LIPID COMPOSITION OF A CITRIC ACID PRODUCING AND A PECTOLYTIC ENZYME EXCRETING *Aspergillus niger* STRAINS

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Received 2.12.1999

Abstract

Lipid synthesis by a citric acid producing *Aspergillus niger* A-60 strain and a pectolytic strain of *Aspergillus niger* A-138 carried out under the same cultivation conditions were compared. High production of individual pectolytic enzyme as well as relatively large amounts of citric acid were determined for the pectolytic strain, whereas citric acid producing strain grown in a pectolytic medium excreted two to four times less pectolytic enzymes with relatively low citric acid and two times more lipids present in fungal mycelia. Regarding lipid classes there were less neutral and more glycolipids determined with the pectolytic strain. A higher degree of fatty acid unsaturation with all lipid classes was observed with the citric acid *A. niger* strain. Both strains cultivated in a pectolytic substrate had similar lipid composition as the citric acid strain cultivated in a citric acid producing medium. On the other hand, citric acid producing strain cultivated in a pectolytic medium and in a citric acid nonaccumulating medium also synthesized a similar lipid pattern. Under conditions of metabolite production there was two times less lipids sinthesized with both *A. niger* strains examined.

Introduction

In industrial processes, aspergilli have been used for many years for the production of citric acid and recently they have been exploited for the production of numerous enzymes. *Aspergillus* cultures are flexible and adaptive and thus capable of utilising a wide range of substrates. To achieve the accumulation of certain metabolite the maintenance of strictly defined cultivating conditions is necessary. It is known that in *Aspergillus niger* trace metal ions, especially manganese and copper essentially influence citric acid accumulation [1, 2, 3]. With pectolytic enzyme accumulation, the maintenance

of proper metal ion concentration is one of the main requirements as well [4]. The lipid composition of microbial fats varies with microbial species, the age of the culture, the composition of the medium, pH and the growth temperature [5, 6, 7]. The degree of unsaturation of fatty acids increases at a temperatures below optimum [8] and by changing the ratio of saturated to unsaturated acids the fluidity of cell membranes is affected [9]. Some work has also been done on *A. niger* lipids concerning citric acid accumulation [10, 11].

During the study of pectolytic enzyme biosynthesis by an *A. niger* mutant, selected by screening after UV irradiation, with the aim to get a pectolytic enzyme mixture suitable for fruit juice clarification, relatively high amounts of citric acid in cultivation media were detected [4]. It is known that under citric acid accumulating conditions there were up to four times less total lipids present in *A. niger* mycelium in comparison with nonaccumulating mycelium [12]. Since the pectolytic *A. niger* strain was capable to produce high amounts of pectolytic enzymes, as well as relatively high amounts of citric acid, we followed the lipid composition of fungal biomass during cultivation.

Material and Methods

*Microorganisms***:** *Aspergillus niger* strain A-60 (NRRL 2270) was selected as a citric acid producing strain. *Aspergillus niger*, pectolytic mutant A-138, selected by screening after UV irradiation in our laboratory and deposited in the Microbial Culture Collection of the National Institute of Chemistry (MZKI) was used as the productive strain. Both strains were maintained on beer wort agar slants. Spores were suspended in sterile tap water (approx. 10^{7} spores ml^{-1}) and 2 ml used for inoculation of 100 ml medium.

Cultivation: Citric acid accumulating (CAA) and nonaccumulating (CANA) media were prepared as previously described [11]. The medium favouring pectolytic enzyme synthesis was prepared as follows: 14% commercial sugar, 1% dry whey, 0.2% NH_4NO_3 and 0.05% $(NH_4)_2SO_4$ in tap water and pH adjusted to 4.5 [13]. Experiments were carried out in 500 ml baffled Erlenmeyer flasks with 100 ml of sterilized medium on

a rotary shaker (100 rpm) at 30 °C. After selected time intervals (3, 5 and 8 days) flasks were removed and the contents analysed.

