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SCIENTIFIC PAPER

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PRODUCTION OF BIOTECHNOLOGICAL USEFUL METABOLITES BY *Mucor racemosus* IN CZAPEK-DOX LIQUID MEDIA SUPPLEMENTED WITH SYNTHETIC DETERGENT

Article Highlights

- *M. racemosus* was tested for production of biotechnologically useful metabolites
- Fungus was grown in liquid medium with addition of detergent at concentrations of 0.3 and 0.5%
- The detergent at 0.3% enhanced α-amylase activity (49.59%) and quantity of arginine (40.38%)
- Amount of arginine (119.09%) and alanine (192.79%) was notably enhanced by 0.5% of detergent
- α-Amylase activity retained full activity in the medium with 0.5% of detergent

Abstract

The capacity of native isolate Mucor racemosus to produce several potentially useful metabolites in a liquid Czapek-Dox medium supplemented with powder anionic-type detergent MERIX (Henkel, Serbia) at concentrations of 0.3% (D3) and 0.5% (D5) was examined in this study. The changes of pH values, the total protein content, activities of acid and alkaline invertase, α-amylase, as well as biomass dry weight were evaluated during fungal growth from inoculation until the 16th day. In addition, the qualitative and quantitative amino acids content of 16 days old fermentation broth was determined by HPLC. D3 considerably enhanced the biomass dry weight (43%), α-amylase activity (49.59%) and the quantity of arginine (40.38%), and also influenced the production of a high amount of proteins (5.32 g/L). D5 significantly enhanced the biomass dry weight (53%), the quantity of arginine (119.09%) and alanine (192.79%) and induced the production of valine, serine and glutamate. In the D5 medium, α-amylase retained 100% of its activity. The acid and alkaline invertase activity was moderately inhibited by D3 and D5. The obtained results may have considerable biotechnological, industrial and environmental potential.

Keywords: amino acids, amylase, detergent, growth, invertase, Mucor racemosus.

During the past decades, the use of products derived from filamentous fungi in fermentation processes has been constantly increasing [1]. Numerous fungal products such as enzymes, alcohols, organic acids and pharmaceuticals have a significant role in the development of modern biotechnology [2]. Enzymes are the most important microbial products obtained for human needs. They have a wide range of application in all industrial sectors including food, environment, pharmaceutical, textile, paper, detergent, *etc*. Two types of fermentation processes commonly used for enzymes production are solid state fermentation (SSF) and submerged fermentation (SmF). In spite of the fact that SSF in comparison with SmF provides higher volumetric productivities, there are some advantages when using SmF. This system

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is easier to engineer by researchers due to the ease of process control and stabilization. Since the production and yield of the aforementioned products depend on the nutritional factors (especially carbon and nitrogen sources) in addition to physicochemical parameters (initial pH, incubation temperature and time) [3,4], the cost of fermentation processes is quite high [5]. From the economic point of view, it is necessary to reduce the production cost through alternative methods. Therefore, the production of different enzymes and other biomolecules in a single fermentation medium could be an efficient strategy [5].

Among commercially important, hydrolytic enzymes, invertase (EC.3.2.1.26) and *α*-amylase (EC.3.2.1.1) have received a great deal of attention because of their extensive biotechnological applications. The spectrum of their use in different industrial areas is summarized in Table 1. In addition to enzymes, industrial production of amino acids is constantly increasing as a result of high volume consumption worldwide. Since they regulate almost all of the metabolic and physiological processes in the body, amino acids are very important organic biomolecules for human health. Potential applications of amino acids range from animal feed additives to flavor enhancers, sweeteners and pharmaceuticals, depending on their type (Table 1).

Mucor racemosus is one of the most common species of the genus *Mucor* and is widely distributed in nature [13]. Nevertheless, there are only few studies focused on their potential application in biotechnology. Generally, *M. racemosus* is utilized in the manufacture of sufu, a fermented cheese-like soybean product common in China and Vietnam [14]. Recent studies have shown that *M. racemosus* may be employed to biotransform some steroid compounds, such as progesterone [15], methyltestosterone [16] and 20(*s*)-protopanaxatriol [17] to intermediates with anti-cancer activities. Likewise, the fungus grown on synthetic and organic substrates may be used for the production of various enzymes such as invertase, alkaline phosphatase, protease and lipase, which are of biotechnological and bioremediation interest [18]. All the previously mentioned findings were obtained during fungal fermentation process using separating solid/liquid media. Nevertheless, there are no reports for the production of different, potentially useful, metabolites such as amino acids and enzymes in single Czapek-Dox liquid medium. Recently, it has been confirmed that Czapek-Dox liquid medium supplemented with commercial detergent enhanced the activity of some fungal enzymes and the quantity of organic acids [19].

The aim of the study was isolation of *M. racemosus* from waste water and investigating the fungal capacity to produce several metabolites: amino acids, acid and alkaline invertase and *α*-amylase in Czapek-Dox liquid medium supplemented with detergent at concentrations of 0.3 and 0.5%. The obtained results could be useful in practical application of the fungus in biotechnology, pharmaceutical, food and detergent industry as well as in bioremediation processes.

