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Characterization and Optimization of Biosynthesis of Bioactive Secondary Metabolites Produced by *Streptomyces* sp. 8812

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Abstract

The nutritional requirements and environmental conditions for a submerged culture of *Streptomyces* sp. 8812 were determined. Batch and fed-batch *Streptomyces* sp. 8812 fermentations were conducted to obtain high activity of secondary metabolites. In the study several factors were examined for their influence on the biosynthesis of the active metabolites-7-hydroxy-6-oxo-2,3,4,6-tetrahydroisoquinoline-3-carboxyl acid ($C_{10}H_9NO_4$) and N-acetyl-3,4-dihydroxy-L-phenylalanine ($C_{11}H_{13}NO_5$): changes in medium composition, pH of production medium, various growth phases of seed culture, amino acid supplementation and addition of anion exchange resin to the submerged culture. Biological activities of secondary metabolites were examined with the use of DD-carboxypeptidase 64–575 and horseradish peroxidase. *Streptomyces* sp. 8812 mycelium was evaluated under fluorescent microscopy and respiratory activity of the strain was analyzed. Moreover, the enzymatic profiles of the strain with the use of Api®ZYM test were analyzed and genetic analysis made. Phylogenetic analysis of *Streptomyces* sp. 8812 revealed that its closest relative is *Streptomyces capoamus* JCM 4734 (98%), whereas sequence analysis for 16S rRNA gene using NCBI BLAST algorithm showed 100% homology between these two strains. Biosynthetic processes, mycelium growth and enzyme inhibitory activities of these two strains were also compared.

Key words: *Streptomyces* sp. 8812, biologically active compounds, media optimization, submerged cultures

Introduction

Streptomyces are Gram-positive bacteria with a remarkably complex developmental cycle. These bacteria are isolated from soil and water in all ecosystems. *Streptomyces* are producers of many secondary metabolites with a wide range of activities, *e.g.* antimicrobial, antitumor and immunosuppressive (Hopwood, 2007). Bioactive secondary metabolites are mostly isolated from submerged cultures, often in discreet amounts. One of the strategies to improve production of secondary metabolites is optimization of chemical and physical conditions of the submerged culture. This process involves defining the composition of the production medium, temperature and pH value (Genilloud *et al.*, 2011).

In this work, the authors present results of a set of experiments performed in submerged cultures for *Streptomyces* sp. 8812 isolated from Brazilian soil. The described research is a continuation of the work on the strain *Streptomyces* sp. 8812 and its metabolites. Previously, two bioactive metabolites with antibacterial activity were isolated and characterized (Solecka *et al.*, 2009a; 2009b; 2012a). One of them had a novel structure. The chemical structure and biological and physico-chemical properties of two secondary metabolites produced by *Streptomyces* sp. 8812 have been determined. The first metabolite is an isoquinoline alkaloid, 7-hydroxy-6-oxo-2,3,4,6-tetrahydroisoquinoline-3-carboxyl acid ($C_{10}H_9NO_4$), with molecular mass of 207.06 Da. The second *Streptomyces* sp. 8812 metabolite is a protoalkaloid, N-acetyl-3,4-dihydroxy-L-phenylalanine ($C_{11}H_{13}NO_5$) with molecular mass of 239.07 Da. Both compounds exhibit antibacterial properties, have the ability to inhibit DD-carboxypeptidase 64–575 activity and are stable to β-lactamase activity. Their chemical structures may serve as lead compounds for modifications enhancing their biological activities (Solecka *et al.*, 2009a; 2009b; 2012a; 2012b).

In the present study, the authors defined the optimum composition and pH value of the production medium for bioactive secondary metabolites biosynthesis. The seed cultures at various growth phases were tested. A fed-batch strategy was used to study the effect of different amino acids on secondary metabolites

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activities. The authors examined whether addition of a resin into the production medium has an effect on the biosynthesis of biologically active compounds. Biological activity of the metabolites was studied under the guidance of DD-carboxypeptidase 64–575 and horseradish peroxidase inhibition reaction. Selected methods were chosen due to their high sensitivity which is significant in the case of secondary metabolites produced in very small quantities (hundredths of permille).

In further steps, genetic classification of *Streptomyces* sp. 8812 using 16S rRNA analysis was performed. Mycelium viability and respiratory activity of *Streptomyces* sp. 8812 during submerged culture was observed and characterized by fluorescent microscopy. The Api® ZYM test was used for biochemical characterization of mycelium during fermentation.

Experimental

Materials and Methods

Microorganism. *Streptomyces* sp. 8812 is a strain isolated from Brazilian soil. The strain is deposited in the Polish Collection of Microorganisms in Wroclaw, with an accession number B/00017. The 16S rRNA gene sequence of *Streptomyces* sp. 8812 has GenBank accession number KT951721.

Morphological and carbon utilization properties of *Streptomyces* sp. 8812 were described in previous paper (Solecka *et al*., 2009a). Spores of *Streptomyces* sp. 8812 were stored at –70°C.

Media and growth conditions. *Streptomyces* sp. 8812 was maintained on yeast-malt agar (ISP2) slants (Shirling and Gottlieb, 1966). The seed medium and initial production medium (M) consisted of (g/l): lactose 10.0, yeast extract 5.0, corn steep liquor (CSL) 10.0, Bacto™ peptone 4.0, Bacto™ tryptone 17.0, $MgCl2 \times 7H_2O$ 0.5, KH_2PO_4 2.0, K_2HPO_4 4.0, $CaCO_3$ 3.0 and microelements: $MnSO_4 \times H_2O$ 0.002, $FeSO_4 \times 7H_2O$ 0.01, $ZnSO_4 \times 7H_2O$ 0.01, $CoCl_2$ 0.0008, $CuSO_4 \times 5H_2O$ 0.001. After sterilization, pH was adjusted to 6.7 value.