Analytical Methods: The activity of individual enzyme of the pectolytic enzyme complex was measured: polygalacturonase activity was determined according to Wang and Keen [14], pectin esterase according to the method described in Product Information Sheet AM-062-80 from Biocon and pectate lyase as described by Ayers et al. [15]. Citric acid was determined according to the method of Saffran and Denstedt as modified by Spencer and Lowenstein [16]. Two enzymes of the tricarboxylic acid cycle were measured after protein extraction from mycelia: citrate synthase (CS) according to Kubicek and Roehr [17] and NADP-isocitrate dehydrogenase (ICDH) as described by Legisa and Mattey [18]. Proteins were determined by the method of Lowry et al. [19]. For lipid analysis, mycelia were crushed in a mortar under liquid nitrogen. Total lipids were determined gravimetrically after extraction according to Folch et al. [20] and further separated on a silica column. Neutral lipids (NL) were eluted with chloroform, glycolipids (GL) with acetone and phospholipids (PL) with methanol. Individual lipid classes were additionally separated by thin layer chromatography. For the TLC separation of a NL solvent system of petrolether- ether- acetic acid (85:15:1) was used; chloroform- acetone- acetic acid- water (10:90:2:3) separated GL and chloroform- methanol- acetic acid- water (120:23:10:4.5) for the separation of PL. Fractions eluted from a silica column were further methylated and individual fatty acids determined by gas chromatography [11].

Results and Discussion

We intended to compare results obtained with both *A. niger* strains cultivated on both substrates; pectolytic and citric acid producing one. Since the pectolytic strain showed nearly no growth in the citric acid accumulating medium, most probably because of the low initial medium pH, we compared results obtained with both strains on pectic substrate with results obtained for citric acid strain cultivated in citric acid accumulating (CAA) and nonaccumulating (CANA)medium.

The effect of cultivation time on pectolytic enzymes, as well as citric acid production and the amount of extracellular proteins for both strains in the pectolytic medium is shown in Figure 1. With the pectolytic A-138 strain, all parameters followed, increased with the time of cultivation. The activities of polygalacturonase and pectin esterase were higher and that of pectate lyase lower than the activities obtained in a 10 l laboratory bioreactor [13]. In the bioreactor, the regulation of aeration and agitation was possible whereas on the rotary shaker, the same shaking conditions (100 rpm) were kept during the whole cultivation period. Varying the aeration/ agitation conditions one could influence the ratio of individual pectolytic enzymes. In addition, citric acid was synthesized in relatively high amounts and could represent a valuable byproduct. Comparing the results obtained with both strains (Fig. 1) there were big differences:

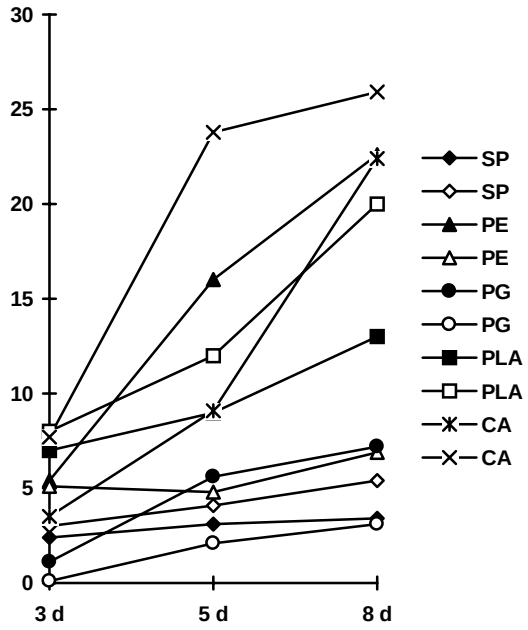

Fig. 1. Pectolytic enzyme and citric acid biosynthesis during cultivation of both *Aspergillus niger* strains (A-138 - full symbols, A-60 - empty symbols) on a substrate favourizing pectolytic enzyme synthesis (SP-soluble protein, mg ml^{-1}:10; PE-pectinesterase activity, U ml^{-1}; PG-polygalacturonase activity, U ml^{-1}; PLA-pectate lyase activity, E_{230}:100; CA-citric acid, g l^{-1}; results are the mean of three individual experiments).