Table 1. An overview of the role and practical applications of tested bioproducts in various industries

Bioproduct	Applications	Industry	References
Amylase (EC.3.2.1.1) (starch hydrolyzing activity)	Glucose and maltose syrups, high fructose corn syrup, starch processing, bread baking, liquefaction, digestive aid, warp-sizing of fibers, delayed staling, mashing, spot removal, removal of starch, clarification, starch modification for paper coating, digestive aids, desizing of fabrics, liquefying purees and soups	Food, baking and milling, brewing, starch, vegetables, distilled beverages, flavors, paper, textile, detergent, dry cleaning, pharmaceutical and clinical	[6,7]
Invertase (EC.3.2.1.1) (sucrose hydrolyzing activity)	Soft center candies and fondants, chocolate coated, high test molasses, enzyme electrode, immune booster, antioxidant, antiseptic, digestive aid tablets	Confectionery, candy, miscellaneous, cosmetic, drug, bioelectronic, paper, detergent	[8,9]
Glutamate	Neural communication, memory formation, learning and regulation, digestion in the stomach	Food, flavor, drug, pharmaceutical, cosmetic	[10-12]
Alanine	Glucose-alanine cycle between tissues and liver, blood pressure, cholesterol level, energy intake		
Valine	Growth of new tissue, metabolic fuel for muscle		
Serine	Catalytic function of trypsin, chymotrypsin, precursor for glycine and cysteine, biosynthesis of purine, pyrimidine, sphingolipids and folate		
Arginine	Cell division, spermatogenesis, embryonic survival, fetal and neonatal growth, removing ammonia, release of hormones, immune function		

EXPERIMENTAL

Materials

Sodium nitrate, potassium phosphate dibasic, magnesium sulfate heptahydrate, sucrose, sodium carbonate, copper sulfate and potassium sodium tartrate were obtained from Merck KGaA (Darmstadt, Germany). 3,5-Dinitrosalicylic acid, glucose standard solution (1mg/mL), starch from potato, bovine serum albumin, sodium acetate, sodium hydroxide, amino acid standard mixtures and Folin-Ciocalteu phenol reagent were obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and reagents were of analytical grade. Commercial detergent "Merix" was kindly supplied by Henkel (Kruševac, Serbia).

All absorption spectra were made using a Perkin-Elmer Lambda 25 UV-Vis spectrophotometer (Shelton, CT, USA). The measurement of pH values of the culture filtrates was carried out by a PHS-3BW digital microprocessor pH/mV/temperature meter model 65-1 (Bante Instruments Ltd., China). Analysis of amino acids was performed using a Dionex Amino Analyzer equipped with an AminoPac PA10 (Dionex, Sunnyvale, CA, USA), an electrochemical detector ISC 5000, with silver reference (pH/Ag/AgCl) and gold (Au) working electrodes.

Isolation, identification and selection of the fungus

The fungal isolate used in this study originated from water samples of the Rasina River (Kruševac, Serbia). The river contains industrial waste water from a factory producing detergents (Henkel, Kruševac). The samples were taken in aseptic glass bottles and transferred to the Microbiological Laboratory at the Institute for Biology and Ecology, Faculty of Science, Kragujevac. The samples were deposited in a refrigerator at 4 °C for 24 h. The next step was the isolation of pure fungal cultures. For this purpose, sterile Petri dishes with potato dextrose agar (PDA) and streptomycin were used. After inoculation, they were placed in an incubator at 28 °C for 5 to 7 days. Pure cultures were obtained by the method of exhausting on poor PDA dishes. The identification of pure fungal cultures was based on morphological characteristics and was carried out by systematic key. The fungal colonies grown on PDA at 28 °C were white at first to brownish to gray with age. Sporangia were 32-54 μm in size and became brownish to gray at maturity. The sporangia contained thousands of ellipsoidal to subglobose sporangiospores. Sporangiophores were 8-14 μm in size and short branches sometimes recurved, with encrusted walls, globose in shape. Sporangiospores were 5-12 μm×4-8 μm in size and broadly ellipsoidal to subglobose, smooth walled, grayish in color. Columella was 27 μm×42 μm variable in size and obovoid, ellipsoidal, cylindrical-ellipsoidal, slightly pyriform, usually with a truncated base and light brown in color. Chlamydospores were numerous in sporangiophores and barrel-shaped when young, subglobose in old cultures, yellowish in color and 8-18 μm in size. The morphological characteristics of the fungus examined in this study were identical to *M. racemosus* described by previous works [20,21].

The pure cultures were cultivated then on Petri dishes with PDA and different concentrations of tested detergent. The fungus with the best colony diameter and the concentration of detergent which provides the best fungal growth were determined. The isolate with the best growth was identified as *M. racemosus* Fresenius and was selected for further study.