In pH-optimization experiment pH of medium M after sterilization was adjusted to several values: 5.5, 5.9, 6.3, 6.7, 7.0, 7.4, 7.8 and was measured every 24 hours during fermentation.

Slants with *Streptomyces* sp. 8812 were incubated at 28°C for 10 days. Spores from fresh solid culture were transferred into 35 ml of the medium M in 500 ml shake flasks. The seed cultures were incubated in a rotary shaker at 220 rpm for: 24, 48 or 72 hours at 28°C. Then, 3.5 ml of seed cultures were transferred to 35 ml of production medium. The biosynthesis process was conducted at 220 rpm for 120 h at 28°C. Samples were taken every 24 h. Yeast-malt broth was used for mycelium growth for genetic analysis. Cultures for genetic analysis were incubated in a rotary shaker at 220 rpm for 24 h at 28°C.

Optimization of the production medium. Several nitrogen sources, like soybean flour, yeast extract, Bacto™ peptone, Bacto™ tryptone, neopeptone, were tested in a set of experiments (Table I). Medium M was modified in three ways: deletion or replacement, and adjustment of the component(s) concentration. Concentrations of macroelements, such as K^+, Mg^{2+}, Ca^{2+}, and microelements were constant in all media. The percentage of C and N values in all tested media was estimated on the basis of Becton Dickinson (BD) tables (BD Biosciences, 2006), for CSL (Keller and Heckmann LPP, 2006). The initial C/N values varied from 0.11 to 2.07.

Statistical method. The most significant component of the production medium was estimated using a statistical method. Each parameter (carbon and nitrogen source in the production medium) was characterized by mass percentage composition. Correlation between the share of each substrate in overall mass of medium and the DD-carboxypeptidase 64–575 inhibitory activity at 120 h was assessed using Spearman correlation coefficient and test of its significance. Calculations were performed using R 3.0.1 statistical software (R Foundation for Statistical Computing, 2013).

Determination of biomass growth. One ml of submerged cultures was taken every 24 h. Probes were centrifuged and the biomass was washed twice with distilled water. Dry biomass was obtained after incubation at 110°C in dryer (Pol-Eco). The drying process was finished when the biomass weight was stable.

Determination of secondary metabolites activities. To determine the optimum conditions for conducting the submerge culture of *Streptomyces* sp. 8812, activity of secondary metabolites was measured. DD-carboxypeptidase 64–575 from *Saccharopolyspora erythraea* 64–575 (Solecka and Kurzątkowski, 1999; Solecka *et al*., 2003) and horseradish peroxidase (HRP, Sigma-Aldrich) were used for enzymatic assays to determine the activity of secondary metabolites.

Inhibition of DD-carboxypeptidase 64–575 activity. The assay was done according to the method previously described by Frère *et al.* (1976), with modifications (Adam *et al.*, 1990; 1991; Solecka *et al*., 2003). The reaction mixture consisted of: 5 µl of DD-carboxypeptidase 64–575, 5 µl of 10 mM 2-((2-benzamidopropanol) thio)acetic acid, 5 µl of supernatant and 85 µl of 0.1 M phosphate buffer pH 8.0. All samples were triplicated. Absorbance was measured at 250 nm for 1200 s at 37°C (Jasco V-630). DD-carboxypeptidase 64–575 inhibition was calculated using the formula shown below:

$$DD - carboxypeptidase\ 64–575\ inhibition\ [\%] = 100 - \left(\frac{A_2 - A_3}{A_0 - A_1} \times 100\right)$$

The initial value of the absorbance of the enzyme control is A_0 whereas final value of the absorbance of the enzyme control is A_1. The initial value of the absorbance of the supernatant sample is A_2 and final value of the absorbance of the supernatant sample is A_3. Due to the method limitation, results were measured with 5% error.

Inhibition of HRP activity. The assay was done according to the method of Lehmann *et al*. (1974) with modifications (Chance and Maehly, 1955). The reaction mixture consisted of: 37.5 µl of 0.01 M phosphate buffer pH 8.0, 2.5 µl of *Streptomyces* sp. 8812 submerged culture supernatant, 5 µl of 0.025 mg/ml HRP, 5 µl of 1.26 mg/ml o-dianisidine and 10 µl of 0.480 mM hydrogen peroxide in 0.01 M phosphate buffer pH 8.0. After 10 min incubation at 37°C, 100 µl of water-methanol-sulfuric acid (5:5:6 v/v) was added and incubated for further 10 min at room temperature. The absorbance was measured at 540 nm (Fluostar Omega, LABTECH). HRP inhibition was calculated using the formula shown below:

$$HRP\ inhibition\ [\%] = \frac{A_{\text{control}} - A_{\text{supernatant}}}{A_{\text{control}}} \times 100$$

The absorbance for the control is A_{control}, whereas the absorbance in the presence of a supernatant is $A_{\text{supernatant}}$. The positive control for enzymatic activity measurements consisted of 2.5 µl of water instead of supernatant and an analogous blank contained phosphate buffer instead of hydrogen peroxide. Sample, control and related blanks were triplicated and carried out under the same condition on the same microliter plate.

Culture supplementation with amino acids. Different concentrations: 0.1, 1, 10 and 100 mM of L-tyrosine, L-tryptophan and L-phenylalanine were separately used in secondary metabolites activity tests. One ml of each concentration was added to shake-flasks cultures every 24 h till the end of fermentation.

