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Morphological Characteristics, Extracellular and Intracellular Protein and Enzyme Patterns of Five *Aspergillus* Species

Katarina Jernejc and Aleksa Cimerman*

National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia

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Dedicated to the memory of Professor Vera Johanides

Summary

Six biotechnologically interesting *Aspergilli*, four of which were isolated lately and included in the culture collection of National Institute of Chemistry MZKI, were examined morphologically and biochemically. Since species may no longer produce typical morphological structures, some nonmorphological methods in classification were used.

Based on both macroscopic and microscopic observations, using an identification key, individual isolates were classified within the genus *Aspergillus*, belonging to three subgenera, four sections, five species and two *A. niger* strains. In different *Aspergilli* differences in the amounts of total lipids, sterols and fatty acids were observed, but they were not significant enough to distinguish between species. Intracellular protein profiles did not differ between the two *A. niger* strains, neither were they different in *A. oryzae* and *A. flavus*, both belonging to the section Flavi. Differences appeared between sections Nigri and Flavi, belonging to the subgenera Circumdati, but the largest differences were observed on the subgenus level.

Enzyme analyses clearly differ among fungi examined on the basis of the number of isozyme bands and their molecular weights. Isocitrate dehydrogenase revealed one band, whereas acid phosphatases two or three bands, in all examined fungi. Malate dehydrogenases showed one and lactate dehydrogenases one band or two isozyme bands. Differences on the subgenus level, with the results obtained, were greatest, but differences at strain level could also be seen.

Key words: morphology, isozymes, taxonomy, *Aspergilli*

Introduction

Aspergillus represents a huge genus of economically, as well as ecologically, important fungi in industry and many fields of applied research. The need for clear taxonomic schemes are increasingly relevant *(1)*. The use of nonmorphological methods, such as wall composition, proteins and enzymes, nucleic acids, aminoacids, hydrocarbon metabolism, and inorganic elements in taxonomy were already discussed more than twenty years ago by Fennel *(2)*, because it is possible that strains no longer produce typical morphological structures. Of all these characteristics, only proteins, enzymes and nucleic acids are still considered. The principal role of chemotaxono-

* Corresponding author; Phone: ++386 (0)1 4760 334; Fax: ++386 (0)1 4259 244; E-mail: katarina.Jernejc@ki.si

my is to provide additional characters to distinguish closely related species. Enzyme electrophoresis has been used for the classification of *Aspergillus* isolates *(3)*, differentiation between *Aspergillus sojae* and *Aspergillus oryzae* by enzyme electrophoretic patterns has been proposed *(4)*, *Aspergillus nidulans* was also electrophoretically tested *(5)* as well as *Aspergillus flavus* and *A. parasiticus (6).* Samson *(1)* made a comprehensive survey of morphological and chemotaxonomical studies applicable in *Aspergillus* classification.

The strains used in this study were obtained in different ways: two of the strains were used from ATCC and NRRL Culture Collections, three of them were isolated from different locations: air, soil, karstic cave and one chosen after screening on pectinases after UV irradiation. Isolates were classified using both conventional morphological and biochemical methods, and are included in our culture collection MZKI.

Materials and Methods

Source and maintenance of Aspergillus species

Five *Aspergillus* species and two *A. niger* strains from the Microbial Culture Collection of the National Institute of Chemistry (MZKI) were examined. Details of selected isolates are listed in Table 1.

All cultures are stored under different conditions: at 4, at –70 °C, under liquid nitrogen, mineral oil, and in the lyophilized state. Cultures cultivated on beer wort agar slants at 30 °C for 7 days were used in the experiments.

