2
Inhibition zone radius (mm)	5	33.12	6.68	9	756.32	84.04	12.57*	0.0062	0.9576
Residual C (g/L)	5	7.27	1.45	9	1423.25	158.14	108.76*	< 0.0001	0.9974
Residual N (g/L)	5	0.02	0.003	9	2.08	0.23	69.34*	0.0001	0.9960
RNA (mg/100g CL)	5	4.468	0.8936	9	114.46	12.72	14.23*	0.0046	0.9624

DF – degree of freedom; SS – sum of squares, MS – mean squares, *Effect significant at $p<0.05$ confidence level.

The results of the statistical analysis are presented in Table 3, where b_0 is the intercept, b_i is the linear, b_{ii} the quadratic and b_{ij} the interaction coefficients and the factor variables are the contents of starch, soybean meal and phosphates (1, 2, 3, respectively). The coefficient of determination (R^2) value of 95.76% for the inhibition zone radius indicates that only 4.24% of total variation cannot be explained by the model, indicating a good agreement between the experimental and predicted values. Relatively high values of R^2, obtained for other examined responses, i.e. residual carbon, residual nitrogen and RNA content, also indicate a good fit of the experimental data to the predicted regression equations. The model F-value of 12.57, 108.76, 69.34 and 14.23 for the inhibition zone radius, residual carbon, residual nitrogen and RNA content, respectively, implies that models for all selected responses are significant at a 95% confidence level.

For the response of residual carbon (Table 3), statistically significant effects are the quadratic effect of the initial starch content (p=0.013329) and the interaction between starch and soybean meal (p=0.005937). Predictably, with the increase in the initial starch content the residual quantities of this nutrient in the fermentation broth are larger.

Table 3 Regression equation coefficients for selected responses

Effects†	Inhibition radius		Residual C		Residual N		RNA	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
Intercept								
b_0	9.796666	0.206441	2.036556	0.546304	0.092122	0.567560	17.18224	0.000941
Linear								
b_1	0.840667*	0.006852	0.109113	0.272833	0.001711	0.703141	0.02419	0.741773
b_2	-0.157500	0.761603	-0.223125	0.375150	-0.038933*	0.016391	1.22608*	0.001032
b_3	4.475000	0.404353	-0.526498	0.827468	0.178025	0.165471	-2.03680	0.308520
Quadratic								
b_{11}	-0.007867*	0.014680	0.003763*	0.013329	0.000043	0.410326	-0.00076	0.377707
b_{22}	0.008333	0.562783	0.004942	0.466680	0.002917*	0.000196	-0.04588*	0.000239
b_{33}	1.833333	0.231148	0.251667	0.704958	0.002208	0.944210	0.37200	0.483640
Interaction								
b_{12}	-0.003000	0.586929	0.011050*	0.005937	-0.000128	0.317704	0.00099	0.622225
b_{13}	-0.110000	0.086680	-0.020600	0.432019	-0.002940	0.051367	0.02528	0.238793
b_{23}	-0.500000*	0.011782	0.006250	0.921469	0.000875	0.773818	-0.04240	0.410804

* Effect significant at $p<0.05$ confidence level, † Numbers 1, 2 and 3 in subscripts represent contents of starch, soybean meal and phosphates, respectively.

A positive interaction between starch and soybean flour contents indicates their synergistic effect while phosphates content in the cultivation medium has no effect on this response. It can be seen from Table 3 that linear (p=0.016391) and quadratic (p=0.000196) terms of the initial soybean meal have statistically significant effects on the residual nitrogen content in the fermentation broth. The same effects are significant for RNA content at the end of fermentation with p-values 0.001032 and 0.000239, respectively (Table 3). When the soybean meal content increases from 5 to 14 g/L, the final RNA content is also increased. On the other hand, a further increase in the soybean meal initial concentration resulted in RNA content decrease.