Addition of anion exchange resin to production medium. Two g of anion exchange resin IRA-400 (OH) (Supelco) was equilibrated twice with 50 ml of 2.0 M acetic acid and rinsed with sterile water till pH value reached 7.0. Resin IRA-400 (2.0 g) was added to 24-h or 48-h old shake-flask cultures. All probes were incubated for 24 h. Next, the resin was removed from flasks and cultivation was continued till 120 h. The resin was rinsed with distilled water and eluted using 100 ml of 2.0 M acetic acid. Material eluted from the resin was lyophilized and compound activity was measured (7 µl of 1 mg lyophilizate in 100 µl of 0.01 M phosphate buffer pH 8.0).

Genetic analysis. Genomic DNA of *Streptomyces* sp. 8812 was obtained using the Wizard® Genomic DNA Purification Kit (Promega). Nearly complete 16S rRNA gene (~ 1500 nucleotides [nt]) was amplified using universal primers: pHr and pAf (Edwards *et al.*, 1989). The amplified product was purified with Wizard® SV Gel and PCR Clean-Up System (Promega). The 16S rRNA gene was sequenced with an automated DNA sequencing system (ABI 3730) and BigDye terminator cycle sequencing kit (Applied Biosystems). Primers used for sequencing are listed by Coenye *et al.* (1999).

A BLAST search service of the GenBank database was used to establish bacterial species most similar to *Streptomyces* sp. 8812 based on the 16S rRNA gene sequence. A phylogenetic tree was constructed using the neighbor-joining, maximum-likelihood, maximum-parsimony tree-making algorithms. An evolutionary distance matrix was generated. Confidence values of branches of the phylogenetic tree were determined in a bootstrap analysis based on 1.000 resampling of the neighbor-joining dataset.

Mycelium observation under fluorescent microscopy. *Streptomyces* sp. 8812 mycelium viability was determined with LIVE/DEAD® *Bac*-Light™ Bacterial Viability Kit (Invitrogen). Centrifuged mycelium samples from submerged cultures were washed gently two times with sterile 0.2% NaCl solution. Then, the harvested mycelium was stained according to the Molecular Probes protocol (15 min at 37°C in dark). The SYTO 9 green fluorescent stain labels cells with intact and damaged membranes. Propidium iodide (PI) only enters bacteria with damaged membranes and affects SYTO 9 fluorescence. Thus, live bacteria appear fluorescent green whereas dead bacteria appear red. The stained mycelium was observed with fluorescent microscope OPTA-TECH MN-800FL immediately after preparation.

The respiration activity of *Streptomyces* sp. 8812 was determined with 5-cyano-2,3-bis(4-methylphenyl)-2*H*-tetrazolium chloride (CTC) (Sigma-Aldrich). During cell respiration CTC is reduced intracellularly to the red fluorescent formazan crystals (CTF). The final CTC concentration in the probe was 5.0 mM. Samples were incubated for 3 h at 37°C in the dark, fixed with 2% formalin (final concentration) and washed twice with sterile 0.2% NaCl solution. Afterwards, probes were observed under the fluorescent microscope OPTA-TECH MN-800FL. Observed pellets were documented on merged photos showing the whole mycelium (light microscopy) and red fluorescent formazan crystals (fluorescent microscopy).

Analysis of enzyme profiles using the api® ZYM test. Mycelium probes from seed and production cultures of *Streptomyces* sp. 8812 conducted in medium M were characterized with api® ZYM tests (bioMérieux SA, France). This semi-quantitative micromethod is designed for determining enzymatic activities. It consists of api® ZYM stripes with enzymatic substrates, incubation boxes, ZYM A and ZYM B reagents. The tested enzymes include phosphatases, esterases, lipases, arylamidases, trypsin, α-chymotrypsin, phosphohydrolase,

α-, β-galactosidases, β-glucuronidase, α-, β-glucosidases, N-acetyl- β-glucosaminidase, α-mannosidase and α-fucosidase. Mycelium from the submerged culture was harvested by centrifugation and washed twice with sterile 0.2% NaCl. Mycelium suspension adjusted to 5–6 McFarland turbidity standard was added to the wells. After 4–4.5-h incubation at 37°C one drop of ZYM A and ZYM B were added to each well. Intensity of the colors that appeared in the wells was estimated on a 1–5 scale, according to the protocol. *Pseudomonas aeruginosa* ATCC 27853 was used as a quality control strain.

Results and Discussion

Medium optimization for bioactive metabolites production by *Streptomyces* sp. 8812

Utilization of nitrogen sources. To establish the optimal conditions of *Streptomyces* sp. 8812 fermentation for secondary metabolites biosynthesis, the production medium M was modified in 17 manners (Table I), with respect to nitrogen sources. The DD-carboxypeptidase 64–575 inhibitory activities of secondary metabolites produced in the different media were compared to those synthesized during *Streptomyces* sp. 8812 fermentation in medium M (Table I). Positive values of correlation coefficients were achieved for soybean flour, Bacto™ peptone, yeast extract, CSL, neopeptone and soytone, indicating that high contents of these nitrogen sources in the medium lead to enhanced production of DD-carboxypeptidase 64–575 inhibitors (Table II). Addition of soybean flour to the medium (medium 11 and 17) had a positive impact on the level of the produced bioactive metabolites. Bacto™ peptone was added to each medium, except medium 4 and 13, and its increasing amounts had a favorable effect on the production of active metabolites. Similar influence was noted for yeast extract, which was present in all media, except 4 and 12; its influence on production of biologically active substances was proportional to its content in the medium.

