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Summary of doctoral thesis

MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR MARKERS IN IDENTIFICATION OF HIGHLY HYDROCARBON PRODUCING STRAINS OF *BOTRYOCOCCUS BRAUNII*

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1. INTRODUCTION

Green microalgae (Chorophyta), organisms capable of oxygenic photosynthesis, contribute with other phytoplanktonic species in producing over half of global oxygen. The diversity of shapes, sizes and metabolic pathways is responsible with the microalgae dispersion in aquatic habitats from all over de word. Structural and functional similarities with vascular plants, the unsophisticated cell structure, the ability to synthesize different types of secondary metabolites with application in pharmaceutics, cosmetic, petrochemical and food industry, etc., are reasons for choosing the green algae as model organisms in ultra structure, biochemical and molecular studies (*Chlorella spp., Chlamydomonas spp., Dunaliella spp.* etc.). This study is focused on *Botryococcus braunii* species, a green alga with biotechnological potential in synthesis of liquid fuels.

Among green algae, *Botryococcus genus* (Class Trebouxiophyceae) is remarkable due to its capacity for synthesizing major amounts of unsaturated hydrocarbons (up to 75% from dry biomass), similar to those from oil deposits (Moldowan and Seifert, 1980). Physical and chemical analysis of kerogen from boghead coals identified in different geographical regions, presented a similar composition with *B. braunii* hydrocarbons. This observation was the starting point for numerous studies which in the first place, focused on the evaluation of chemical diversity of strains. In this direction, the studies of Pierre Metzger, Claude Largeau, Eliette Casadevall and others from the Laboratory of Bioorganic Chemistry and Organic Physics in Paris, are well known as primary observations in classification of these strains into three chemical races, A, B and L. The A race produces linear and odd-numbered *cis* and *trans* (C_{23}-C_{31}) *n*-alkadienes and C_{27}-C_{31} *n*-alcatrienã, meanwhile the B race is the producer of triterpenoids called botyococcenes and squalene (C_{30}-C_{37}) of general formula C_nH_{2n-10}. *B. braunii* strains belonging to both races are wide spread, being reported in temperate, tropical and alpine regions. The few strains from L race were collected only from tropical regions (Metzger et al, 1988). Among the three, the B race seems to be the most promising as a source of liquid fuel because the content of hydrocarbon is generally higher than in other races, but the slow rate of growth is the major inconvenient in this matter. Numerous attempts have been made to improve the growth process and to corroborate it with hydrocarbon synthesis. A doubling time of approximately 3 days was the best time obtained in air-lift (1% CO_2) and permanent stirring conditions, a much lower value with respect to other green algae (*Chlorella spp., Scenedesmus spp.*).

For the moment, the ability of synthesize up most a certain type of hydrocarbons, represents the single criterion in discriminating the strains. The high degree of morphological

diversity makes impossible the strains classification from this point of view, a known fact in literature in which a genus of 13 species (Komárek and Marvan, 1992) or 2 species (Zalessky, 1926; Komárková, 1991) was proposed. The phylogenetic approach based on molecular markers (18S rDNA) sustains a genus with a single species, *B. braunii* being the only one member (Plain et al, 1993).

This study holds forth: 1) morphological, biochemical and molecular characterization of some *B. strains* derived from the Algal and Cyanobacterial Collection form the Institute of Biological Research, Cluj-Napoca (AICB); 2) the estimation of biotechnological potential meaning the growth rate and hydrocarbon synthesis in some *B. braunii* strains.

The originality at national and international scale emerges from multiple aspects. In the first place, this study takes advantages of the AICB collection, unique in our country due to its impressive number and biodiversity of photosynthetic microorganisms; 30 strains (A and B chemical races), isolated from different parts of Transylvania, were used in this study.

Internationally, the novelty of this thesis is emphasized by the great number of analysis made for the 30 *B. braunii* AICB strains but also derives from the multiple aspects that were studied. These aspects meant morphological aspects, growth process monitoring in batch system with or without CO_2 administration, qualitative and quantitative analyses of hydrocarbons, carotenoids, chlorophylls and fatty acids. Based on 18S rDNA markers the phylogenetic analysis focused on identifying molecular markers that would sustain the strains classification into two chemical races, A and B. Taking into account the biotechnological potential of this alga, a set of AICB strains capable of synthesizing major amounts of hydrocarbons (botryococcenes and squalenes) in a grown process with a minimum doubling time was proposed.