The response of the inhibition zone radius is mainly influenced by the starch content and the interaction between the soybean meal and phosphates content. In order to gain a better understanding of the effects of variables on antimicrobial production, the predicted model for inhibition zone radius was presented using three-dimensional response surface plots (Fig. 2 A-C). Figure 2A shows that production of an antifungal compound by *Streptomyces* sp. MS1 is positively affected by increasing starch concentration in the range of 10-40 g/L, while higher starch concentrations resulted in a reduction of the analyzed response. On the other hand, an increase in soybean meal content resulted in a decrease of active components production at all starch concentrations. The effects of starch and phosphates concentrations on the inhibition zone radius are given in Fig. 2B. With low quantities of starch in the cultivation medium, the increase in phosphates content in the selected range has no significant effect on the analyzed response. However, when higher concentrations of starch were applied, the decrease in phosphates content resulted in higher values of the inhibition zone radius. Maximum values of the observed response were achieved with starch and phosphates content of 40-60 g/L and 0.5-1.0 g/L, respectively. The results presented in Fig. 2C imply that the effect of soybean meal and phosphates concentration on the inhibition zone radius values can be analyzed. The interaction between soybean meal and phosphates amount has a statistically significant effect (p=0.012) on the observed response. The negative interaction between these two factors (Table 3) indicates their antagonistic effect. With an average starch content of 35 g/L maximum values of the inhibition zone radius are achieved with a lower amount of soybean meal and a higher amount of phosphates.
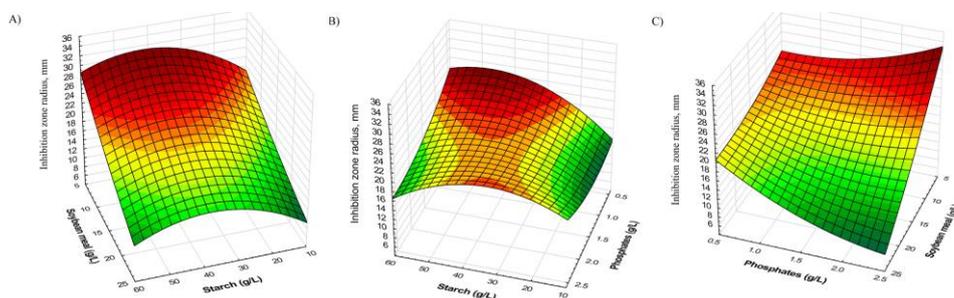
Fig. 2 Effects of starch, phosphates and soybean meal concentrations in the cultivation medium on the inhibition zone radius when *C. albicans* was used as a test organism. (A-Effects of starch and soybean meal; B- Effects of starch and phosphates; C-Effects of soybean meal and phosphates).