A negative value of correlation coefficient was obtained for Bacto™ tryptone (Table II), which was present in all tested media. High amounts of this nitrogen source in the medium, impaired enzyme inhibitory activities. For media 2 and 3, containing 5 g of Bacto™ tryptone, which were additionally enriched with 5 g of neopeptone and 5 g of soytone, respectively, secondary metabolites activities were as high as after fermentation in medium M. However, Bacto™ tryptone, when used as the only source of nitrogen in the production medium, was not sufficient for secondary metabolites production (medium 4). A decrease in inhibitory activity of bioactive metabolites was also noticed when the amounts of Bacto™ tryptone, Bacto™ peptone and yeast extract in the production medium were reduced by 50% (medium 5). In comparison to the composition of medium M, increasing the amounts of these three components by 50% (medium 6) did not enhance biosynthesis of bioactive metabolites. Additionally, when the yeast extract and Bacto™ peptone were separately removed from the initial medium (medium 12 and 13, respectively), a decrease in secondary metabolites activity was also observed.

Further on, the influence of CSL in the production media on the biosynthesis of enzyme inhibitors was examined. CSL (10 g/l) was found to be an important component of medium M in obtaining biologically active secondary metabolites. Elimination of CSL (medium 7) resulted in low inhibitory metabolites activities. As a rich source of amino acids, vitamins and glucose, CSL is often used as a component of microbial fermentation broth (De Azeredo *et al.*, 2006; Zou *et al.*, 2009). It is produced in the maize steeping process. The low costs of CSL production are an important factor standing in favor for its utilization in pharmaceutical industry (De Azeredo *et al.*, 2006).

DD-carboxypeptidase 64–575 inhibitory activities were comparable to the levels observed for medium M cultures after fermentation in medium 11, which contained a reduced amount of Bacto™ tryptone (from 17 g to 5 g), 20 g of soybean flour and which was deprived of CSL. It was shown that soybean flour is more nutritional for bacteria in submerged cultures than other soya extracts (Ortiz *et al.*, 2007). Overall, the conducted studies show clearly the great importance of complex nitrogen sources on production of bioactive metabolites by *Streptomyces* sp. 8812. Moreover, statistical analysis revealed that higher amounts of each nitrogen source (except Bacto™ tryptone) tested had a positive impact on the production of bioactive metabolites.

Utilization of carbon sources. The influence of α-lactose in the production media on the biosynthesis of enzyme inhibitors was examined. Statistical analysis showed that higher DD-carboxypeptidase inhibitory activity was observed when the percentage of α-lactose ($rho = -0.489$, $P = 0.040$, $n = 18$) was lower in the production medium (Table II). Therefore, increase of the lactose content to 15 g (medium 15) did not improve the activity of bioactive metabolites.

The relationship between the C/N ratio values in the different media and the activity of secondary metabolites (Table I) was analyzed. Efficient production of bioactive metabolites during fermentation processes was determined to occur at moderate C/N ratio values for some *Streptomyces* strains (Zhinan and Peilin, 1999). Statistically significant correlation for the value of C/N ($rho = -0.508$; $P = 0.031$, $n = 18$) (Table II) was observed. In spite of similar initial C/N ratio values

Table I
The 17 modifications of initial medium M.

	α-Lactose	Soybean flour	Bacto™ Peptone	Yeast Extract	Bacto™ Tryptone	Corn Steep Liquor (CSL)	Neopeptone	Soytone	C/N*	Activity at 120 h (%)***
M	10	–	4	5	17	10	–	–	1.27	100
1	10	–	4	5	5	10	–	–	2.04	65.2
2	10	–	4	5	5	10	5	–	1.62	100
3	10	–	4	5	5	10	–	5	1.92	100
4	10	–	–	–	17	–	–	–	1.87	50.2
5	10	–	2	2.5	8.5	10	–	–	2.07	39.1
6	10	–	6	7.5	25	10	–	–	0.93	100
7	10	–	4	5	17	–	–	–	1.34	72
8	10	–	4	5	17	15	–	–	1.24	87.5
9	10	–	4	5	17	10	–	5	1.27	79.1
10	10	–	4	5	17	10	5	–	1.09	87.5
11	10	20	4	5	5	–	–	–	nc**	100
12	10	–	4	–	17	10	–	–	1.36	67.2
13	10	–	–	5	17	10	–	–	1.49	65.5
14	–	–	4	5	17	10	–	–	0.26	100
15	15	–	4	5	17	10	–	–	1.77	95.5
16	–	–	4	5	17	–	–	–	0.11	66.7
17	–	10	4	5	5	10	–	–	nc	100

* C/N means percentage contribution of C and N in medium
** nc means not calculated
*** activity is a percent of DD-carboxypeptidase 64–575 inhibition

of medium M (1.27), 7 (1.34), 8 (1.24), 9 (1.27) and 12 (1.36), medium M gave the highest activity of secondary metabolites after fermentation. In the light of the above, it seems that for *Streptomyces* sp. 8812 nitrogen sources were more important than α-lactose for biosynthesis of bioactive metabolites.

Characterization of mycelium growth and biosynthesis of secondary metabolites – DD-carboxypeptidase 64–575 inhibitors. The most intensive *Streptomyces* sp. 8812 mycelium growth rate was observed during the first 24 hours of fermentation (0.38 mg/ml/h). Mycelium biomass concentration after 24 h was maintained at a similar level until the end of fermentation (to 144 h). Production of DD-carboxypeptidase 64–575 inhibitors began at the same time as biomass formation (Table III) and reached the maximum level after 48 h. The overlap of trophophase (intensive growth) and idiophase (secondary metabolites formation) resulted in an uniphasic culture. It was shown in other

Table II
Correlation between the percentage share of each substrate in overall mass of medium and the DD-carboxypeptidase 64–575 inhibitory activity at 120 h.