2. THE AIMS OF THE STUDY

❖ The characterization of the growth process based on specific parameters (growth curve, pH, optical density, exponential growth rate, doubling time, chlorophylls) with or without CO_2 administration;

❖ Morphological and ultra structural characterization of 30 strains of *B. braunii* alga, belonging to A and B chemical races, with the purpose of highlight specific features of a certain chemical race;

❖ The identification and quantification of hydrocarbons synthesized by the AICB strains, from A and B races;

❖ The qualitative and quantitative determination of fatty acids marking out the differentiations between strains of the two races;

❖ The qualitative and quantitative analyses of carotenoids identified in the extracts of AICB strains, A and B races;

❖ The phylogenetic analysis based on 18S rDNA nuclear markers in order to find a possible genetic prove in sustaining the chemical classification the *B. braunii* strains.

3. MATHERIAL AND METHODS

3.1   Biologic material

The biologic material studied in this thesis contained 30 *B. braunii* strains, deposited in the Algal and Cyanobacterial Collection (AICB) of the Institute of Biological Research from Cluj-Napoca (tab. 1). These strains were collected from different counties of Transylvania (Cluj, Mureş, Bihor and Sibiu). With a single exception, AICB 476 derived from a saline habitat (Ocna Sibiului), all the strains were collected from fresh and slightly saline waters. For the moment, all the *B. braunii* AICB strains investigated are incubated in BG 11 medium.

Table no. 1

The list of *B. braunii* AICB strains investigated morphologically, biochemically and molecularly.

No.	Strain code	Race	Habitat	Geographical localisation	Growth medium	The actual stage
1	AICB 53	A	dam reservoir	Sălicea (Cluj)	BG 11	unialgal
2	AICB 413	B	dam reservoir	Cheile Turului Cluj)	BG 11	unialgal
3	AICB 414	B	fishpond	Mărtineşti (Cluj)	BG 11	unialgal
4	AICB 415	A	fishpond	Turda (Cluj)	BG 11	unialgal
5	AICB 416	B	fishpond	Mărtineşti (Cluj)	BG 11	unialgal
6	AICB 418	B	fishpond	Mărtineşti (Cluj)	BG 11	unialgal
7	AICB 434	B	dam reservoir	Mărtineşti (Cluj)	BG 11	unialgal
8	AICB 438	B	fishpond	Mărtineşti (Cluj)	BG 11	unialgal
9	AICB 440	B	fishpond	Mărtineşti (Cluj)	BG 11	unialgal
10	AICB 441	A	brackish pond	Turda (Cluj)	BG 11	unialgal
11	AICB 442	B	fishpond	Mărtineşti (Cluj)	BG 11	unialgal
12	AICB 462	A	brackish pond	Turda (Cluj)	BG 11	unialgal
13	AICB 464	A	brackish pond	Turda (Cluj)	BG 11	unialgal
14	AICB 472	A	brackish pond	Turda (Cluj)	BG 11	unialgal
15	AICB 475	A	brackish pond	Turda (Cluj)	BG 11	unialgal
16	AICB 476	A	saline pond	Ocna Sibiului (Sibiu)	BG 11	unialgal
17	AICB 749	A	dam reservoir	Turda (Cluj)	BG 11	unialgal
18	AICB 851	A	fishpond	Ţaga Mare (Cluj)	BG 11	unialgal
19	AICB 855	A	fishpond	Tăureni (Mureş)	BG 11	unialgal
20	AICB 856	A	lake	Lacul Steluţei (Bihor)	BG 11	unialgal
21	AICB 857	A	fishpond	Tăureni (Mureş)	BG 11	unialgal

22	AICB 858	A	fishpond	Tăureni (Mureş)	BG 11	unialgal
23	AICB 859	A	fishpond	Tăureni (Mureş)	BG 11	unialgal
24	AICB 860	A	fishpond	Tăureni (Mureş)	BG 11	unialgal
25	AICB 861	A	fishpond	Tăureni (Mureş)	BG 11	unialgal
26	AICB 862	A	fishpond	Tăureni (Mureş)	BG 11	unialgal
27	AICB 870	B	fishpond	Cristuru (Bihor)	BG 11	unialgal
28	AICB 872	B	fishpond	Tăureni (Mureş)	BG 11	unialgal
29	AICB 874	A	dam reservoir	Turda (Cluj)	BG 11	unialgal
30	AICB 878	A	dam reservoir	Turda (Cluj)	BG 11	unialgal