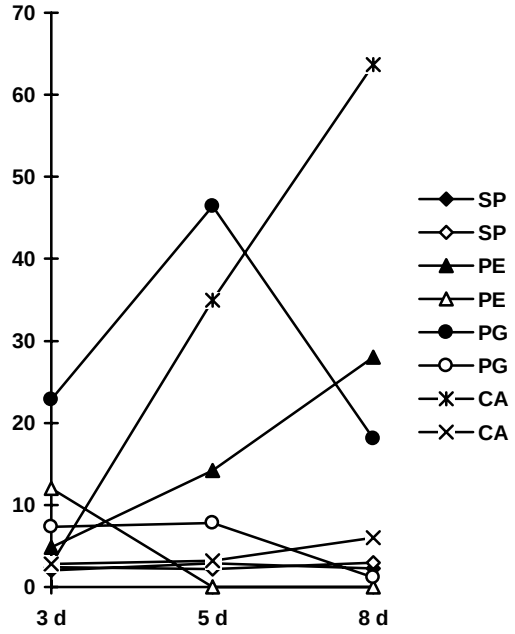

Fig. 2. Enzyme and citric acid synthesis during cultivation of *A.niger* A-60 strain in citric acid accumulating (CAA-full symbols) and in a citric acid nonaccumulating (CANA-empty symbols) substrate (SP-soluble protein, mg ml^{-1}:10; PE-pectinesterase activity, U ml^{-1}:10; PG-polygalacturonase activity, U ml^{-1}:10; CA-citric acid, g l^{-1}; results are the mean of two individual experiments).

pectin esterase activities were four times lower, more than two times less polygalacturonase activity was determined and pectate lyase activities were higher with the citric acid strain A-60. After 8 days of cultivation both strains excreted similar amounts of citric acid.

In Figure 2 the results obtained in a citric acid accumulating and a nonaccumulating medium with the citric acid producing *A. niger* strain A-60 are gathered. High amounts of citric acid were detected in the accumulating medium with individual enzyme activities much lower than in the pectolytic substrate, indicating that the pectolytic substrate induces enzyme synthesis and excretion. In the nonaccumulating medium, the synthesis of pectolytic enzymes as well as citric acid was negligible, whereas the biosynthesis of lipids was two times higher (see Figure 4B). The difference between both media was only in the trace metal presence (copper in the accumulating and manganese in the nonaccumulating medium) which confirms the big influence of trace metals on fungal metabolism.

Following enzyme activities of selected intracellular enzymes involved in citric acid biosynthesis the measured activities of citrate synthase did not follow any explainable rule (Table 1).

Table 1. Specific activity of citrate synthase during cultivation of two *Aspergillus niger* strains on different substrates.

	specific activity (mU/mg protein)			
	PS	**PS**	**CAA**	**CANA**
Day	**A-138**	**A-60**	**A-60**	**A-60**
2	275	165	225	361
3	110	250	130	314
4	90	125	187	179
7	123	130	210	223

(PS-pectolytic substrate, CAA-citric acid accumulating substrate, CANA-citric acid nonaccumulating substrate, A-138 pectolytic *Aspergillus niger* strain, A-60 citric acid *A. niger* strain)

For NADP-isocitrate dehydrogenase it is known that it oxidizes isocitrate to 2-oxoglutarate, and its inhibition results in increased citrate levels, since the chemical equilibrium of cis-aconitase lies in favour of citrate formation [21]. Data presented in Table 2 show specific activities of NADP-isocitrate dehydrogenase which were low in the cases of considerable citric acid excretion, and higher when only negligible biosynthesis was observed as in the case of cultivating strain A-60 in the citric acid nonaccumulating medium.