Experimental conditions

The fungus was grown in 250 mL Erlenmeyer flasks with 200 mL of modified Czapek-Dox liquid nutrient medium with the following composition (g/L): $NaNO_3$ – 3.0; K_2HPO_4 – 1.0; $MgSO_4{\cdot}7H_2O$ – 0.5 and sucrose – 30.0, and distilled water up to 1000 mL (control-C). The pH value of the control (C) medium was measured by pH-meter and adjusted at 4.80 with 0.1 M HCl. The detergent at concentrations of 0.3 and 0.5% was then added to the C medium and pH values of these media (D3 and D5) were measured again and recorded as initial pH values. The Erlenmeyer flasks were autoclaved at 121 °C for 20 min. Each flask with the exception of negative controls (ncD3 and ncD5) was inoculated with 1 mL spores suspension (10^7 spores/mL). Afterward, all bottles were incubated on an electric shaker (Kinetor-*m*, Slovenia) at 150 rpm and room temperature for 16 days. Sampling was started on the 3rd day after inoculation and repeated in the same intervals until the end of experiment. In order to separate mycelium from the fermentation broth, the content of each Erlenmeyer was filtered through Whatman filter paper No. 1.

Determination of mycelium dry weight biomass

The biomass of the fungal mycelium in the fermentation broth was estimated in terms of dry weight. The mycelium separated on filter paper was washed with distilled water several times. Thereafter, both the filter paper and mycelium were dried at 80 °C until a constant weight was obtained and re-weighed. The mycelium dry weight was calculated using Eq. (1):

$$DW\,(\mathrm{g/L}) = 5(Wcf - Wif) \quad (1)$$

where *DW* is total mycelium dry weight biomass, *Wcf* is weight of mycelium with filter paper and *Wif* is initial weight of filter paper.

Preparation of crude enzyme extract

The fermentation broth previously collected by filtration was then centrifuged at 12000*g* (at 4 °C) and used as the crude extract for the measurement of pH values, proteins, amino acids and enzyme activities.

Measurement of pH values

The measurement of pH values of the culture filtrates was carried out by digital PHS-3BW microprocessor pH/mV/temperature meter model 65-1 (Bante Instruments Ltd., China). The glass electrode was initially standardized with appropriate buffer solution of pH 4.0, 7.0 and 10.0. The electrode was inserted into the fermentation broth, and pH value was read and recorded.

Protein assay

Protein was determined according to Lowry [22]. The sample test tube contained 0.2 mL of fermentation broth and 3 mL of the prepared alkaline reagent (2% Na_2CO_3 was dissolved in 0.1 M NaOH, 0.5% $CuSO_4 \cdot H_2O$ and 1% solution of the K-Na tartrate). The control test tube contained 0.2 mL of distilled water and 3 mL of alkaline reagent. The reaction mixtures of both test and control tubes were mixed and kept at room temperature for 15 min. Thereafter, 0.6 mL of Folin-Ciocalteu phenol reagent (dissolved in distilled water in the ratio of 1:2) was added in each tube. They were mixed and kept at room temperature for 30 min. The protein concentration in the sample was determined spectrophotometrically (Perkin-Elmer Lambda 25 UV-Vis spectrophotometer, USA) by reading the absorbance at 750 nm. Bovine serum albumin was used as standard.

Determination of amino acids

Amino acid content of the fermentation broth of the control and both detergents' media was analyzed at the end of the experimental period (on the 16th day) by anion-exchange chromatographic method, also known as AAA-Direct gradient method [23] in the Laboratory Sojaprotein (Bečej, Serbia). Sample injection was via a Dionex auto-sampler equipped with a 25 µL sample loop, after filtration through a membrane filter of 0.2 µm. Amino acids were separated on an AminoPac PA10 analytical (2×250 mm) and an AminoPac PA10 guard column (2×50 mm) (Dionex, Sunnyvale, CA, USA), at a flow rate 0.25 mL/min., at temperature of 30 °C. The mobile phase consisted of deionized water, 0.250 M sodium hydroxide, 1 M sodium acetate gradient system. Detection was by electrochemical detector ISC 5000 (Thermo Scientific) with a silver reference electrode (pH/Ag/AgCl) and a gold (Au) working electrode. Chromatographic data were collected and plotted using the Dionex Autolon 300 Software.

Assays of invertase activity (EC 3.2.1.26)

Acid invertase activity was determined by incubating 300 µL of fungal enzyme source and 700 µL of sucrose dissolved in 0.06 M citrate buffer (pH 4.5), at 55 °C for 30 min. For determination of alkaline invertase activity, the enzyme source and sucrose dissolved in 0.02 M of phosphate buffer (pH 8.0) were mixed and incubated at 37 °C for 30 min. To stop the reaction, 1 mL of 3,5-dinitrosalicylic acid reagent was added and heated for 5 min in a boiling water bath. The absorbance was read at 410 nm on the spectrophotometer [24]. Glucose was used as the standard. One unit (IU) of invertase activity was defined as the amount of enzyme that catalyzed the production of 1 µmol of reducing sugar as glucose under the assay conditions.