3.3. Optimization of medium composition

Previously described and developed models were used to determine the optimal composition of a culture medium. When a single optimization aim is defined i.e. to provide maximal antifungal components production, the highest value of desirability function (D=1.00) is obtained with starch, soybean meal and phosphates contents of 40.52, 5.10 and 2.21 g/L, respectively (Fig. 3A). Under these conditions, the obtained models predict the maximum inhibition zone radius of 33.12 mm while the expected contents of residual carbon, residual nitrogen and RNA are 12.20 g/L, 0.23 g/L and 21.28 mg/100g CL, respectively. According to these results, effluents from the process, obtained after active components separation, would contain significant amounts of unused nutrients, particularly starch. In order to decrease these amounts, we added two more individual desirability functions that would minimize residual carbon and residual nitrogen content. As it can be seen from Fig. 3B, the second optimization set with a total desirability function (D) of 0.896, predicts that the maximal inhibition zone radius, minimal residual carbon and minimal residual nitrogen are achieved when starch, soybean meal and phosphates contents in the cultivation medium are 20.68, 5.00 and 2.43 g/L, respectively. In this case the values of the observed responses would be the inhibition zone radius 33.00 mm, residual carbon 5.30 g/L, residual nitrogen 0.32 g/L and RNA content 20.44 mg/100 g CL. These results indicate that if the initial starch content is reduced from 40.52 to 20.68 g/L then cultivation broth will contain an approximately equal content of antifungal components and a significantly lower amount of residual carbon. To validate the developed mathematical models, an additional experiment was conducted in triplicate tests. A cultivation medium with optimized starch, soybean meal and phosphates contents of 20.68, 5.00 and 2.43 g/L, respectively, was prepared and biosynthesis. It was carried under the same conditions applied in optimization experiments. Under the optimized conditions, the observed inhibition zone radius was 32.87 mm and the standard deviation was 0.23.
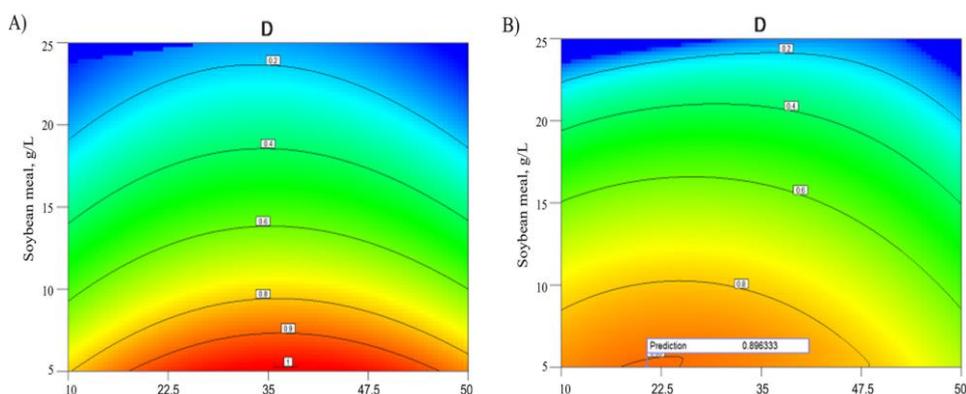
Fig. 3 Contour plots of the overall desirability function (D) for the applied contents of starch and soybean meal for the constant content of phosphates of 1.5 g/L (A-inhibition zone radius maximization; B-inhibition zone radius maximization and residual carbon and nitrogen minimization).

For the residual sugar, residual nitrogen and RNA content, the average values of additional experiments were 5.78 g/L, 0.34 g/L and 22.49, with the matching standard deviations 0.24, 0.02 and 0.85, respectively. These experimental results, therefore, verified the predicted values and the effectiveness of the developed models.

3.4. Purification and identification of antifungal compounds produced by *Streptomyces* sp. MS1

Streptomyces sp. MS1 isolate was grown in the optimal production medium, as determined through bioassay guided experiments. A seven-day-old whole culture was initially extracted with ethyl acetate and the mycelium residue was subsequently extracted with methanol. The antimicrobial activity of *Streptomyces* sp. MS1 crude extracts was tested in disc diffusion assay (data not shown). No zones of inhibition in disc diffusion assay occurred when *Escherichia coli* and *Staphylococcus aureus* were used as test organisms. On the other side, the crude methanolic extract of the *Streptomyces* sp. MS1 mycelia showed higher antifungal properties against *Candida albicans*, with a zone of growth inhibition of 5 or 8 mm in diameter when 0.05 or 0.5 mg of the extract was applied to the disc, in comparison to the ethyl acetate extract of the whole culture (data not shown). The MIC, defined as the minimal concentration at which no growth of *C. albicans* occurred in a liquid culture, was 8 μg/mL for crude methanolic extract.

The methanolic extract of *Streptomyces* sp. MS1 mycelium residue was further purified by flash chromatography and the fractions were tested for the anti-*Candida* activity. Starting with 400 mg of *Streptomyces* sp. MS1 methanolic extract (mycelium obtained from 500 mL of Streptomyces sp. MS1 culture) six fractions were obtained. Two of these fractions, namely F2 and F5, exhibited antifungal properties against *C. albicans* but the zones of growth inhibition were notably different (Fig. 4 and Fig. 5). In the case of F2 fraction, the zone of growth inhibition was quite moderate, 2 mm in diameter when 0.5 mg of the F2 fraction was applied to the disc (Fig. 4). A larger zone of growth inhibition, 4.5 mm in diameter, was detected with 0.5 mg of F5 fraction applied on the disc (Fig. 5). There was 80 mg of F2 and 23 mg of F5 collected during the first round of chromatography. In order to determine the active antifungal principle of the fractions F2 and F5, they were further refined by one more round of flash chromatography, followed by NMR or LC-MS analysis (Fig. 4 and 5).