Media and growth – morphological tests

For the identification of selected *Aspergilli* we used the identification system for the genus *Aspergillus (6).* The determination was performed on three culture media, malt extract agar MEA, Czapek yeast extract agar CYA, and CYA with 20 % sucrose CY20S, at two different temperatures, 25 or 37 °C. Individual media were sterilized at 121 °C for 15 min and poured into 90 mm Petri dishes. For each culture, four Petri dishes, two with CYA, and one of each of the other media were used, and inoculated at three points. One CYA plate was incubated at 37 °C and the other three at 25 °C. After 7 days of incubation all plates were observed for macroscopic characteristics, such as colony diameter, conidial color, mycelial color, exudate, soluble pigment, colony reverse, and microscopic characteristics: vesicles, metulae, phialides, conidia. Cultivation procedures and measurements were repeated three times and the results represent the mean values with deviations of less than 15 %.

Submerged cultivation

To follow intracellular proteins within selected *Aspergilli*, they were cultivated under submerged conditions, as a liquid culture is generally a more homogenous system than a solid one. The medium was prepared as described by Paterson and Bridge *(7).* After inoculation of 100 mL sterile media in 500 mL Erlenmeyer flasks, each fungus was examined after 24 hours of cultivation on a rotary shaker at 30 °C.

Analysis of fungal mycelia and cultivation media

After 24 hours the flasks were removed and the mycelia quickly separated by suction filtration. In the filtrate pH, extracellular proteins *(8)* and glucose *(9)* were determined. The mycelium separated from the known volume of the cultivation medium was thoroughly washed and dried to constant weight for the calculation of dry biomass. Known amounts of individual fungal mycelia were frozen in liquid nitrogen and thoroughly crushed in a mortar. Intracellular proteins were extracted into Tris-glycine buffer pH=8.3 (Tris-HCl 3 g, glycine 14.4 g/L) and their amount determined *(8).* A known amount of mycelium crushed in liquid nitrogen was used for the

Table 1. Details on *Aspergillus* species studied

Species Section Subgenus	Code No. MZKI	Location	Date of isolation	Important metabolite production
A. niger van Tieghem Nigri Circumdati	A-60	NRRL 2270 Peoria, USA	obtained 1971	submerged citric acid production
A. niger Nigri Circumdati	A-138	MZKI, Ljubljana, SI	UV mutant 1986	pectolytic enzyme production
A. oryzae Flavi Circumdati	A-109	ATCC 11601 Rockville, USA	obtained 1982	alpha-amylase producer
A. terreus Terrei Nidulantes	A-160	MZKI, Ljubljana, SI isolated from soil	1992	production of lovastatin
A. flavus Flavi Circumdati	A-168	MZKI, Ljubljana, SI isolated from air Besançon, France	1993	extracellular proteases
A. fumigatus Fumigati Fumigati	A-213	MZKI, Ljubljana, SI isolated in karstic cave Škocjan	1993	secondary metabolites

extraction of total lipids *(10)* and the determination of sterols and fatty acids *(11).*

Protein electrophoresis

A 10 % polyacrylamide electrophoresis (SDS-PAGE) according to Laemmli was performed *(12).*

Isozyme analysis

In selected species esterases, acid and alkaline phosphatases, catalases and dehydrogenases (malate, lactate and NAD-isocitrate dehydrogenase) were determined. Isozyme electrophoresis was carried out on polyacrylamide gels with buffer systems as described by Paterson and Bridge *(7).* Staining procedures used for esterases, catalases and phosphatases were as described *(7)*, lactate dehydrogenases were stained according to Dietz and Lubrano *(13)*, whereas malate and isocitrate dehydrogenases were stained according to Zervakis, Sourdis and Balis *(14).*

Results

Morphological characteristics

In Table 2 macroscopic characteristcs: colony diameters, conidial colors, mycelial colors, and colony reverse colors are presented; microscopic characteristics such as the shape and size of vesicles, metulae, phialides and conidia are given in Table 3. Results were compared with the identification key for *Aspergillus* species *(6)*. Previously obtained species were reidentified and the new ones, that is A-160, A-168, A-213 were isolated and determined.