Assay of α-amylase activity (EC 3.2.1.1)

The enzyme activity was determined according to Bernfeld [25] by incubating a reaction mixture of 1 mL 1% starch dissolved in 0.02 M phosphate buffer (pH 6.8) and 0.5 mL of aliquot of fungal enzyme source and phosphate buffer, at 55 °C for 10 min. Afterwards, the reaction was stopped by adding 2 mL of a solution of 3,5-dinitrosalicylic acid followed by boiling for 10 min. The reducing sugar released from the enzymatic reaction was measured spectrophotometrically by reading the absorbance at 540 nm, with glucose as standard. The amylase activity can be defined as the amount of enzyme that released 1 µmol of reducing sugar as glucose per min (IU/mL).

Statistical analysis

The data of this study were analyzed using SPSS statistical software package (SPSS for Windows, ver. 13.0, Chicago, IL, USA). For testing of the normality of distribution, means and standard deviation, Student's *t*-test was used. Comparison between growth media were performed with Mann-Whitney and Kruskal-Wallis tests. Pearson's correlation coefficient was used for the measurement of the strength of the association between tested variables. All significance tests were two-tailed (0.05 and 0.01). A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Biomass formation

The formation of fungal biomass during 16-day cultivation in C, D3 and D5 media is shown in Table 2. The growth of the fungus was influenced by the time interval and chemical composition of media. During first 3 days the fungal biomass formation was very low in C medium (0.088 g/L), but increased about 2- and 7.8-fold in the presence of D5 and D3 medium, respectively. Obviously, the fungus had better response to D3 than D5 in the early phase of growth. From the 3rd to the 6th day, a considerable amount of biomass was produced in C and D5 media which can be explained by intensive metabolic activity of the fungus. At the same time interval, the biomass formation in D3 medium was very low and indicates on stationary phase in fungal growth. The significant changes in fungal growth were observed between the 6th and the 9th day. Over a mentioned period, the growth of fungus was a very low in C medium and indicates on stationary phase and beginning of autolysis, since the biomass was reduced after that period. The biomass formation in D3 media was increased until the 12th day, on which the highest biomass was obtained, but afterward the biomass decreased as a result of autolysis. In D5 medium, the decreasing of biomass was observed between the 6th and the 9th day; after that the biomass was increased until the end of experiment. The biomass was estimated at the end of the experimental period in C, D3 and D5 media was 0.620, 0.884 and 0.944 g/L, respectively. Statistical analysis confirmed significant differences in biomass DW amount between C and D3 ($t = 0.031$, $p < 0.05$) and D5 ($t = 0.025$, $p < 0.05$) media. According to statistics, a moderate correlation revealed between biomass amount and cultivation time, which can be explained by the above-mentioned autolysis. Regardless of autolysis, total biomass DW measured in D5 and D3 media on the 16th day was about 53 and 43% higher compared to C, respectively. The differences in biomass between the two growth media were due to the fungal ability to degrade the detergent and to incorporate the products of degradation into biomass. This finding is in line with the result of our previous study [18], which showed that *M. racemosus* has the capacity to degrade commercial detergent at concentration of 0.5%. The results of this study, showed higher growth ability of fungus in D5 than in D3 medium, probably as a result of better biodegradation rate of the detergent at higher concentration. In existing literature data, only few fungi species are identified as better degraders of higher concentrations of pollutants than lower ones. For example, Mohsenzadeh *et al.* [26] found that *Alternaria* sp. and *Penicillium* sp. are better at decreasing efficiency of petroleum at 8 than 2%. Taking into consideration these facts, the current study can be very important from the aspect of mycoremediation of the environment and provide support for further investigation in this direction.

Changes in pH value of media

Table 2 shows the changes in pH values during fungal growth in C, D3 and D5 media. The pH values before inoculation in C, D3 and D5 media were 4.80, 9.35 and 9.80, respectively. During cultivation of fungus, the pH values of media were changed according to the type of media and cultivation time. Statistical data showed a low and positive correlation between pH and type of media whereas negative correlation was found between pH and cultivation time, biomass amount, and protein content. The pH value of the C medium was slowly increased during the growth of mycelium (until the 6th day). Thereafter, the pH value of medium was decreased during the stationary phase and autolysis. In contrast to C, the pH values of D3 and D5 media strongly decreased during the intensive growth of fungi; after that the changes in the pH value were less pronounced. The most significant decrease in pH value was noted in D3 medium from 9.35 to 6.24 and in D5 medium from 9.36 to 6.46. These changes could be explained by the production

Table 2. Total biomass dry weight, total protein content and pH of M. racemosus during 16 days of cultivation in C (control medium), D3 (medium with 0.3% detergent) and D5 (medium with 0.5% detergent)