All signals of the purified fraction F2 in a complex ^{1}H-NMR spectrum were grouped in two areas δ 4.40 to 3.35 and δ 2.50 to 0.80, which led to the conclusion that the fraction F2 does not have any aryl or vinyl protons. The analyses of ^{13}C-NMR spectrum in combination with DEPT-135 (positive CH and CH_3 signals, negative CH_2 signals) and DEPT-90 ^{13}C-NMR (only CH signals) showed the presence of 10 CH_3, 10 CH_2, 15 CH and 5 quaternary carbons. The HSQC technique was used for further analysis of F2 fraction. From this analysis it could be concluded that several CH_2 and CH protons were present in area 4.40 to 3.35 (Fig. 4). The greater chemical shift of these protons (relative to region δ 2.50 to 0.80) was probably attributable to the fact that they were attached to heteroatom connected carbons. The presence of one CH_3 group (δ 3.35) in this area indicated a methyl ether group. The molecular formula of F2 was determined to be $C_{40}H_{68}O_{11}$ on the basis of a M^+-1 peak at *m/z* 723.5 (data not shown).
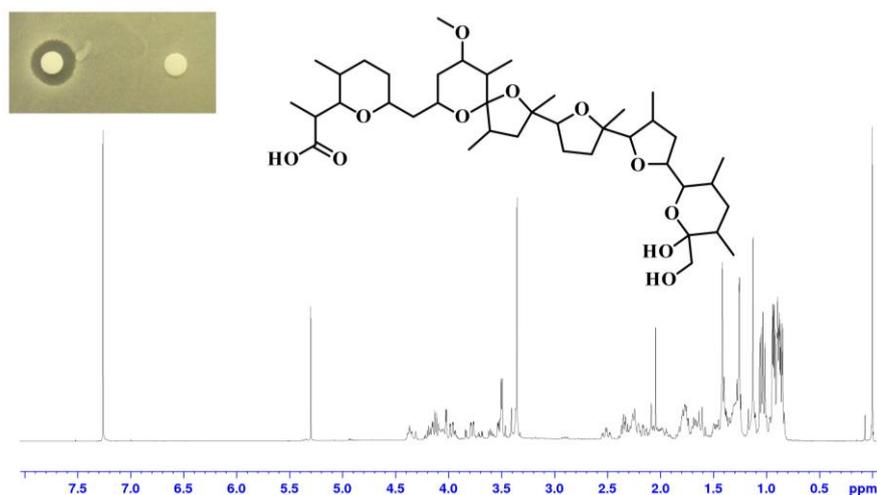
Fig. 4 Chemical and structural identification of nigericin from fraction F2 that exhibited anti-*Candida* activity by NMR. The inhibition zone of the purified compound (200 μg/disc) is shown.
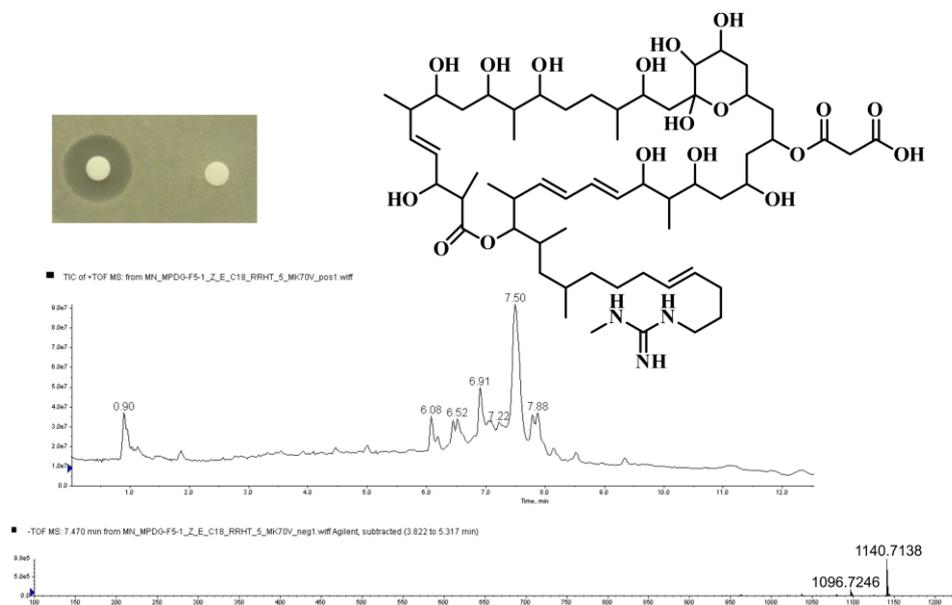
Fig. 5 Chemical and structural identification of niphimycin and niphimycin-like compounds from fraction F5 that exhibited anti-*Candida* activity by LC-MS. The inhibition zone of the purified compound (200 μg/disc) is shown.