Submerged cultivation

After 24 hours of cultivation the amounts of fungal biomass, total lipids, sterols, fatty acids, and proteins were determined in the mycelia, whereas glucose, extracellular proteins, and pH were measured in the filtrate. Results presented in Table 4 show the differences between individual *Aspergilli,* but they were not significant enough to distinguish between species with certainity.

Intracellular protein electrophoresis

SDS-PAGE of intracellular proteins extracted from 1 or 2 days old mycelia is shown in Fig. 1. Individual protein bands were determined densitometrically and the results are given in Table 5. Thirty seven protein bands were recorded. With all the fungi the protein bands no. 3, 27 and 35, marked with arrows in Fig. 1, were present. In individual *Aspergillus* samples 18-24 protein bands were recorded. Table 6 shows the number of characteristic

Table 2. Macroscopic characteristics of *Aspergillus* species examined after 7 days of growth at 25 °C on CYA, MEA and CY20S media, respectively

Characteristic	*Aspergillus* Species	CYA	MEA	CY20S
Colony diameter	A60	54.0	36.9	36.2
(mm)	A138	66.6	34.9	70.6
	A109	62.5	60.3	47.8
	A168	66.2	62.0	63.9
	A160	51.0	43.3	64.6
	A213	48.2	44.5	48.7
Conidial color	A60	dark brown	almost black	dark brown to black
	A138	dark brown to black	dark brown to black	dark brown to black
	A109	green brown to green yellow	yellow green or yellow grey	olive or yellow green
	A168	olive green	olive green	yellow-olive
	A160	light brown to brown-orange	light ochre to orange brown	light brown
	A213	blue green to grey blue	grey blue	grey blue
Mycelial color	A60	white to yellow white	whitish	whitish
	A138	white	whitish	whitish
	A109	white to yellow	whitish	whitish
	A168	whitish-yellowish	whitish-yellowish	whitish-yellowish
	A160	white	whitish, almost colorless	whitish
	A213	whitish	whitish-greyish	whitish
Colonial reverse	A60	white-yellowish	colorless to pale yellow	yellowish
	A138	yellow to yellow brown in centre	yellowish	yellowish
	A109	whitish to grey yellowish	yellowish	yellowish, yellow orange centre
	A168	brown-orange	whitish-yellowish	grey yellowish
	A160	yellow-orange to brown	grey-yellow centre orange yellow	yellow-orange centre brown orange
	A213	yellow green	yellow green	yellow brown

Table 3. Microscopical characteristics of selected *Aspergillus* species grown for 7 days, at 25 °C on CYA and MEA, respectively

Structures	*A.* sp.	Characteristic					
		Shape	lenght / μm		width / μm		Color
		CYA	CYA	MEA	CYA	MEA	CYA
Vesicles	A60	round			28.1	30.1	yellow brown
	A138	round			58.3	61.2	brownish
	A109	round			31.1	33.3	pale yellow, brown or not colored
	A168	round			34.1	32.9	yellow-brown to pale green
	A160	round			14.4	15.2	yellow brown
	A213	round			22.6	16.2	
Metulae	A60		6.8	7.0	3.5	3.6	pale
	A138		11.0	17.6	3.9	4.9	
	A109						
	A168		9.7		4.5		brown or grey green
	A160		6.2	6.2	2.4	2.8	green-brown, also yellow
	A213						
Phialides	A60		6.5	7.3	3.2	3.5	pale
	A138		8.6	9.5	3.4	4.3	
	A109		8.1	8.9	3.3	4.2	
	A168		8.9	7.5	3.3	4.3	brown or grey green
	A160		5.3	5.7	2.0	2.2	brighter than metulae
	A213		6.4	7.0	2.8	3.1	
Conidia	A60	round			4.9	4.7	brownish
	A138	round			5.1	5.1	brown orange brick
	A109	round			5.7	5.9	yellow-green
	A168	round			4.8	5.0	mostly green
	A160	round			3.4	3.3	brown and yellow green
	A213	round			2.9	3.3	