3.2   Cultivation methods and growth analysis

The growth protocol implied the use of an exponential phase inoculum. BG-11 medium in 250 ml or 500 ml vesels was used in this purpose. The cultures were maintained in steady conditions of temperature ($20\pm2^{o}C$), permanent illumination with white fluorescent light (2500 lux), continuous stirring in air flux. The cultivation period was about 30-100 days. All the 30 strains were grown in this way at least one time.

A second option for growing meant the use of 100 ml algal samples, in an Applikon bioreactor with proper annexes in order to assure a permanent illumination 24 hours a day ($630 \ \mu mol \cdot m^{2} \cdot s^{-1}$). Some AICB strains were grown in this bioreactor with or without CO_2 administration. The CO_2 was administrated as air-CO_2 mixture, in different percentages. Regardless of CO_2 concentration in the mixture, due to the possibility of debit regulation, exact amount of 0,02 l/min of CO_2 was introduced in the algal culture (1 litre). The gas exchange between air and algal culture was facilitated through a permanent mechanical stirring (400 rpm). The temperature was maintained at a steady value of $25^{o}C$. The pH value was monitored daily.

The growth corves were calculated after Sorokin (1973), based on optical density. The bulk values were transformed in base-2 logarithms, differences against the inoculum (Δlog_2) being graphically represented. Computing the base-2 logarithms was necessary because is permits an easy calculation of growth rate and mostly the doubling time.

The pigments extraction was done in acetone. The identification of pigments was based on maximal wave length absorption, 663 nm for chlorophyll *a*, 645 nm for chlorophyll *b* and 480 nm for the carotenoids, respectively. The quantitative representation used the specific absorption coefficients after Arnon method (1949) for chlorophylls and Goodwin (1976) and Britton et al (1995) methods for carotenoids.

3.3 Light and electron microscopy methods

As for the light microscopy, the protocol consisted in microscopic observation of each strain using an Olympus BX-41 light microscope, digital photography and making of necessary measurements (cells and colonies size, etc.).

For SEM protocol, the algal samples were analyzed with an electron microscope, Jeol JSM 5510LV, using an acceleration voltage of 10 kV with a 5 size spot.

The TEM investigations permitted the obtaining of some ultrafine sections using a TEM microscope, Jeol JEM 1010.

3.4 The quantitative and qualitative analysis of hydrocarbons using GC-MS

In the case of *B. braunii* we used an extraction mix consisting of chloroform, methanol and water in ratio of 1:1:0.5 (v/v/v). The mix separation in 2 specific phases made possible the hydrocarbon extraction in chloroform, which represented the inferior phase.

The gas chromatography-mass spectrometry coupling method proved to be not only a very sensitive detection method, but a most precise method for the identification of separated compounds. In the mass spectrometer, the molecules separated through gas chromatography break up into small ionized fragments which would be accelerated and subsequently detected based on their specific electric charge. The separation was done using the linear programming of column temperature from $60^{\circ}C$ to $280\text{-}300^{\circ}C$. Nitrogen was used as mobile phase (for flame ionization detector) and helium also (in the mass spectrometer). In this way, an Agilent Technologies 6890 N gas spectrometer coupled with an Agilent Technologies 5973 N mass spectrometer was used.

3.5 The quantitative and qualitative analysis of fatty acids using GC-MS

C_{12}-C_{22} saturated and unsaturated fatty acids with various degrees of unsaturation were identified. The fatty acids were determined from the same algal extracts that were used for hydrocarbons analyses. For the fatty acids separation, a capillary column containing a polar stationary phase was used. An accurate separation was assured by linear programming of temperature in the HP 4890 gas chromatograph. The quantitative analysis of fatty acids was based on the area normalisation method. The chromatographic pick areas were then calculated.