Based on all spectral data presented, the F2 structure could be characterized as polyether carboxylic antibiotics nigericin, which was in good agreement with literature data (Wu et al., 2009).

During HPLC/MS analysis of the purified fraction F5, the major product eluted at 7.50 min. The major peak the pseudo-molecular ion observed at m/z 1142.7866 $[M+H]^+$ was in agreement with the $C_{59}H_{103}N_3O_{18}$ molecular formula for guanidyl-polyol macrolide antibiotic compound, niphimycin (m/z 1142.7315 [M+H]+, calc.). However, from the HPLC trace it was evident that this fraction was eluted as a family of structurally similar products (Fig. 5). Therefore, NMR spectra of this fraction were of lesser quality in comparison to F2, but still in good agreement with those described in literature for niphimycin (Ivanova et al., 1998; Kim et al., 2013).

4. DISCUSSION

On the basis of certain morphological, biochemical and physiological properties of streptomycetes, soil isolate MS1 was classified as *Streptomyces hygroscopicus* (Grahovac et al., 2014). Still, the molecular identification based on 1410 nucleotides long 16S rDNA sequence did not fully confirm this conclusion (Fig. 1). Soil isolate MS1 was affiliated with genus *Streptomyces* indeed, but 16S rDNA sequence analysis could not resolve whether MS1 was *S. hygroscopicus* or some other closely related strain (*S. castelarensis*, *S. sporoclivatus* or *S. violaceusniger*). Genomic DNA-DNA hybridization between these strains should be performed before making a final decision.

In an earlier study, the antifungal potential of *Streptomyces* sp. MS1, as well as the enhanced production of the antifungal metabolite in the optimized cultivation medium, were demonstrated (Grahovac et al., 2014). To further optimize the composition of the cultivation medium, the experimental factorial design and response surface methodology were employed. A search for the optimal combination of major components of the medium (contents of starch, soybean meal and phosphates) was performed according to the experimental plan presented in Table 1. The result of the response surface model fitting in the form of ANOVA demonstrates that the models for all selected responses (inhibition zone radius, residual carbon, residual nitrogen and RNA content) are highly significant (Table 2). The high values of F-tests indicate that the developed models can adequately explain the variations in responses values with the designed levels of variables. Regression equation coefficients for selected responses are presented in Table 3. The most important parameters for the response of residual carbon are the initial starch content and the interaction between starch and soybean meal. On the other hand, the initial soybean meal content has a statistically significant effect on the residual nitrogen content in the fermentation broth. Initially, RNA content in the fermentation broth follows the increase in soybean meal initial concentration (5 to 14 g/L), but a further increase results in the declining of RNA content. The most significant coefficients in the regression equation that describes the response of the inhibition zone radius are linear and quadratic coefficients of starch content and the interaction between soybean meal and phosphates contents. The model that predicts the effects of starch, phosphates and soybean meal contents on the inhibition zone radius is presented by three-dimensional response surface plots (Fig. 2 A-C).