	N	Spearman correlation coefficient (rho)	P-value
α-Lactose	**18**	**–0.489**	**0.040**
Soybean flour	18	0.386	0.114
Bacto™ Peptone	18	0.338	0.170
Yeast extract	18	0.302	0.224
Bacto™ Tryptone	18	–0.136	0.590
Corn Steep Liquor	18	0.014	0.956
Neopeptone	18	0.204	0.418
Soytone	18	0.154	0.542
C/N	**18**	**–0.508**	**0.031**

Table III
DD-carboxypeptidase 64–575 inhibitory activity of *Streptomyces* sp. 8812 metabolites and biomass concentration during 144 h of cultivation.

Time of cultivation (h)	DD-carboxypeptidase 64–575 inhibition (%)	Mycelium biomass concentration (mg/ml)
24	70	9.14
48	82.6	6.3
72	87.4	5.5
96	90	5.6
120	92.4	5.4
144	90	5.7

studies that when defined media support the rapid growth of *Streptomyces coelicolor* A3(2), the strain produced actinorhodin in a fully biphasic fermentation profile. Many environmental factors (media composition, temperature, *etc.*) determine the correlation between biomass growth and bioactive metabolite production (Liao *et al.*, 1995).

Optimization of pH value of the production media. The pH value during the fermentation process affects bacterial cell growth and bioactive product formation, as well as stimulates or inhibits the activity of enzymes (Chen *et al.*, 2011). HRP inhibitory activities of secondary metabolites were tested during *Streptomyces* sp. 8812 fermentation in medium M at different initial pH values. Highest metabolites activities were obtained when initial pH values of the media did not exceed 7.4 (Supplementary Fig. 1(A)S).

During the first 24 hours of fermentation the pH value rose from 6.7 to 8.2, and maintained at similar level to the end of the cultivation (Supplementary Fig. 1(B)S). Due to the glycolysis process in bacterial cells and the release of pyruvate, the pH value typically decreases during the first 24 h of fermentation (Desai *et al.*, 2002; Chen *et al.*, 2011). In the present studies, the pH value increased during the first 24 h of fermentation. Possibly, *Streptomyces* sp. 8812 showed a low intensity of glycolysis process under submerged culture conditions. Perhaps, this strain intensively utilizes peptides and amino acids as nitrogen and carbon sources, generating ammonia as a side product which causes the increase of pH value. Furthermore, *Streptomyces* sp. 8812 biomass growth and bioactive secondary metabolites biosynthesis were not affected by high pH values resulting from the 24-hour fermentation (Table III).

Optimization of the age of the seed culture. The optimum age of the seed culture and its physiological state, including the morphological form and metabolic activity, are important factors for efficient production of secondary metabolites (Zou *et al.*, 2011). Inhibitory activities of *Streptomyces* sp. 8812 metabolites during fermentation cultures that were initiated with seed cultures at various age (at 24, 48 or 72 h) are presented on Supplementary Fig. 2S. The 24- and 48-hour seed cultures, at late logarithmic phase and middle stationary growth phase, respectively, contributed short lag phases of the submerged cultures. So, the optimal age of seed culture for the production of bioactive metabolites was 24 h or 48 h. The 72-hour seed culture needed more time to adapt to new conditions and entered once more the logarithmic growth phase. This resulted in a delay in the reaching the maximum activity of secondary metabolites.

Influence of amino acid supplementation on secondary metabolites activities. Fed-batch culture conducted by supplementation amino acids to the fermentation broth is a process aiming at efficient biomolecule production (Zhang *et al.*, 2012). In the present work, the authors studied the stimulatory effect of amino acids on secondary metabolites production by *Streptomyces* sp. 8812 (Supplementary Fig. 3(A)S-(C)S). Among bioactive metabolites isolated during *Streptomyces* sp. 8812 fermentation was a novel isoquinoline alkaloid (Solecka *et al.*, 2009a; Solecka *et al.*, 2009b). Biosynthesis of isoquinoline alkaloids in nature starts from three amino acids: L-phenylalanine, L-tyrosine and L-tryptophan (Kegg Pathway Maps, 2014). Thus, these three amino acids were chosen for evaluating their influence on bioactive metabolites activities (HRP inhibition). The rising concentrations of L-tryptophan in the media led to a slight decrease of metabolites activities (Supplementary Fig. 3(A)S). Cultures supplemented with 1 mM L-tyrosine were characterized by high secondary metabolites activities at 24 h proceeded by a sudden drop (Supplementary Fig. 3(B)S). L-tyrosine at 100 mM concentration had a stimulatory effect on secondary metabolites production. Cultures containing 10 mM L-tyrosine (Supplementary Fig. 3(B) S) as well as 0.1, 1, 10 and 100 mM L-phenylalanine (Supplementary Fig. 3(C)S) had comparable secondary metabolites activities to control cultures. In summary, the authors observed a concentration-dependent stimulation of metabolites activities during L-tyrosine feeding. Below the optimal 100 mM concentration of L-tyrosine the activity was equal or lower in respect to the control fermentation.

In bacteria, the three tested amino acids are involved in biosynthesis of phosphorylated nicotinamide adenine dinucleotide [NAD(P)] and melanin pigment (Arai and Mikami, 1972). Biosynthesis of bioactive metabolites appeared to be closely related to melanin production. *Streptomyces* sp. 8812 colonies obtained from regenerated protoplasts lacking the ability to produce melanin showed no bioactive metabolites activities (data not shown). The progressive concentration of L-tyrosine clearly influenced melanin production in *Streptomyces* sp. 8812 (data not shown). It seems that bioactive metabolites have similar precursors as melanin. Probably, when L-tryptophan is added to the *Streptomyces* sp. 8812 culture, the primary metabolism pathway tends more toward NAD(P) biosynthesis than secondary metabolites production. In comparison to the tested strain, *S. coelicolor* produces blue antibiotic-actinorhodin. *S. coelicolor* mutants, defective at different stages of actinorhodin biosynthesis pathway were all impaired in blue antibiotic production (Hopwood, 2007).