Day	Total biomass dry weight, g/L			pH			Total protein content, g/L		
	C	D3	D5	C	D3	D5	C	D3	D5
0	0	0	0	4.80	9.35	9.85	0	0	0
3	0.088	0.676	0.150	5.24	6.24	9.36	2.060	3.185	0.092
6	0.648	0.700	0.876	6.13	6.37	6.46	1.560	2.720	2.110
9	0.650	0.816	0.800	6.01	6.14	6.89	5.130	5.320	2.490
12	0.624	0.924	0.876	5.87	5.36	6.31	5.320	2.740	2.490
16	0.618	0.884	0.944	5.80	5.50	5.62	5.130	2.740	3.740

of strong organic acids in media as a result of intensive metabolic activity. It is important to mention that the final pH of culture media was identical, although the initial pH values differed significantly. This ascertainment implicates a good mechanism regulation of external pH and the slightly acidophilus nature of the fungus. The obtained results were in accordance with the results of several authors who have demonstrated that a decrease in pH values of the fungal cultures was due to the production of organic acids [27]. Vrabl *et al*. [28] pointed out that high external pH values strongly enhance the production of organic acid by *Penicillium ochrachloran*. Moreover, this phenomenon might be a general response of filamentous fungi to alkali stress.

Protein content

The total protein content secreted in fermentation broth of C, D3 and D5 is represented in Table 2. The quantity of protein in C medium was increasing to 2.06 g/L from inoculation until the 3rd day and then decreasing to 1.56 g/L until the 6th day. However, the rapid increase of protein content was observed from the 6th to the 9th day, with the highest value, *i.e.*, 5.32 g/L noted on the 12th day. The quantity of proteins in D3 medium was higher compared to C medium until the 9th day. The maximal value of proteins secreted in this medium (5.32 g/L) was identical as in C medium. Therefore, the total protein content was significantly decreasing until the end of experimental period. In D5 medium, the total protein content was very low on the 3rd day; afterwards it was considerably increasing until the 6th day. From that point on, the quantity of protein was slightly increasing until the 12th day. Thereafter, total protein content was significantly increasing and achieved the highest value (3.74 g/L) on the 16th day. The ratio between highest protein content in C and in both D3 and D5 media revealed more efficient secretion in D3 media than in D5 medium, probably as a result of different growth rates of mycelia in these media. The obtained results agree with the study of Yücesoy *et al*. [29] which confirmed correlation between protein amounts and biological activity. According to the statistics, a moderate correlation was found between total proteins and α-amylase activity, whereas a low correlation was found between total proteins and invertase activities (AlkI and AcI). This statistical data implicates that a low connectivity between total proteins and invertase activities could be the result of the inhibitory effect of the tested detergent (or some of its degradation products) on invertase activities.

Qualitative and quantitative composition of amino acids

Qualitative and quantitative content of amino acids produced by *M. racemosus* in Czapek-Dox liquid medium without and with addition of detergent at concentrations of 0.3 and 0.5% was determined on the 16th day. The results are presented in Table 3 and Figure 1. In C and D3 media, the fungus produced only 2 amino acids: alanine and arginine. However, the addition of detergent at a concentration of 0.5% into the growth medium caused the production of 5 different amino acids: alanine, arginine, valine, serine and glutamate. Based on obtained results, arginine was the most represented amino acid found in all tested media. Quantitatively, the addition of detergent in the medium enhanced the production of arginine for 40.38 (D3) and 119.09% (D5) compared to the control medium. It is important to note that the amount of alanine produced in growth media was significantly influenced by a concentration of detergent. The D3 had an effect on the production of lower quantity of alanine (for 25.25%) whereas the D5 strongly enhanced the quantity of alanine (for 192.79%) compared to the control medium. Three amino acids: valine, serine and glutamate were specific only for D5 medium. The quantity of valine was 7-fold, and serine and glutamate 13-fold lower in relation to arginine.

Table 3. The qualitative and quantitative amino acids content (mg/L) of 16 days old fermentation broths of M. racemosus determined in C (control medium), D3 (medium with 0.3% detergent) and D5 (medium with 0.5% detergent)

Medium	Qualitative composition of amino acids	Quantitative composition of amino acids, C / mg L^{-1}	Retention time, t / min
C	Alanine	2.048	1.77
	Arginine	0.305	6.47
D3	Alanine	2.875	1.77
	Arginine	0.228	6.47
D5	Alanine	4.487	1.77
	Arginine	0.893	6.47
	Valine	0.637	8.73
	Serine	0.338	10.20
	Glutamate	0.344	31.15