MS1 is positively affected by increasing the starch concentration up to 40 g/L, while the higher increase resulted in a reduction of the analyzed response. Generally, quickly metabolized substances like glucose evince a negative effect on the biosynthesis of antimicrobial components i.e. for catabolic repression (Singh and Rai, 2012). On the other hand, complex carbon sources like polysaccharides (starch), polyhydric alcohols (glycerol) or oligosaccharides (lactose) provide significantly better yields of bioactive metabolites (Sanchez and Demain, 2002). Unlike starch content, the increase in soybean meal content resulted in a decrease of antifungal components production at all starch concentrations. This can be explained by the fact that higher amounts of nitrogen source cause directing a production microorganism metabolism towards the synthesis of biomass and, consequently, negatively affecting the production of secondary metabolites. The effects of starch and phosphates concentration on the inhibition zone radius are in good agreement with the literature data, which suggest that when carbon or nitrogen source is a limiting factor, growth is rapidly reduced and antimicrobials biosynthesis takes place in the stationary phase (Gesheva et al., 2005). While the quantity of starch in the cultivation medium is low, the increase in phosphates content has no significant effect on the inhibition zone radius. However, with a simultaneous increase in the concentration of starch and a decrease in phosphates content, higher values of the inhibition zone radius are observed. Finally, an antagonistic effect of soybean meal and phosphates concentrations on the inhibition zone radius values was indicated. With an average starch content of 35 g/L maximum values of the inhibition zone radius are achieved with a lower amount of soybean meal and a higher amount of phosphates.

This can be explained by the fact that nitrogen in the media stimulates biomass growth but not the biosynthesis of secondary metabolites. On the other hand, phosphorus is recognized to have a profound effect on bioactive metabolites production by actinomycetes as it is commonly the major growth-limiting nutrient (Sanchez and Demain, 2002).

The final goal of response surface methodology application is the optimization of biosynthesis, so the developed models were used to define the optimal composition of the culture medium for growth of *Streptomyces* sp. MS1 (Fig. 3). With the first optimized culture medium, where the only one desirability function was used (to obtain maximal production of the antifungal component); the predicted maximum inhibition zone radius was 33.12 mm. Still, considerable amounts of the initial components, particularly starch, would remain unused after active components separation. Considering that costs of cultivation medium preparation participate in the overall costs of the process with a significant share, two more individual desirability functions were added aiming to minimize residual carbon and residual nitrogen content. By meeting these additional criteria, the process would also be more environmentally friendly. In the second attempt of optimization the inhibition zone radius was 33.00 mm, while the amounts of residual sugar and nitrogen were decreased. These results indicate that the reduction in the initial starch content by even 51.03% provides approximately equal content of antifungal components in the cultivation broth and a significantly lower residual carbon content. An additional experiment, conducted in triplicate tests, confirmed the predicted values and the effectiveness of response surface methodology in mathematical representation of anti-*Candida* metabolites production.