Table 4. Amounts of biomass, proteins, lipids, remaining sugar, and pH of media of different *Aspergillus* species after 24 hours of submerged cultivation at 30 °C

Fungus	dry mass	*w* (cell protein)	*w* (total lipids)	*w* (sterol)	*w* (fatty acids)	*γ* (glucose)	γ (proteins)	pH
	g/L	%	%	%	%	g/L	g/L	
A60	5.36	23.9	6.33	27.6	50.6	3.2	0.82	2.9
A138	3.37	11.8	6.41	29.0	43.1	7.8	1.88	4.3
A160	3.37	31.6	5.68	21.1	51.6	4.1	1.57	3.7
A109	4.52	31.6	6.38	21.1	51.0	2.5	0.90	3.1
A168	3.87	26.5	5.38	37.0	33.3	3.7	1.09	3.4
A213	4.09	17.5	6.59	21.1	49.0	6.6	1.42	3.8

protein bands within taxonomic groups after SDS-PAGE of intracellular proteins.

Isozyme analysis

The following intracellular enzymes: dehydrogenases, catalases, esterases, and phosphatases were determined. Malate, lactate and isocitrate dehydrogenases as well as acid phosphatases were detected, whereas alkaline phosphatases and esterases were not. We were able to detect catalases, but there were no differences among the fungi examined, as the bands remained at the start. Fig. 2 shows the number and position of individual isozyme bands of all *Aspergilli* examined. After 24 hours of submerged cultivation, there were two or three bands for acid phosphatases, one for malate dehydrogenases, one for NAD-isocitrate dehydrogenase, and one to two for lactate dehydrogenases.

Discussion

Morphological characteristics

Recent work has brought several significant changes in the taxonomy of the genus *Aspergillus*, as well as in

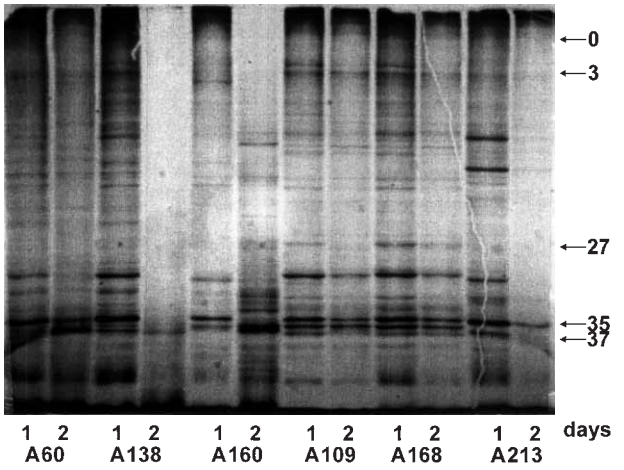
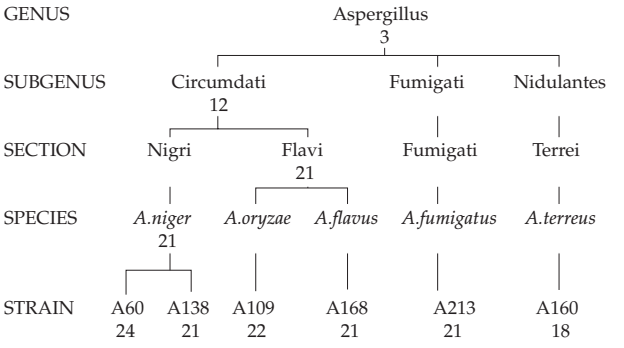


Fig. 1. SDS-PAGE of intracellular proteins extracted from 1 and 2 day old mycelia grown in submerged culture (A60 – *A. niger*, A138 – *A. niger*, A160 – *A. terreus*, A109 – *A. oryzae*, A168 – *A. flavus*, A213 – *A. fumigatus*)

