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FATTY ACID PROFILING: ITS USEFULNESS IN THE EVALUATION OF MICROBIAL ASSOCIATIONS WITH THE GREEN MICROALGA *Apatococcus constipatus*

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In memory of Dr. Johannes Eichel

Abstract: To determine differences in microbial community structures, fatty acids from two strains of the green microalga *Apatococcus constipatus* were isolated and identified by instrumental means. The main fatty acid s found were 16:0 and 14:0. These predominant acids represented more than 53% of the total fatty acid (content in both algal isolates. In addition, saturated fatty acids were present in much greater quantity than unsaturated ones. Differences between the strains in the composition of other, modified fatty acids were also evident. The occurrence of fatty acid biomarkers characteristic of certain taxonomic groups confirmed the presence of Gram-positive and Gram-negative bacteria, and fungi. Those observed variations were undoubtedly due to distinct community structures of symbiotic microorganisms living in close associations with the alga. The results presented here indicate that different isolates of the same alga might exhibit different microbial community structures.

Key Words: *Apatococcus constipatus,* Green Microalgae, Fatty Acid Profiles, Biomarkers, Microbial Community Structure

INTRODUCTION

"Green algae" is the most diverse systematic group of lower plants, containing more than 7000 species [1]. It includes some of the most common, ecologically important genera, such as *Chlamydomonas*, *Volvox* or *Ulva*. Microalgae are the main suppliers of organic matter and energy in various ecosystems, e.g. as a

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Abbreviations: GC - gas chromatography; FA - fatty acid; FAME - fatty acid methyl ester.

source of essential FAs. Due to this, a formation of lichen-like structures between those lower plants, bacteria and fungi is possible and in fact desirable. Such lichen-like structures are efficient and self-sufficient micro-ecosystems, where each of the symbionts supplies others with essential compounds. At the same time, it should be noted that each of those individual organisms is able to function separately.

Green algae mainly grow in a variety of freshwater habitats, often attached to other living organisms or to the ground. They are frequently found in various soils and muds, and some, such as the green microalgae of the genus *Apatococcus*, may be found in places where moist conditions prevail. *A. constipatus* occurs, among other locations, in the cover crusts surrounding needles of Norway spruces [2], in bright green patches on some Spanish monuments [3], or on various insulating elements of African electric tractions [4].

Although this cosmopolitan microalga occurs worldwide, it is not the subject of intense scientific study. Thus, very little is known about the chemistry and ecology of this species. Recently, the identification of the homologue series of exclusively saturated 5-n-alkylresorcinols in *Apatococcus* cells was reported [5]. Based on these data, considerable biochemical diversity among the algal isolates was estimated. Significant variations in FA compositions were also observed during the developmental process of this organism [6]. To date, none of the natural compounds synthesised by *Apatococcus* cells have been described in the literature.

In this study, the compositions of the total FAs within two isolates of *A. constipatus* are documented. Based on these results, a presumable structure of microbial communities inseparably associated with the studied alga is also discussed.

MATERIALS AND METHODS

Plant material, media and culture conditions

For the purpose of this study, two isolates (marked as A and H, respectively) of the green microalga *A. constipatus* Printz (*Chaetophraceae* family and *Leptosiroideae* subfamily) were used. The algae were collected from silicone-rubber insulators in Tanzania in 1999 [4]. The medium of Slamer and co-workers [7] was applied to the alga cultivation. The cultures were grown on slants in glass tubes in daylight at 25°C. In order to compare FA patterns in the stationary phase of algal growth, the strains were cultured for 3 months. Afterwards, the algal cells were rinsed off the slants with 0.1 M $MgSO_4$, and freeze-dried.