3.6 The quantitative and qualitative analysis of carotenoids using HPLC

The carotenoids extraction form algal culture was done by direct saponification approach with a 30% solution of KOH in ethanol. It was used a Pekin-Elmer Lambda 3 connected to an Perkin Elmer R 100 A recorder to measure the absorbance of carotenoids extracts (in order to determine the total content of carotenoids) and those of standard solutions (in order to determine with accuracy the carotenoids concentrations). The monitoring of separations was made at 450 nm, separations being conducted at $35^{o}C$; the system pressure remained constant. A HPLC Agilent 1100 was used. Quantitative analysis of carotenoids restricted to those carotenoids that possessed standards and was based on external standard method, a specific procedure of Hart (1995).

3.7 The phylogenetic analysis based on 18S rDNA

Obtaining the 18S rDNA sequences presumed the genomic DNA extraction, using the commercial kits useful in obtaining a high degree of purity. Using specific primers (Senousy et al., 2004) we proceeded for the standard PCR reaction. The DNA fragments were directly sequenced or cloned in cloning vectors (pGEM-T) prior to sequencing. We used the ABI Prism 310 and Beckman Coulter 8800 genetic analysers (ICEI). The DNA fragments were assembled using the *Vector NTI Advanced v 9.0* software. The sequences validation was done using blastn (BLAST-NCBI). For multiple sequence alignment, 42 18S rDNA sequences were extracted from GenBank (NCBI). In this way, the *Mega 4.1* software was used.

Phenetics or distance (Minimum Evolution) and cladistic methods (Maximum Parsimony) were two distinct methods used in constructing phylogenetic trees, based on 18S rDNA sequences.

4. RESULTS AND DISSCUTIONS

4.1 Characterisation of AICB strains growth process with or without CO_2 administration

The evaluation of AICB strains growth process based on calculated parameters sets off major differences, which depended on strain and growth conditions. The aerated cultures with ambient air and without stirring, presented growth rates values between 0.03 and 0.31 OD/day and a doubling time between 3.27 and 33.14 days (tab. 2).

Table no. 2

The growth parameters calculated based on optical density measured for AICB algal cultures.

Calculated parameter	AICB strains									
	53		851	416		418		442		440
	$+ CO_2$	$-CO_2$	$+ CO_2$	$+ CO_2$	$-CO_2$	$+ CO_2$	$-CO_2$	$+ CO_2$	$-CO_2$	$-CO_2$

Exponential growth rate	1.72	0.1	0.17	0.3	0.31	2.01	0.24	0.25	0.08	0.03
Doubling time	0.58	9.69	6.05	3.35	3.27	0.5	4.12	4.05	12.21	33.44

The doubling time is measured in days and the exponential rate in relative units of OD/day.