Table 2. Specific activity of NADP-isocitrate dehydrogenase during cultivation of two *Aspergillus niger* strains on different substrates

	specific activity (mU/mg protein)			
	PS	**PS**	**CAA**	**CANA**
Day	**A-138**	**A-60**	**A-60**	**A-60**
1	104	60	73	74
2	54	23	63	107
3	43	27	51	110
4	18	29	54	87
5	9	40	45	104
7	20	32	26	110

(PS-pectolytic substrate, CAA-citric acid accumulating substrate, CANA-citric acid nonaccumulating substrate, A-138 pectolytic *Aspergillus niger* strain, A-60 citric acid *A. niger* strain)

Total lipids were extracted from 3, 5 and 8 days old *A. niger* mycelia and separated on silica column. The sum of individual lipid classes obtained after silica column separation comprised more than 90% of total lipids applied on a column, indicating a fairly good separation method.

Results of total lipid amounts, as well as individual lipid classes (NL, GL and PL) from the pectolytic medium are shown in Figure 3A. Figure 3B composes results obtained for the citric acid strain grown in citric acid accumulating and nonaccumulating media. In the pectolytic substrate an initial increase of total mycelial lipids on day 5 was

followed by the decrease in the pectolytic strain, whereas the citric acid strain synthesized two times more total lipids. Among lipid classes, NL comprised two thirds of total lipids, about one fifth of PL, with the remainder representing GL.

Further separation of lipid classes by TLC showed that among NL, triacylglycerols and free fatty acids predominated; monogalactosyldiacylglycerol was the most abundant glycolipid, whereas phosphatidylethanolamine predominated among PL. Results obtained were consistent with those obtained with different *A. niger* strains by other authors [10, 11, 12], as well as with some other fungal species [23].

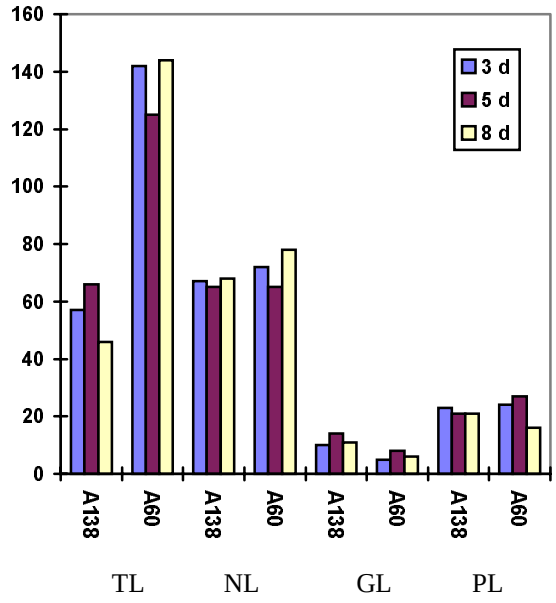

Fig. 3A. Amount of total lipids (TL-mg g^{-1} dry mycelium) and individual lipid classes (% of TL) in mycelia of both fungi during growth on pectolytic substrate (NL-neutral-, GL-glyco- and PL-phospho-lipids).