Effect of anion exchange resin on bioactive metabolites production. Bioactive metabolites produced by *Streptomyces* sp. 8812 were shown to bind to a strong anion exchange resin added to the fermentation broth.

Substances eluted from the resin added to 24-hour old cultures showed 46.6% of HRP enzyme inhibition. When resin was added at 48 h to the fermentation broth, inhibition of the HRP enzyme by eluted substances reached 56.6%.

The authors examined the supernatant from submerged cultures of *Streptomyces* sp. 8812 supplemented with the anion exchange resin. Addition of the resin to the 24- and 48-hour cultures resulted in a decrease of metabolites activities by 14.5% and 21.4%, respectively (Supplementary Fig. 4S). In both cases, the microorganism did not produce more bioactive metabolites after the resin was removed from the fermentation broth. Thus, it seems that reduction of the amount of bioactive metabolites in the culture broth is not a stimulating factor for further biosynthesis. An opposite effect of adding resin was observed for cultures which result in pristinamycin (streptogramin family), teicoplanin (glycopeptide family) and proteasome inhibitor NPI-0052 production (Lee *et al.*, 2003; Jia *et al.*, 2006; Tsueng and Lam, 2007). In all mentioned cases, synthesis of bioactive secondary metabolites was shown to increase.

The choice of resin type was made based on its ionic and hydrophobic/hydrophilic properties. Environmental conditions, such as temperature and pH value, are also important factors for active substance absorption (Casey *et al*., 2007). It has been well documented that addition of resin to the fermentation broth can reduce feedback repression, eliminate the toxic effect of antibiotics on growth, stabilize the structure of active compounds (prevention from hydrolysis) and, consequently, increase the production of secondary metabolites (Lee *et al.*, 2003; Jia *et al.*, 2006; Tsueng and Lam, 2007). What more, addition of the resin to the medium facilitates further recovery of bound metabolites.

Phylogenetic study of *Streptomyces* sp. 8812. Sequence analysis for 16S rRNA gene using NCBI BLAST algorithm showed 100% homology of *Streptomyces* sp. 8812 to *Streptomyces capoamus* JCM 4734. Phylogenetic analysis of *Streptomyces* sp. 8812 indicated that its closest relative is *Streptomyces capoamus* JCM 4734 (98%) (Supplementary Fig. 5S). *S. capoamus* is a known producer of antitumor and antifungal compounds (Goncalves da *et al.*, 1968; Hayakawa *et al.*, 1985; Singh *et al*., 2008).

Mycelium observation under fluorescent microscopy. Mycelium viability and development of *Streptomyces* sp. 8812 cell clumps are shown in Fig. 1(A)-(F). The clumps are irregular in shape, unlike pellets, and smaller (Denser Pamboukian *et al.*, 2002). In 24-hour seed cultures, single live cells were observed at the periphery of the clumps (Fig. 1(A)-(B)). After 17 h of production culture live and dead cells were observed in the clumps. At 24 h, the edge of the clumps consisted of thick live mycelium and a decaying center (Fig. 1(D)). The hyphae inside older clumps were dying, probably due to its high density and low permeability to nutrient substances. At 48 h and 72 h dead cells occupied most of the clump area (fluorescent red) (Fig. 1(E)-(F)). Remaining of live cells was seen in the center of the clumps.

Fig. 2(A)-(F) depicts changes in the respiratory level of *Streptomyces* sp. 8812 under submerged culture. The intensity of red fluorescence is indicative of the respiratory level of bacterial cells. Red areas on the figures represent high respiratory level. Mycelium which formed clumps showed the highest respiratory activity at 17 h (Fig. 2(C)). High respiratory activity is correlated with high biomass production and active uptake of nutritious substances from the culture broth. After 17 h of cultivation, cultures enter the stationary phase and a drop in respiratory activity was observed (Fig. 2(D)). At 48 h, there were single red spots seen inside the clumps (Fig. 2(E)). No detectable respiratory activity was observed after 72 h of cultivation. The primary metabolism of the clumps at 72 h was low and could not be detected by the CTC fluorescent method (Fig. 2(F)). The CTC assay allows precisely establishing the logarithmic growth phase as well as early and late stationary phase. Using both fluorescent staining methods, the authors determined that the most intensive growth of *Streptomyces* sp. 8812 mycelium under submerged culture occurs during the first 17 h of cultivation.

In summary, mycelium observations allowed to establish that the intensity of production of secondary metabolites with DD-carboxypeptidase 64–575 and HRP inhibitory activity by *Streptomyces* sp. 8812 was correlated with high respiratory level and mycelium viability.