Table 6. Number of characteristic bands inside a taxonomic group obtained with SDS-PAGE of intracellular proteins



other fungal genera. Species isolated and included in our culture collection represent commonly isolated *Aspergillus* species of economic importance. Six isolates were examined in detail. They were grown on three media at two temperatures. Morphological characteristics are gathered in Table 2 and Table 3. Additionally, we distinguished between uniseriate *Aspergilla* such as *A. oryzae*, *A. flavus* and *A. fumigatus* and biseriate ones, both *A. niger* strains and *A. terreus*. All the parameters measured fitted the descriptions in relevant references *(6).*

Submerged cultivation

Liquid culture is recomended for physiological studies because of more homogenous conditions compared with solid culture. In liquid media growth is much quicker and adequate aeration has to be maintained to provide good growth and oxidative biochemical pathways. It was presumed that following dry biomass, lipids, sterols, fatty acids, intracellular proteins and glucose would give us additional parameters showing differences between isolated *Aspergilli*. From Table 4, where the mean results of three individual cultivations under the same conditions were gathered, differences among *Aspergilli* appeared, but they were not significant enough to provide help in identification.

Table 5. Intracellular proteins of *Aspergilli* after one day of submerged cultivation at 30 °C (A60 – *A. niger*, A138 – *A. niger*, A160 – *A. terreus*, A109 – *A. oryzae*, A168 – *A. flavus*, A213 – *A. fumigatus*)

Band no.	A60	A138	A160	A109	A168	A213
1						*
2	*	*	*	*	*	
3	*	*	*	*	*	*
4			*			
5	*	*		*	*	*
6	*	*	*	*	*	
7			*	*	*	*
8	*	*	*			*
9			*	*	*	
10	*	*				*
11	*			*	*	
12				*	*	*
13	*					*
14	*	*	*	*		
15						*
16	*	*				
17			*	*	*	*
18	*	*	*			*
19			*	*	*	
20	*	*				*
21				*	*	
22	*	*	*	*	*	
23	*					*
24	*	*		*	*	*
25				*	*	*
26	*	*				
27	*	*	*	*	*	*
28	*	*				
29						*
30	*	*		*	*	
31			*			*
32	*	*	*			
33				*	*	*
34	*	*	*	*	*	
35	*	*	*	*	*	*
36	*	*	*	*	*	
37	*	*		*	*	*

Intracellular proteins

Intracellular protein electrophoresis can help with doubtful classical identification. Most isolates showed species specific protein profiles but with possible differences in density, while species within the same genus differed significantly. At the generic level, protein profiles demonstrated possible existence of one or more characteristic generic bands common to all species *(15).*