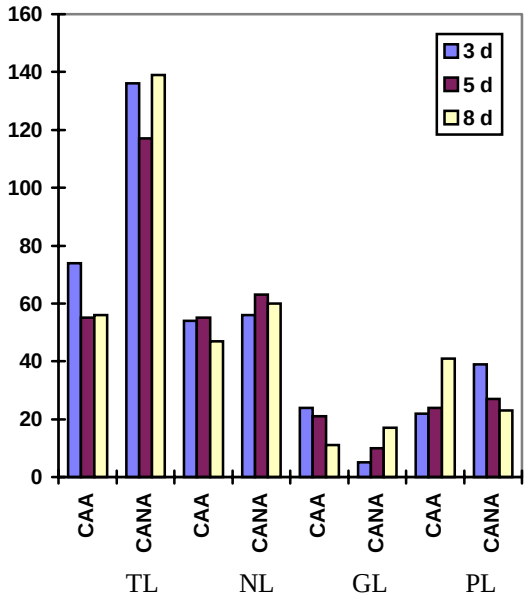

Fig. 3B. Amount of total lipids (TL-mg g^{-1} dry mycelium) and individual lipid classes (% of TL) in a citric acid producing *A. niger* strain A-60 cultivated in citric acid accumulating CAA and nonaccumulating CANA conditions (NL-neutral-, GL-glyco- and PL-phospho-lipids).

Regarding results obtained for both strains and in the two media (Figures 4A and 4B), in medium unfavourable for the production of defined metabolite the fungal metabolism turned to higher lipid biosynthesis, as it was in the case of citric acid strain A-60 on pectolytic and on citric acid nonaccumulating media (Figure 4B). Both strains synthesized two times less lipids when metabolite excretion was enhanced (Figure 4A).

The fatty acid composition of individual lipid classes for the pectolytic *A. niger* strain grown in the pectolytic medium is given in Table 3 and for the citric acid strain A-60 under the same cultivation conditions in Table 4. With both strains oleic, linoleic and palmitic acid comprised the bulk of fatty acids during the whole cultivation period. Unsaturated acids, mainly oleic and linoleic, increased with time and represented around 70% of total fatty acids. Palmitic acid was the only saturated acid present in considerable amounts. In all cases the ratio of saturated to unsaturated fatty acids diminished during cultivation time, indicating that activites of desaturases increased with the time of cultivation. The desaturases in microorganisms so far examined are

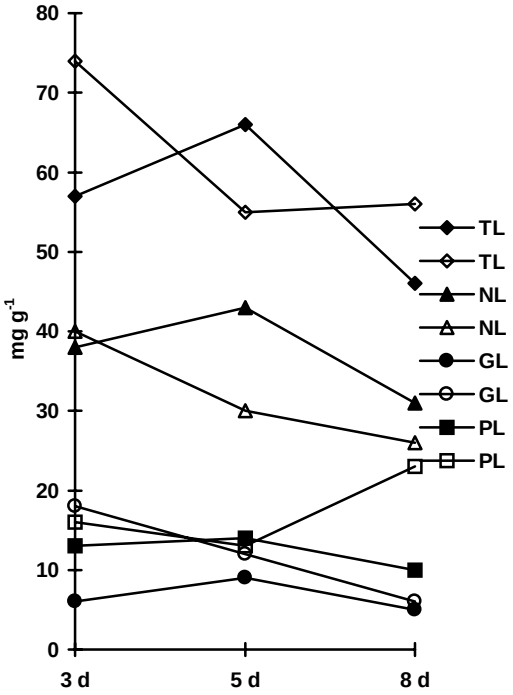

Fig. 4A. Amount of total lipids and individual lipid classes in fungal mycelium grown in accumulating conditions (A-138 strain grown on pectolytic medium- full symbols; A-60 strain on citric acid accumulating medium- empty symbols; TL-total lipids, NL-neutral, GL-glyco, PL-phospholipids)