Extraction and preparation of FAME

Total lipids were extracted from the dry material using the modified method of Bligh and Dyer [8]. Briefly, the sample was dispensed into a separating funnel

and extracted three times. 5 ml of chloroform and methanol (1:2 by vol.) was added. The content was shaken for 1 min and allowed to stand for 1 hr. 5 ml of chloroform was added, followed by 5 ml of distilled water. The lower layer that separated was filtered through a SP-1 filter (Whatman, UK) in order to remove any solid material. The solution was transferred into a flask, the extracts were combined and the solvent was removed under nitrogen. The obtained fractions were converted into FAME with small modifications as described previously [9]. Briefly, the hydrolysis of the lipids was achieved by resuspending the material in 1 ml of solution 1 (45 g of NaOH in 300 ml of 50 % MeOH) and boiling it for 30 min at 100°C in a water bath. Methylation of the lipids was done by adding 2 ml of solution 2 (325 ml of 6N HCl and 275 ml of MeOH) and incubating for 10 min at 80°C, followed by rapid cooling in an ice bath. The extraction of FAME was carried out by adding 1.25 ml of solution 3 (200 ml of hexane and 200 ml of methyl-*tert*-butyl ether), and shaking the whole mixture for 10 min. For the separation of the phases, the tube was incubated for 1 hr at -20°C. Afterwards, the lower phase was discarded, and the upper phase was transferred into a new tube and 3 ml of solution 4 (10.8 g of NaOH in 900 ml of H_2O) was added. The mixture was shaken for 5 min in order to remove potential contamination, and the upper phase was used directly for instrumental analyses.

GC conditions

FAME profiles of the studied alga were determined by gas chromatography. One microliter of each sample was injected into a Shimadzu GC-17A gas chromatograph equipped with an AOC-1400 autosampler, an AOC-17 autoinjector and a CBM101 controller. A Permabond OV-1-DF fused silica capillary column (Macherey & Nagel, Germany) was used, and the column oven temperature was programmed as follows: 100°C for 2 min, 100-210°C for the next 38 min. The flow rate of the carrier gas (He) was 1 ml per min. The chromatograms obtained were analysed using the Windows 3.11 PC-programme Class GC. FAs were identified by comparing the relative retention times of their FAME with authentic standards, and their relative compositions were estimated from the area of the peaks in the chromatogram. Additional identification of particular FAs was achieved using FAB- and EI-MS. In this case, low-resolution spectra were recorded on an AMD 402 two-sector mass spectrometer (AMD Intectra, Germany) of B/E geometry. Operating procedures were the same as described elsewhere [6]. FAs are designated by the number of carbon atoms followed by a colon and the number of double bonds in the molecules. Cis and trans isomers are indicated by *c* or *t*, whereas the suffix *cyc* indicates cyclopropane FAs. Hydroxylated FAs are marked with the OH suffix, where the preceding number indicates the carbon atom substituted with this group.

FA profiling and estimation of microbial community structures

The profiles were interpreted using FA biomarkers (Table 1) that had been already identified as either sole or principal compounds in particular groups of

organisms [10]. The complexity of microbial communities associated with the alga was evaluated by determining the proportions between the sum total content of biomarkers characteristic for a specific taxonomic group and the total saturated FA content [11].

Tab. 1. Fatty acid biomarkers*

Taxonomic group	Fatty acids
Biomass of all microorganisms	14:0, 16:0, 18:0
Gram-positive bacteria	i and a-branched FAs
Gram-negative bacteria	OH FAs
Fungi	18:2

* on the basis of [10]

Tab. 2. Relative composition of fatty acids in *A. constipatus*

Fatty acid	Isolate A %	Isolate H %
14:0	14.2	25.2
15:0 a	1.6	1.4
15:0	3.2	2.2
14:0 3-OH	4.6	n.d.
16:0 i	5.0	2.2
16:1	n.d.	4.8
16:0	32.5	17.9
17:0 i	1.3	n.d.
17:0 a	7.6	0.8
16:0 2-OH	3.0	n.d.
18:2	10.9	1.5
18:1 c	n.d.	8.9
18:0	8.2	10.2
19:0 cyc	0.8	n.d.
20:0	n.d.	21.8
Others (including PUFA)	7.1	3.1