Mycelium enzymatic profiles. Results of the api® ZYM test for *Streptomyces* sp. 8812 revealed the highest activity of two enzymes: leucine arylamidase and α-chymotrypsin. Both enzymes confirmed that *Streptomyces* sp. 8812 assimilates nitrogen during the whole fermentation process. Other enzymes from the aminopeptidase group: valine and cystine arylamidases, and trypsin showed low activity at the initial stages of cultivation. Arylamidases and trypsin were active till 48 h and 120 h, respectively. The api® ZYM test revealed low activity of hydrolases, such as esterase or esterase lipase (color intensity 3). The activity of esterase and esterase lipase was noted at 0 h and 24 h of cultivation, respectively. There was no esterase activity observed after 72 h. Esterase lipase was active till the end of fermentation. Acid phosphatase showed a high level of activity during the first 17 h, between 24 h and 120 h its activity was reduced. The api® ZYM results revealed that acid phosphatase could be an enzymatic marker to precisely determine the logarithmic phase of *Streptomyces* growth. The most intensive phase of

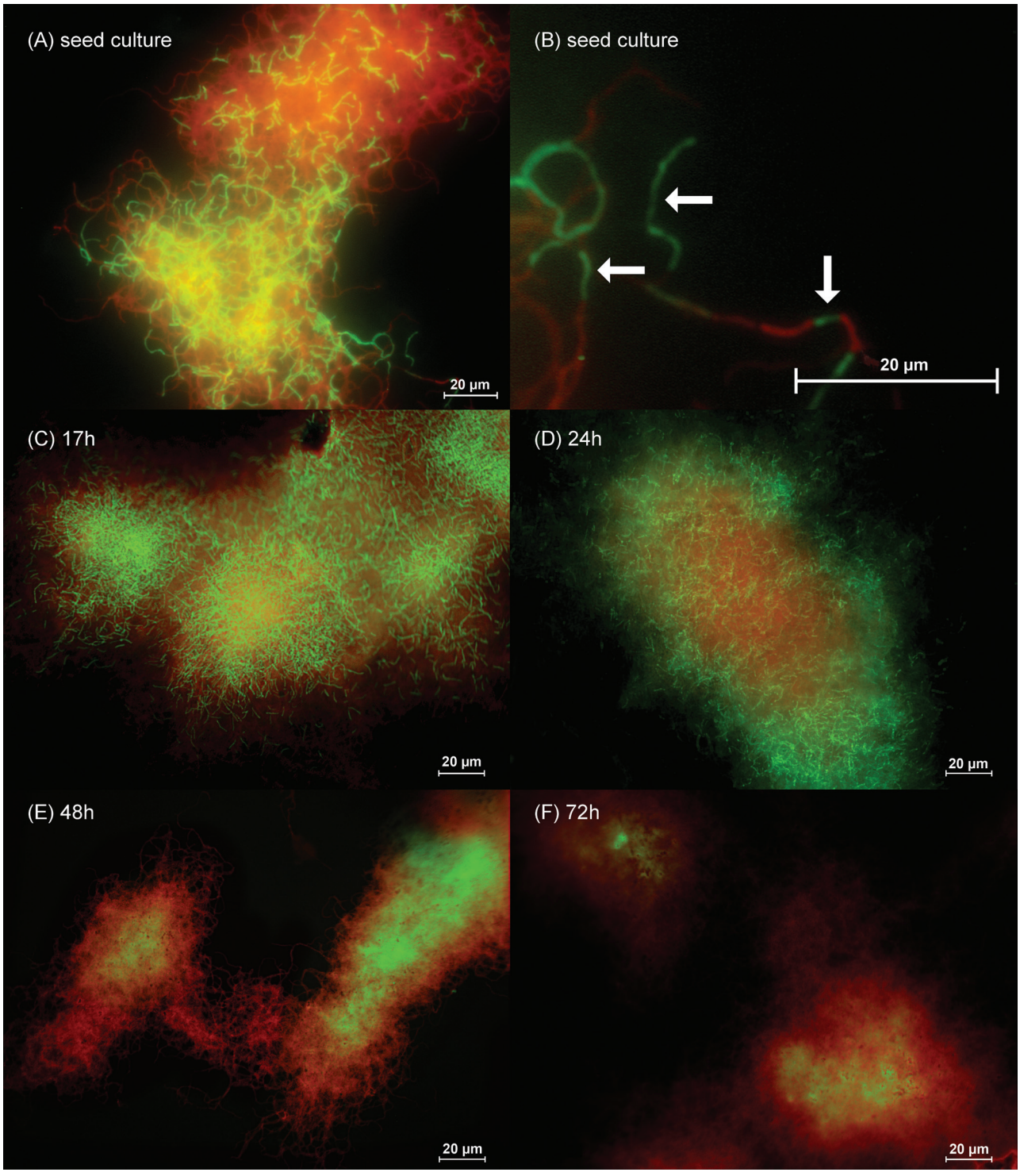

Fig. 1 (A)-(F). Analysis of the development and cell death processes of mycelium in clumps of *Streptomyces* sp. 8812 in submerged cultures. Images correspond to mycelium stained with SYTO 9 and PI. (B) Single live cells in hyphae from seed culture are indicated with arrows.

Streptomyces sp. 8812 growth was between 17 h and 24 h of cultivation. An alkaline phosphatase was active at low level from 48 h to 120 h. The activity of naphthol-AS-BI-phosphohydrolase was high only at 120 h and could result from release of this enzyme from the cytosol of dead bacterial cells. No activity was detected for lipase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase during the whole process of *Streptomyces* sp. 8812 fermentation.

Comparison of *Streptomyces* sp. 8812, *S. capoamus* and their metabolites. *S. capoamus* and *Streptomyces* sp. 8812 cultured for 14 days on ISP2 agar showed similar phenotypic characteristics of aerial mycelium.