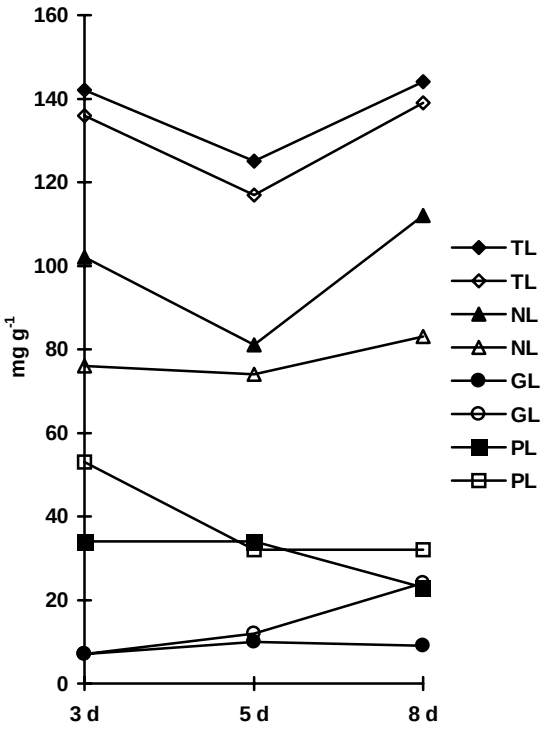

Fig. 4B. Total lipids and individual lipid classes in *A. niger* strain A-60 mycelia grown in conditions not favourable for citric acid synthesis (full symbols- pectolytic substrate; empty symbols- citric acid nonaccumulating medium CANA; TL-total lipids, NL-neutral, GL-glyco, PL-phospho lipids)

Table 3. Fatty acid composition of individual lipid classes in *Aspergillus niger* A-138 mycelia grown in the pectolytic substrate

NL									
Day	**$C_{14:0}$**	**$C_{16:0}$**	**$C_{16:1}$**	**$C_{18:0}$**	**$C_{18:1}$**	**$C_{18:2}$**	**$C_{18:3}$**	**sat/unsat**	**UI**
3	2.4	29.1	1.6	6.0	35.9	23.6	1.4	0.60	0.89
5	1.3	18.8	1.3	3.8	41.4	32.3	1.1	0.31	1.11
8	2.8	19.0	0.9	2.2	41.8	33.1	0.2	0.32	1.10
GL									
Day	**$C_{14:0}$**	**$C_{16:0}$**	**$C_{16:1}$**	**$C_{18:0}$**	**$C_{18:1}$**	**$C_{18:2}$**	**$C_{18:3}$**	**sat/unsat**	**UI**
3	1.4	25.6	1.5	2.3	37.1	31.2	0.9	0.41	1.04
5	1.5	20.7	2.1	2.9	34.1	37.6	1.1	0.33	1.15
8	1.4	16.7	0.6	4.5	51.8	25.0	n.d.	0.29	1.02
PL									
Day	**$C_{14:0}$**	**$C_{16:0}$**	**$C_{16:1}$**	**$C_{18:0}$**	**$C_{18:1}$**	**$C_{18:2}$**	**$C_{18:3}$**	**sat/unsat**	**UI**
3	0.8	24.9	1.9	4.6	44.4	23.1	0.3	0.43	0.93
5	1.7	19.0	1.5	2.9	34.3	31.6	n.d.	0.31	0.99
8	1.4	23.6	1.1	1.2	40.1	32.6	n.d.	0.35	1.06

(NL-neutral lipids, GL-glycolipids, PL-phospholipids; $C_{14:0}$-miristinic acid, $C_{16:0}$-palmitinic acid, $C_{16:1}$-palmitinoleic acid, $C_{18:0}$-stearic acid, $C_{18:1}$-oleic acid, $C_{18:2}$-linoleic acid, $C_{18:3}$-linolenic acid; n.d.-not determined, sat/unsat-ratio between saturated and unsaturated fatty acids, UI-unsaturation index, UI=(%monounsaturated+2x%diunsaturated+3x%triunsaturated acids)/100)

membrane bound [24, 25]. The higher degree of unsaturation of fatty acids in polar lipids may be a structural necessity of their function as membrane components. Regarding the unsaturation index, *A. niger* A-60 strain had higher value with polar lipid fractions (GL and PL) than the neutral lipid fraction which is in agreement with the results of other

workers [26]. With the pectolytic strain, however, the unsaturation index did not vary significantly among individual lipid fractions. It seem that with the

Table 4. Fatty acid composition of lipid classes in citric acid producing *Aspergillus niger* A-60 strain cultivated in the pectolytic substrate