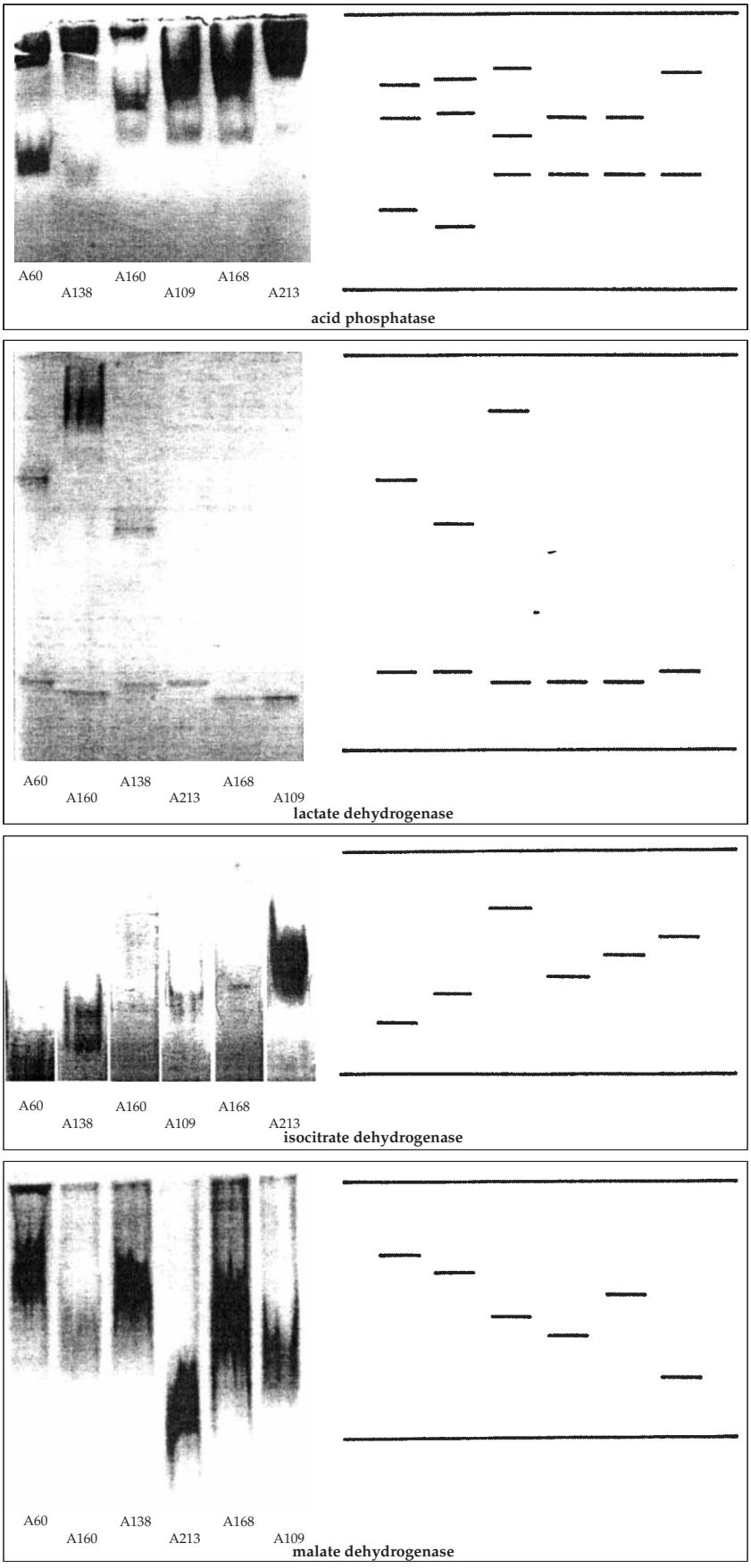


Fig. 2. Intracellular isozymes of different *Aspergilli* after 24 hours of cultivation

SDS-PAGE electrophoresis of intracellular proteins of the isolates is shown in Fig. 1. Silver staining was characteristic for each individual protein and did not correlate to its concentration *(16).* For differentiation, the possition of the individual protein band was considered (Table 5). Thirty seven different protein bands were observed with 18 to 24 protein bands belonging to each individual fungus. From the Table 5, it is seen that 3 protein bands were present with all fungi tested and might represent characteristic bands of the genus *Aspergillus.* Section Nigri (both *A. niger* strains) and section Flavi (*A. oryzae* and *A. flavus*) both belonging to the subgenus Circumdati had about 50 % similar bands. *A. oryzae* and *A. flavus* had the highest similarity (95 %), as well as both *A. niger* strains (88 %). Differences on the species level, for *A. terreus* and *A. fumigatus*, were significant as well (Table 6). On the basis of similarity species could be differentiated, with still more pronounced differentiation on the subgenus level. This could serve as an additional parameter for differentiation.