The CO_2 administration, in permanent mechanical stirring, ameliorated the growth process. In these conditions, the growth rate ranged between 0.17-2.01 and the doubling time had a minimum of 0.5 and a maximum of 6.05 days (tab. 2, fig. 1-4).
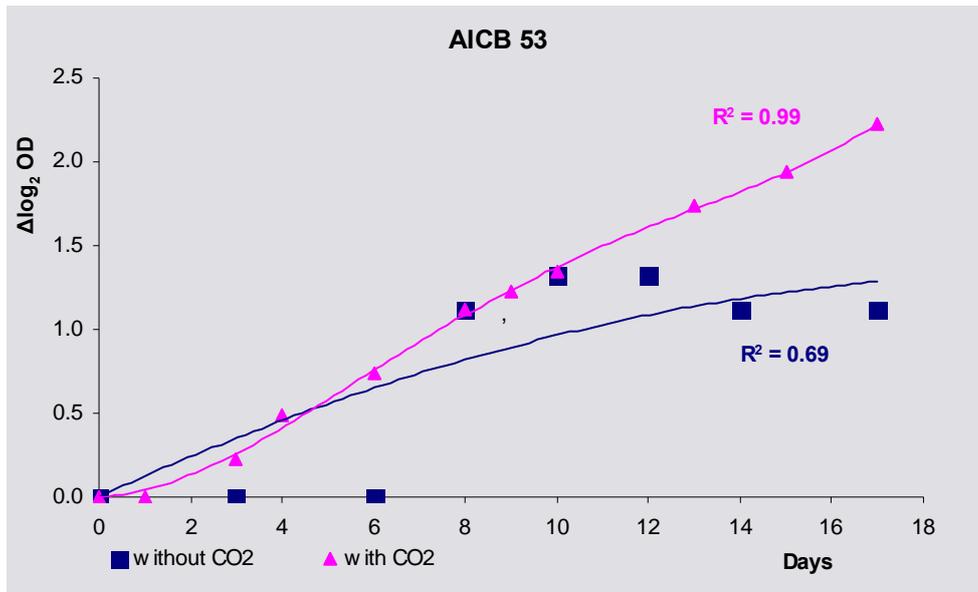
Fig. 1. The growth curves based on OD (600 nm) for the AICB 53 strain (A race), cultivated in bioreactor, with or without CO2 (0.02 l/min).
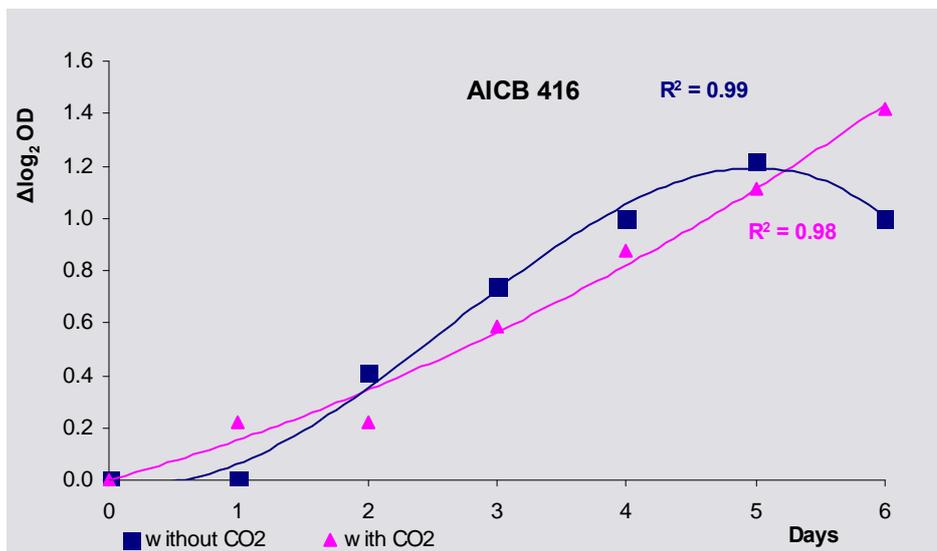
Fig. 2. The growth curves based on OD (600 nm) for the AICB 416 strain (B race), cultivated in bioreactor, with or without CO2 (0.02 l/min).
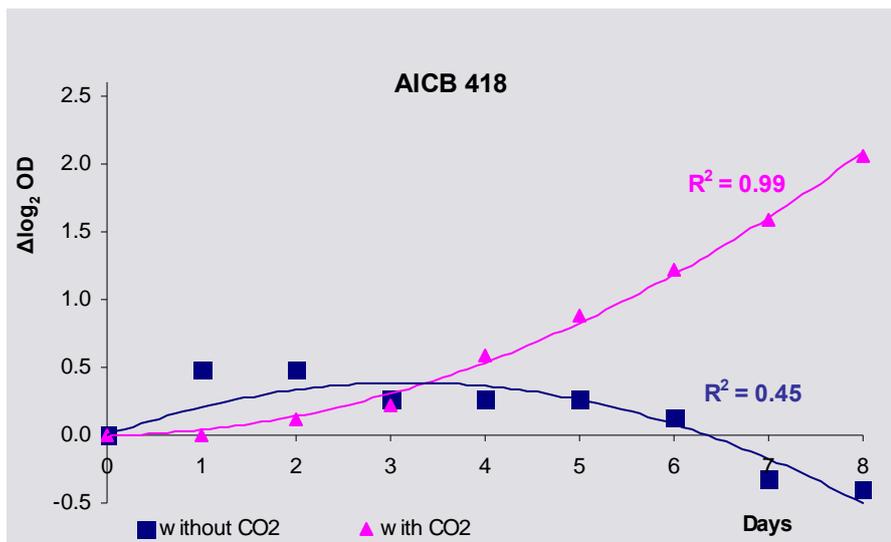
Fig. 3. The growth curves based on OD (600 nm) for the AICB 418 strain (B race), cultivated in bioreactor, with or without CO2 (0.02 l/min).
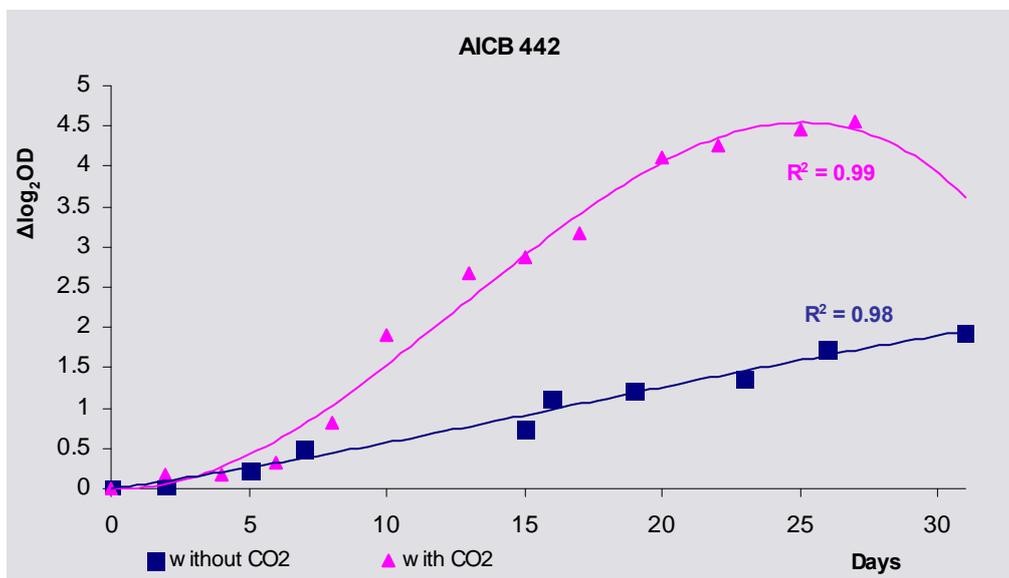
Fig. 4. The growth curves based on OD (600 nm) for the AICB 442 strain (B race), cultivated in bioreactor, with or without CO2 (0.02 l/min).