NL									
Day	**$C_{14:0}$**	**$C_{16:0}$**	**$C_{16:1}$**	**$C_{18:0}$**	**$C_{18:1}$**	**$C_{18:2}$**	**$C_{18:3}$**	**sat/unsat**	**UI**
3	1.0	20.6	1.7	7.1	33.8	33.5	2.3	0.40	1.09
5	0.6	18.4	1.4	5.6	34.0	38.0	2.1	0.33	1.18
8	1.9	16.0	1.9	3.2	29.3	44.6	3.0	0.27	1.29
GL									
Day	**$C_{14:0}$**	**$C_{16:0}$**	**$C_{16:1}$**	**$C_{18:0}$**	**$C_{18:1}$**	**$C_{18:2}$**	**$C_{18:3}$**	**sat/unsat**	**UI**
3	n.d.	18.7	n.d.	3.1	15.3	56.6	6.3	0.28	1.47
5	0.7	17.9	1.1	1.0	31.1	46.6	1.5	0.24	1.30
8	0.1	15.9	1.2	0.6	27.2	53.8	0.6	0.20	1.38
PL									
Day	**$C_{14:0}$**	**$C_{16:0}$**	**$C_{16:1}$**	**$C_{18:0}$**	**$C_{18:1}$**	**$C_{18:2}$**	**$C_{18:3}$**	**sat/unsat**	**UI**
3	0.7	20.7	0.8	2.7	26.2	47.1	1.7	0.32	1.26
5	0.7	17.7	1.3	1.0	28.9	48.1	2.2	0.24	1.33
8	3.8	16.6	7.1	4.0	21.4	43.9	3.3	0.32	1.26

(abbreviations as in Table 3)

pectolytic strain desaturases were still active after the necessary desaturation of polar lipids had occured, and the newly synthesized unsaturated acids were distributed to other cellular lipids. Comparing the results with the previous ones with the citric acid producing *A. niger* strain A-60 [11, 27], similarities as well as differences were observed. When *A. niger* A-60 was grown in conditions hindering citric acid production (CANA), there were

more than 60% of the NL fraction and the ratio of saturated to unsaturated fatty acids was around 0.30 [11] what was comparable with the results obtained with *A. niger* A-138 as well as with A-60 strain cultivated in the pectolytic substrate (Table 3 and Table 4). Strain A-60, however, producing citric acid contained 50% of NL with a saturation ratio of 0.20. Since it is known, that a lower ratio of saturated to unsaturated fatty acids gives rise to better membrane permeability [28] what was observed also for the citric acid producing *A. niger* A-60 strain, one could predict that with altered fatty acid composition of the pectolytic *A. niger* strain A-138 the excretion of pectolytic enzymes as well as citric acid could be enhanced with concomitant consideration of the influence of other parameters such as aeration, temperature, medium composition etc.

Acknowledgements

The financial support of the Ministry of Science and Technology, Republic of Slovenia is gratefully acknowledged.

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Povzetek

Primerjali smo biosintezo lipidov citronskega in pektolitičnega seva glive *Aspergillus niger*. V substratu ugodnem za produkcijo pektolitičnih encimov je pektolitični sev sintetiziral visoke množine posamznih pektolitičnih encimov, kot tudi znatne množine citronske kisline, medtem ko so bile množine pri citronskem sevu nižje ob dvakrat višji množini celokupnih celičnih lipidov. Delež nevtralnih lipidov je bil nižji, glikolipidov pa višji pri pektolitičnem sevu, medtem ko je pri citronskem sevu opazen večji delež nenasičenih maščobnih kislin. Oba seva gojena v pektolitičnem substratu sta imela podobno lipidno sestavo kot citronski sev gojen v substratu, ki omogoča akumulacijo citronske kisline. V pogojih, ko je bila biosinteza posamznih metabolitov visoka, smo zasledili dvakrat manj celokupnih celičnih lipidov.