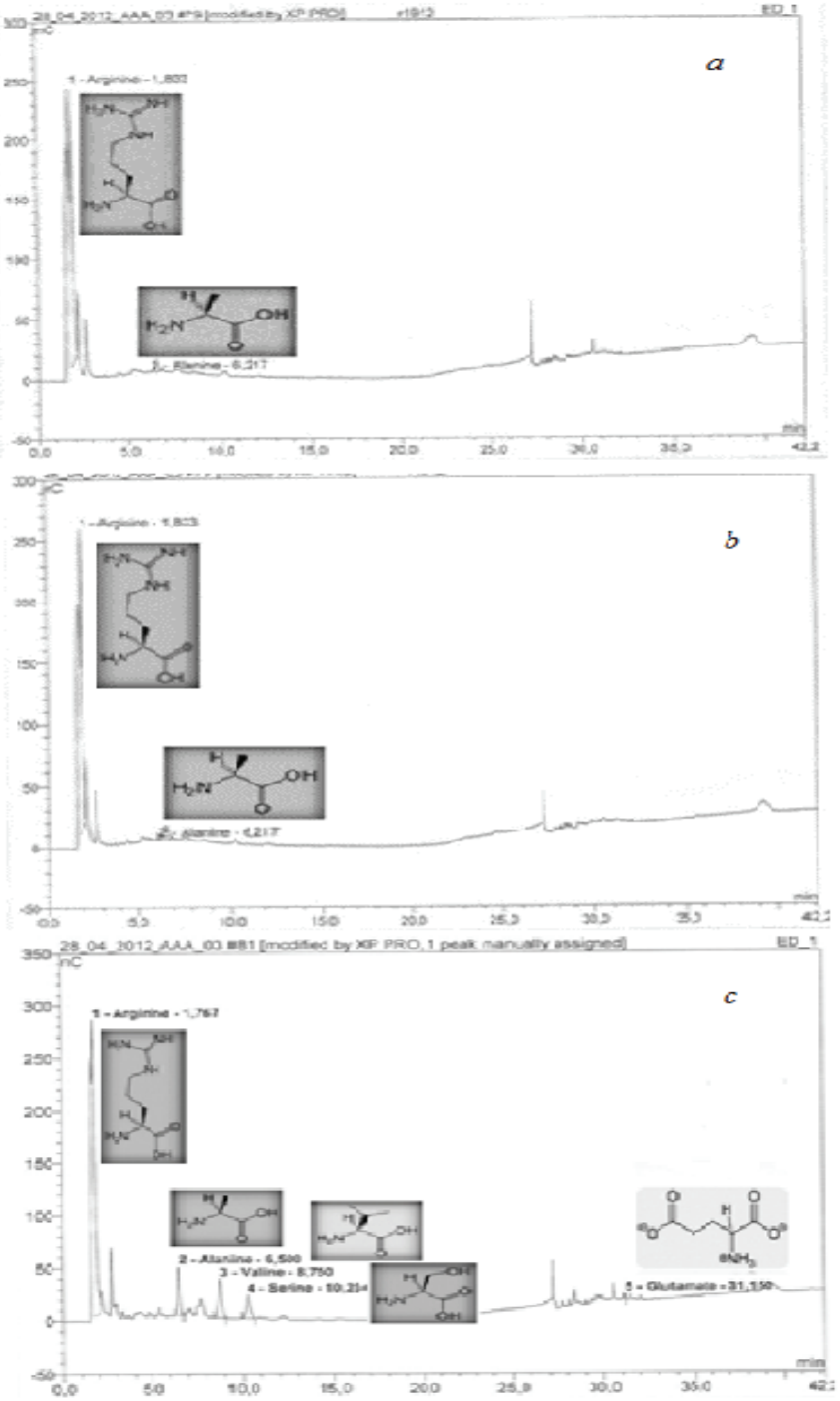

Figure 1. The chromatogram of amino acids: a) determined in C (control medium), b) in D3 (medium with 0.3% detergent) and c) in D5 (medium with 0.5% detergent) after 16 days of fungal cultivation.

Recently, Adedayo and Sani [30] have investigated amino acid composition of baobab fermented with *M. racemosus*. They revealed that the fermented sample contained a high amount of glutamate and about 3-fold smaller amount of arginine, valine, alanine and serine. The observation of mentioned authors is opposite to current results which revealed the different amino acids content of *M. racemosus*. These differences in production of amino acids can be explained by the influence of the experimental conditions (composition of growth medium, conditions of growth, duration of experiment, etc.) and fungus origin. Stojanovic and coworker [31] investigated the effect of the Czapek-Dox liquid medium (without and with 1% D) on the secretion of amino acids by several filamentous fungi. They revealed that the composition

of this medium has good properties for the production of a high yield of amino acids by *F. oxysporum* and *T. roseum*. On the other hand, *A. niger*, *A. tenuis* and *P. verrucosum* produced a lower quantity of amino acids in this medium. The aforementioned authors demonstrated that the addition of 1% D in the medium reduces or enhances the quantity of amino acids, depending on the fungal species or type of amino acids. For example, the detergent caused the sreduction in amino acids yield by *A. tenuis*, *P. verrucosum* and *T. roseum,* and stimulation in amino acids yield by *A. niger* (especially glutamic acid, leucine, and asparagine acid) and *F. oxysporum* (alanine, glutamic acid, and asparagine acid) [31]. The current results partially agree with results obtained by the mentioned authors. Moreover, this is the first study which identified the qualitative and quantitative amino acids compositions of *M. racemosus* in applied experimental conditions and provides a good base for further research toward the optimization of cultivation conditions and quantification of amino acids.