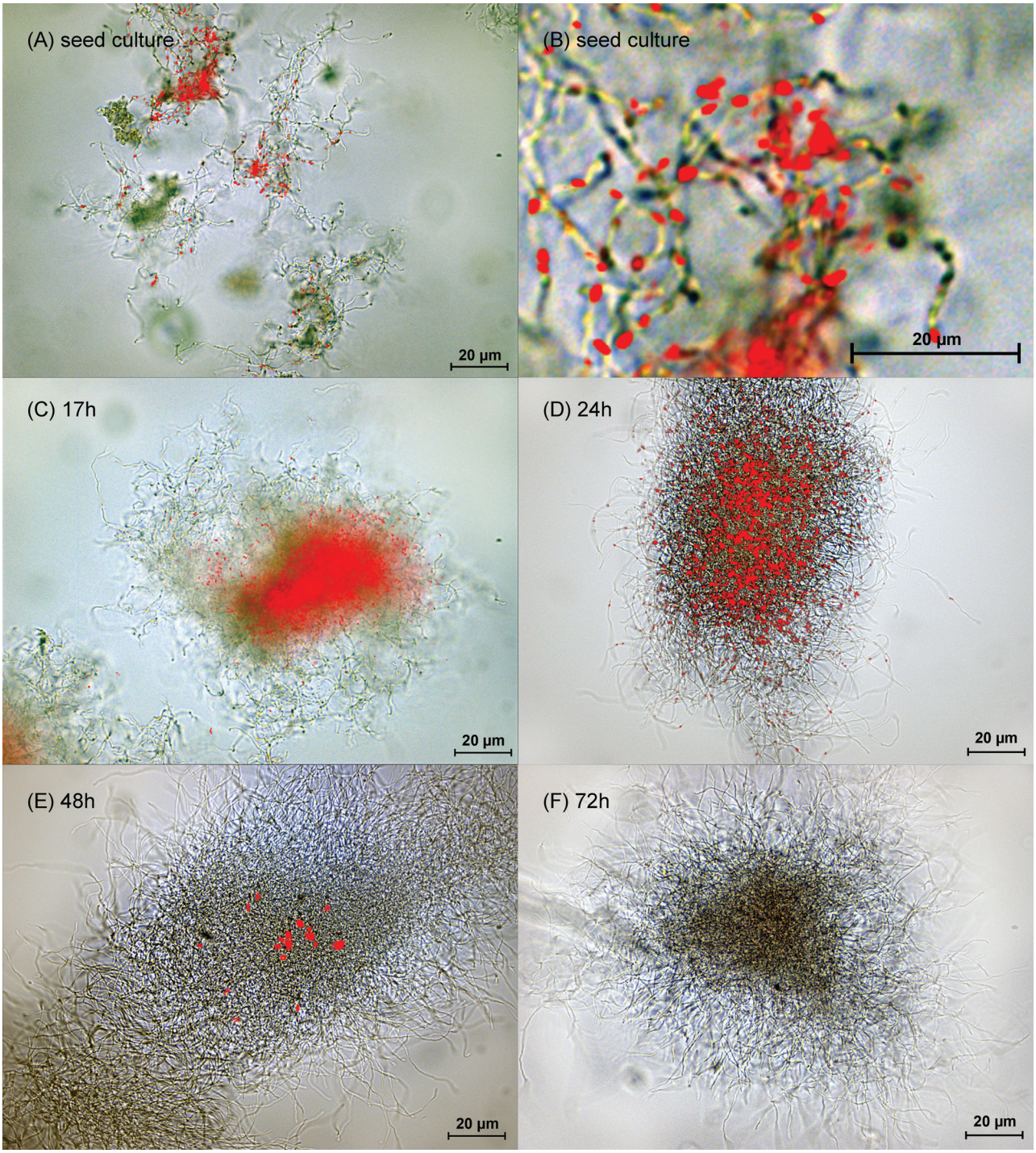

Fig. 2 (A)-(F). Analysis of respiratory processes of mycelium in clumps of *Streptomyces* sp. 8812 in submerged cultures. Images correspond to mycelium stained with CTC.

When both strains were cultivated in medium M, their secondary metabolites exhibited very similar DD-carboxypeptidase 64–575 inhibitory activities (Supplementary Fig. 6S). Until now, the only antitumor and antifungal metabolites isolated from *S. capoamus* fermentation broth were capoamycin and ciclacidine (Goncalves *et al.*, 1968; Hayakawa *et al.*, 1985). In the present study, it was determined that *S. capoamus* produces also DD-carboxypeptidase 64–575 inhibitors.

Conclusions. Conducted studies confirmed that biosynthesis of bioactive metabolites by *Streptomyces* sp. 8812 is a bioprocess influenced by various external factors (pH, medium composition, temperature, feeding). Statistical analysis showed that for *Streptomyces* sp. 8812 increasing amounts of complex nitrogen sources (except Bacto™ tryptone) in the tested media was important for biosynthesis of DD-carboxypeptidase 64–575 inhibitors. Higher DD-carboxypeptidase

64–575 inhibitory activities of secondary metabolites were achieved when the C/N ratio was lower. Production of DD-carboxypeptidase 64–575 inhibitors began at the same time as biomass formation (Table III) and reached the maximum level after 48 h. Production of secondary metabolites was not affected by pH value of the production medium in the measured pH range of 5.5 to 7.4. The optimal age of seed culture for the production of bioactive metabolites was 24 h or 48 h. The authors also observed a concentration-dependent stimulation of metabolites activities during L-tyrosine feeding. Phylogenetic analysis of *Streptomyces* sp. 8812 indicated that its closest relative is *S. capoamus* JCM 4734 (98%), whereas sequence analysis for 16S rRNA gene using NCBI BLAST algorithm showed 100% homology between these two strains.

Development studies of *Streptomyces* sp. 8812 in submerged culture were performed by means of LIVE/DEAD® Bac-Light™ Bacterial Viability Kit, respiratory activity CTC test and api® ZYM test. Obtained results allowed to separate precisely the logarithmic and stationary phase of growth. Intensive production of secondary metabolites with DD-carboxypeptidase 64–575 and HRP inhibitory activity ceased when the respiratory level and viability of the mycelium dropped. Overall, it was determined that vital functions determine the production of secondary metabolites in *Streptomyces* sp. 8812.

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