Response surface methodology proved to be a useful tool for bioassay guided optimization of culture medium composition but for further process development the chemical identification of the antifungal metabolites produced by *Streptomyces* sp. MS1 is important. The methanolic extract of the mycelium residue was purified by flash chromatography, and anti-*Candida* activity was demonstrated for two fractions. It was shown that each of these two fractions contains a different antifungal metabolite (Fig. 4, 5). These two concurrently produced antifungal compounds are identified by NMR and LC-MS methodology as polyether carboxylic antibiotic nigericin and guanidyl-polyol macrolide, niphimycin. Nigericin has been previously isolated from several *Streptomyces* sp. strains (David et al., 1985). It is considered as an ionophore antibiotic because of the ability to transport alkali ions across lipid barriers (Wu et al., 2009). Nigericin was firstly used for its anticoccidial activity as a complement in animal foods (Bogaert et al., 1990). The biosynthetic pathway of nigericin in *Streptomyces* sp. has been well described (Mouslim et al., 1995) and its production has been optimized up to 100 mg/L by combining a high producing strain with an adapted medium (Grabley et al., 1990). Then again, a polyol macrolide niphimycin exhibited a potent fungicidal effect by disrupting the plasma membrane but its mode of action is clearly distinguishable from that of the polyene macrolide antibiotic like amphotericin B. A synergistic combination of direct plasma membrane damage and oxidative stress as a cause of the antifungal activity of niphimycin at a higher dose is proposed (Wu et al., 2009). It is highly likely that the alkyl guanidinium chain of niphimycin plays a major role in the fungicidal activity of niphimycin in cooperation with the polyol lactone ring as its enhancer (Ogita et al., 2007). Another representative of streptomycetes, *Streptomyces hygroscopicus* ATCC 29253 produces rapamycin (macrocyclic polyketide), elaiophylin (a 16-membered macrolide with C2-symmetry) and nigericin. It was observed that antifungal bioassays of fermentation extracts against *C. albicans* by the agar diffusion method did not agree quantitatively with the more accurate method of HPLC analysis (Fang et al., 2000). In all cases, bioassay data were higher than HPLC values. Both antibiotics, elaiophylin, originally characterized as an antibacterial agent, and antifungal agent nigericin, showed the potential to increase the antifungal activity of rapamycin. This could be the case with two antibiotics from *Streptomyces* sp. MS1 isolate, nigericin and niphimycin, two structurally different antifungal agents that might act synergistically against *C. albicans*. Similarly, in a chemical screening of secondary metabolites produced by *Streptomyces* sp. DSM3816, two different types of antibiotics with antifungal activity were detected, nigericin and niphymicin (Grabley et al., 1990). It was also shown that *Streptomyces* sp. DSM3816 produces elaiophylin and two novel niphimycin analogs, amycins A and B, besides nigericin and niphymicin. It was implied that amycin B contains a malonyl-monoester less and amycin A has one more than niphimycin. The demalonyl product amycin B had an increased activity against *C. albicans* compared with amycin A and niphimycin. The presence of multiple antifungal metabolites with different spectra of activity, and whose production may respond to changes in the environment ensure the supremacy of the producing strain over other bacterial and fungal competitors in rhizosphere that are sensitive to their antimicrobial metabolites.

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ZEMLJIŠNA STREPTOMICETA MS1, PROIZVOĐAČ NIGERICINA I NIFIMICINA: BIOESEJ SA KANDIDOM KAO OSNOV ZA OPTIMIZACIJU SASTAVA HRANLJIVE PODLOGE METODOM ODZIVNE POVRŠINE

U cilju povećanja proizvodnje antifungalnih jedinjenja, metabolita zemljišnog izolata MS1, optimizovan je sastav hranljive podloge, pri čemu je za praćenje antifungalne aktivnosti korišćen bioesej sa kandidom. Na osnovu morfoloških, fizioloških i biohemijskih osobina, kao i sekvence 16S rDNK, ovaj izolat je svrstan u rod streptomiceta. Optimizacija sastava hranljive podloge je rađena primenom Box-Behnken-ovog eksperimentalnog dizajna i metodom odzivne površine. Korišćenjem podloge koja sadrži 40.52 g/L skroba, 5.10 g/L sojinog brašna i 2.21 g/L fosfata dobijen je maksimalan prinos antifungalnih komponenti. Primenom hromatografije i 1H i 13C NMR spektroskopije prečišćena su i identifikovana dva antifungalna metabolita streptomicete MS1, polietarski antibiotik nigericin i makrolid nifimicin.

Ključne reči: *antifungalna aktivnost, nigericin, nifimicin, streptomicete, optimizacija kultivacione tečnosti*