Invertase (EC 3.2.1.26) activity of *M. racemosus*

Figure 2 shows the activities of acid (AcI) and alkaline (AlkI) invertase during 16-day-cultivation of *M. racemosus* in C, D3 and D5 media. In the C medium, the activity of AcI rapidly increased from the 6rd to the 9th day, when it achieved the maximum value of 0.03 IU/mL. At the beginning of the stationary phase, the enzyme activity was decreased and the lowest value was achieved on the 12th day (autolysis phase). Regardless of the concentration, the presence of detergent in the growth medium inhibited AcI activity compared to C medium. Thus, the maximal

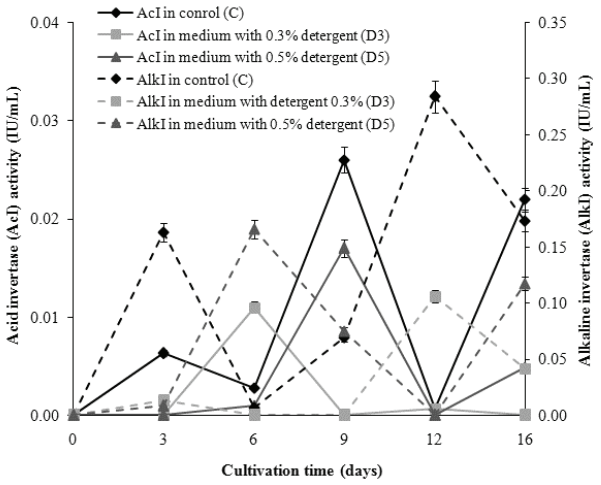

Figure 2. Acid (AcI) and alkaline (AlkI) invertase activity (IU/mL) of M. racemosus noted in C (control medium), D3 (medium with 0.3% detergent) and D5 (medium with 0.5% detergent) during 16 days of cultivation.

values of AcI measured in D3 and D5 media were 0.01 and 0.02 IU/mL, respectively. The AlkI activity in C medium rapidly increased from the 6rd to the 12^{h} day, when the enzyme achieved maximum activity (0.28 IU/mL). Evidently, the both detergents' concentrations had inhibitory effect on AlkI activity. In D3 and D5 media, the enzyme reached maximal values of 0.11 and 0.17 IU/mL on the 12th and the 6th day, respectively. In other words, D5 and D3 inhibited about 37.32 and 58.45% of the AlkI activity, respectively, compared to C medium.

The effect of pure surfactants and commercial detergents on invertase activity of filamentous fungi is insufficiently examined and described in literature. According to El Atar [32], invertase activity of *Penicillium chrysogenum* in the presence of surfactants sodium dodecyl sulfate (SDS), Tween 20, 40, 60 and 80 and Triton X-100 enhanced for 21.3, 18.8, 17.5, 13.8 10 and 6.3%, respectively. In contrast to these results, Shankar *et al*. [33] revealed that relative invertase activity of *Saccharomyces cerevisiae* MTCC 170 decreased with increase in concentration surfactants (1, 3 and 5%) and by time of exposure. Moreover, in the presence of 1% polyethylene glycol (PEG), SDS, Tween 80 and 20 and Triton X-100, relative enzyme activity was as follows: 35, 30, 25, 15 and 10%. Recently, Jakovljević *et al*. [34-36] have investigated the invertase activity of *P. chrysogenum*, *Penicillium cyclopium* and *Trichoderma harzianum* during submerged fermentation in Czapek-Dox liquid medium supplemented with commercial detergent "Merix" at a concentration of 0.3%. The detergent showed stimulatory effect on AlkI activity of *T. harzianum* but inhibitory effect on enzyme activity of *P. chrysogenum* and *P. cyclopium.* On the other hand, AcI of *P. cyclopium* was stimulated by the detergent. Taking into consideration the results of the previous and current studies, it could be concluded that the effect of the detergent on invertase activities of fungi depends on their morpho-physiological characteristics.

α-Amylase activity (EC 3.2.1.1)

Figure 3 presents data of fungal *α*-amylase activity determined in C, D3 and D5 media during 16-day cultivation. As the figure shows, *α*-amylase activity in all types of growth media was very low on the 3rd day of cultivation. Afterwards, the enzyme activity rapidly increased until the 9th (in D3 medium) and the 12th day (in C and D5 media). The highest enzyme activity was achieved in D3 (16.23 IU/mL) followed by in C and D5 media (10.85 IU/mL). In other words, the enzyme retained full activity (100%) in D5 medium,

whereas its activity was enhanced 49.59% in D3 medium, compared to control.

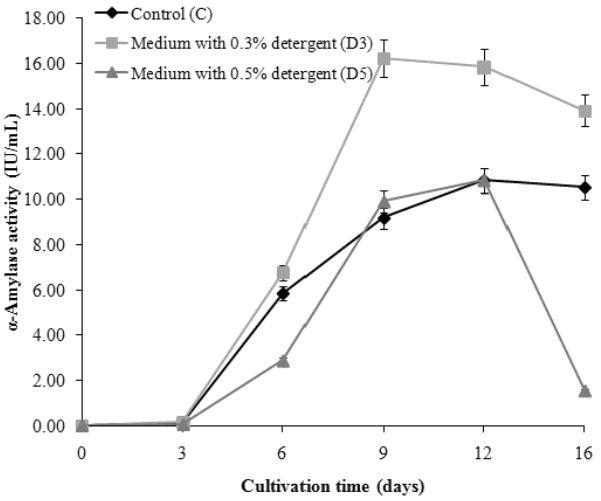

Figure 3. Alpha-amylase activity (IU/mL) of M. racemosus determined in C (control medium), D3 (medium with 0.3% detergent) and D5 (medium with 0.5% detergent) during 16 days of cultivation.