Isozyme analysis

A wide variety of enzyme systems have been used in the study of fungi as well as with the taxonomy of *Basidiomycettes (17).* Isozymes in both intra and extracellular samples have been used extensively in filamentous fungi. With *Aspergilli* mainly extracellular enzymes, especially pectinases and amylases have been determined *(18).*

In our work we determined intracellular enzymes such as dehydrogenases (malate, lactate, isocitrate), esterases, catalases, alkaline and acid phosphatases. Dehydrogenases and acid phosphatases were detected (Fig. 2). We failed to detect alkaline phosphatases and esterases. Regarding catalases, we detected them, but there were no differences between the fungi examined since catalase bands remained at the start. In future less dense gels should be tested. As shown in Fig. 2, there were two or three bands for acid phosphatases, one band for isocitrate dehydrogenases and malate dehydrogenases, and one or two bands for lactate dehydrogenases. Similar results had also been obtained by other authors. With acid phosphatases one to three isozyme bands were visible *(19,20),* one to two bands with malate dehydrogenases *(21,22)* and one to five bands with lactate dehydrogenases *(23-25).* Both *A. niger* strains showed the same number of isozyme bands at different positions with the exception of one lactate dehydrogenase isozyme, the same in both strains. *A. oryzae* and *A. flavus* showed three bands at the same position, with a different malate dehydrogenase and isocitrate dehydrogenase band position. Regarding the isozyme patterns of each individual *Aspergillus* one can clearly distinguish not only between species, but also between strains. Isozyme analysis has also been used for distinguishing between *Fusarium* species and strains, and was described as a rapid and accurate method *(26,27).*

Conclusions

The Microbial Culture Collection of the National Institute of Chemistry holds mainly industrially important filamentous fungi, especially *Aspergilli*. Besides the species obtained from different collections abroad, we have also isolated a number of strains ourselves. Classification of the new isolates was mainly based on morphological characteristics. Since these may be sometimes variable or atypical, or even changed, we verified the applicability of additional biochemical characters.

All *Aspergilli* examined in this work produced typical morphological characteristics. Electrophoretic banding patterns demonstrated reliability of separated proteins in distinguishing species, with 3 common protein bands observed and proposed to be characteristic for the genus. Enzyme electrophoresis has been shown to be another valuable tool, particulary when morphological characteristics overlap. On the basis of the results obtained, isozyme patterns may serve as an additional method for the comparison and classification of *Aspergillus* isolates.

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Morfološke osobine, ekstracelularni i intracelularni proteini i enzimi u pet sojeva *Aspergillus*

Sažetak

Šest biotehnološki interesantnih sojeva roda *Aspergillus*, od kojih su četiri nedavno izolirana i uključena u zbirku kultura Nacionalnog instituta za kemiju (MZKI), ispitano je morfološki i biokemijski. Kako se događa da sojevi više ne proizvode tipične morfološke strukture, u klasifikaciji je potrebno koristiti nemorfološke postupke. Na osnovi makroskopskih i mikroskopskih opažanja, koristeći ključ za identifikaciju, klasificirani su pojedini izolati unutar roda *Aspergillus*, i to u tri podroda, četiri sekcije, pet vrsta i dva soja *A. niger*. U pojedinim aspergilima opažene su različite količine ukupnih lipida, sterola i masnih kiselina, ali nisu bile dovoljno signifikantne da bi se razlikovali pojedini sojevi. Profil intracelularnih proteina nije se razlikovao između dva soja *A. niger*, niti je bio različit u *A. oryzae* i *A. flavus* iako oba pripadaju u sekciju Flavi. Razlike su postojale unutar sekcija Nigri i Flavi koje potpadaju podrodu Circumdati, dok su najveće razlike opažene na razini podroda. Analiza enzima unutar ispitanih funga razlikuje se u broju izozimskih vrpca i njihove molekularne mase. Izocitrat dehidrogenaza davala je jednu vrpcu, dok su kisele fosfataze imale dvije ili tri vrpce u svim ispitivanim fungima. Malat dehidrogenaze pokazuje jednu, a laktat dehidrogenaze jednu ili dvije izozimske vrpce. S dobivenim rezultatima najveće su bile razlike na razini podroda, ali su se one mogle opaziti i na razini sojeva.