RESULTS AND DISCUSSION

Two strains of the green microalga *A. consitipatus* isolated previously from separate locations were examined to obtain their FA compositions. Total lipids were isolated from the cell cultures using continuous extraction with an appropriate mixture of organic solvents and water. The thus-obtained lipidic extracts were hydrolysed, methylated and then processed by GC and MS. FAs

were identified based on their peak retention times (GC) as well as on their low-resolution mass spectra compared to those of authenticFAs (EI-MS) or on their calculated molecular ion masses (FAB-MS). The mean FAs compositions found in *A. constipatus* are presented in Table 2. As shown, those strains contained some major saturated FAs, namely 16:0 and 14:0 acids. In total, the saturated FAs comprised 54.9 and 53.3% of the total FA contents, respectively. Monounsaturated FAs were estimated to be present in minor amounts, but only in the extract from isolate H. On the other hand, considerably higher concentrations of diunsaturated FAs (only one homologue 18:2 *c*) were found in both isolates, although the content in isolate A was up to seven times higher than in isolate H. Also, a few unusual hydroxylated FAs were detected in the studied alga. Unlike the unsaturated FAs, this type of FAs derivative was only present in isolate A. Moreover, relatively spurious amounts of cyclopropane FAs were detected in strain A, whereas they were not again detected in strain H. On the basis of the previous studies on *A. constipatus* [6], a practical lack of polyunsaturated FAs in cells seems to be a characteristic feature of this organism. The presence of FAs characteristic of bacterial and fungal organisms indicated microbiological contamination of the cultures. Similarly to algae, most bacteria have a specific FA profile, which is distinct and different from that of algae. However, getting rid of those microbes from cultures using different doses of various fungicides and ß-lactam antibiotics prevented the algae from further normal growth. Thus, the statement that these unidentified microbes live in close symbiosis with the studied alga seems to be completely justified [6].

Tab. 3. Proportions of taxonomic-specific FAs in extracts from *A. constipatus*[*]

Isolate	Gram +	Gram -	Fungi
A	0.28	0.13	0.20
H	0.08	0.04	0.03

[*] Specific fatty acid biomarkers characteristic of taxonomic groups were normalised by dividing them by the saturated fatty acids found in each sample

The saturated FA contents (14:0, 16:0 and 18:0) were summed to evaluate a yield of a biomarker for biomass [10]. However, it should be stressed that the values reported here only reflected the proportions of biomarkers found, but not absolute numbers of microbial cells. This measure revealed an almost similar biomass for the two studied algal isolates, which suggests similar quantities of associated microbes. Other biomarkers were expressed as proportions of the total saturated FAs (Table 3). Biomarkers of Gram-positive and Gram-negative bacteria were found in generally higher proportions in isolate A than in isolate H. Moreover, the proportion of Gram-positive to Gram-negative bacteria was lower in the case of the H isolate. Similarly, the presence of fungal organisms was considerably higher in isolate A (*ca.* 33%) than in isolate H, in which fungi constituted about 20%. In general, bacteria were present in higher amounts than

fungi in both isolates. Furthermore, particular taxonomic groups were significantly different for each of the algal isolates.

It is not completely clear why the examined algal isolates have dissimilar microbial associated communities. In all likelihood, the competition between microbes for resources to be converted into energy and cellular components essential for bacterial growth is a crucial factor limiting and affecting the compositions of alga-associated microbial communities [12]. Moreover, the structure of such communities may change successfully as a function of e.g. substrate utilisation, which depends on the physiological status of the plant [13]. Additionally, those physiological features may directly influence upon the quality and quantities of plant exudates that can be utilised by bacterial and fungal associates. Thereby, some changes in FA profiles can be observed, directly resulting from shifts in the microbial community structure. Undoubtedly the results presented in this paper effectively contribute to our present knowledge on the biology and microbial ecology of *A. constipatus*.

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