Literature data about the impact of pure surfactants and commercial detergents on amylase activity of microbial original are contradictory. Chakraborty *et al*. [37] revealed 80-97% of *α*-amylase activity from *Streptomyces* A3 in the presence of surfactants (Tween 40, 60 and 80 and SDS) at a concentration of 0.5%. However, the enzyme retained lower percentage activity (from 35 to 70%) in the presence of commercial detergents in the following order: Tide > Surf > Rin > Ariel (all from India). According to Menon et al. [38], amylase activity of *Bacillus subtilis* JS-16 enhanced about 2-fold by SDS, whereas it retained a very low residual activity of 27 and 13% when Surf and Tide (India) were used as additives, respectively. Yang *et al*. [39] found that activity of *α*-amylase from *Alkalimonas amylolytica* was stable in the presence of washing powder detergents - Tide (USA) and Nice. Nevertheless, enzyme activity was less than 50% in the presence of laundry (Blue Moon and Liby) and liquid detergents (On Nice and Liby) (all from China). Ali *et al*. [40] observed relative stability of amylase from *Aspergillus penicillioides* TISTP 3639 in the presence of a liquid and two type powdered detergents (all from Thailand). Recently, Jakovljević and Vrvić [19] have revealed that commercial detergent "Merix" at a concentration of 0.3% enhanced the activity of *α*-amylase from *Aspergillus niger* for 12.7%. The results of the current study are far better than results obtained in the previous study with *A. niger*, and provide a good base for further investigations in terms of practical application of *M. racemosus* in the detergent industry.

CONCLUSIONS

Briefly, the current study highlights the ability of *M. racemosus* to produce several biotechnological useful metabolites: amino acids, proteins, and enzymes such as acid and alkaline invertase and *α*-amylase in single Czapek-Dox medium supplemented with commercial detergent. The production level of metabolites was affected by concentration of detergent. At a concentration of 0.3%, the detergent enhanced the fungal biomass (43%), *α*-amylase activity (49.59%), and the quantity of arginine (40.38%) and affected the production of a high amount of proteins (5.32 g/L). At a concentration of 0.5%, the detergent significantly enhanced the fungal growth and total biomass (53%), the quantity of arginine (119.09%) and alanine (192.79%), and induced the production of valine, serine, and glutamate, whereas *α*-amylase retained full activity. The presented results indicate that the addition of commercial detergent in Czapek-Dox liquid medium inoculated with *M. racemosus* could be a useful strategy for the production of the mentioned biomolecules for the purpose of their practical application in different industrial areas.

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NAUČNI RAD

PRODUKCIJA BIOTEHNOLOŠKI KORISNIH METABOLITA GLJIVOM *Mucor racemosus* U TEČNOJ CZAPEK-DOX PODLOZI SA DODATKOM SINTETIČKOG DETERDŽENTA

U ovom radu ispitivan je kapacitet prirodnog izolata Mucor racemosus za produkciju potencijalno korisnih metabolita u tečnoj Czapek-Dox podlozi sa dodatkom praškastog deterdženta MERIX (Henkel, Srbija) u koncentracijama 0,3 (D3) i 0,5% (D5). Promene pH vrednosti, sadržaj proteina, aktivnost kisele i alkalne invertaze, α-amilaze i količina suve biomase ispitivani su tokom 16-dnevnog uzgajanja gljive. Takođe, kvalitativni i kvantitativni sastav aminokiselina u fermentacionoj tečnosti gljive, nakon 16 dana kultivacije, određen je HPLC metodom. Deterdžent navedenih koncentracija uticao je na dvofazni rast gljive. U podlozi D3, deterdžent je uticao na povećanje suve biomase (43%), aktivnosti α-amilaze (48,59%) i količine arginina (40,38%), a takođe je uticao i na produkciju veće količine proteina (5,32 g/L). U podlozi D5, deterdžent je značajno uticao na povećanje suve biomase (53%), količine arginina (119,09%) i alanina (192,79%), i indukovao je produkciju valina, serina i glutamata. α-Amilaza je zadržala 100% aktivnosti u podlozi D5. Aktivnost kisele i alkalne invertaze bila je umereno inhibirana u obe podloge sa deterdžentom. Dobijeni rezultati mogu imati značajan uticaj na potencijalnu primenu gljive u biotehnologiji, industriji i ekologiji.

Ključne reči: amino kiseline, amilazna aktivnost, deterdžent, invertazna aktivnost, kriva rasta, Mucor racemosus.