Whereas, the chlorophyll pigments minimum content was relative constant for the two races strains (0.52% B race and 0.50% A race), the upper value was higher for the A race strains. The major differences between the chlorophylls/carotenoids ratio of the two races demonstrated the capacity of the A race strains to synthesize greater amounts of chlorophylls and lower amounts of carotenoids respectively (fig. 5). The last ones were present in greater percentages in B race strains which in stead produced lower quantities of chlorophylls.
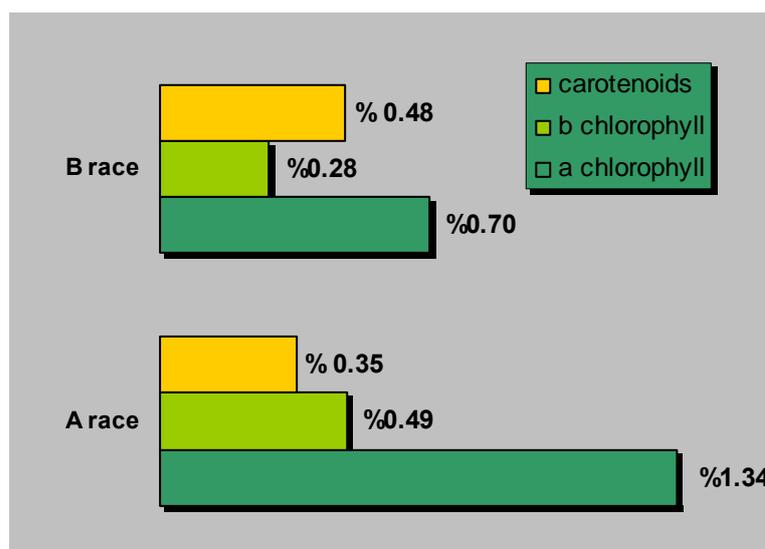
Fig. 5. The contents of *a* chlorophyll, *b* chlorophyll and carotenoids in AICB strains belonging to both A and B chemical races.

Although, all the strains were cultivated in conditions of CO_2 administration, they all showed higher exponential growth rates, the chlorophyll content decreased in some strains of A race and in one strain of B race (fig. 6). With the exception of AICB 53, the increasing/decreasing tendencies of the chlorophyll content followed the same tendencies with the hydrocarbon synthesis.
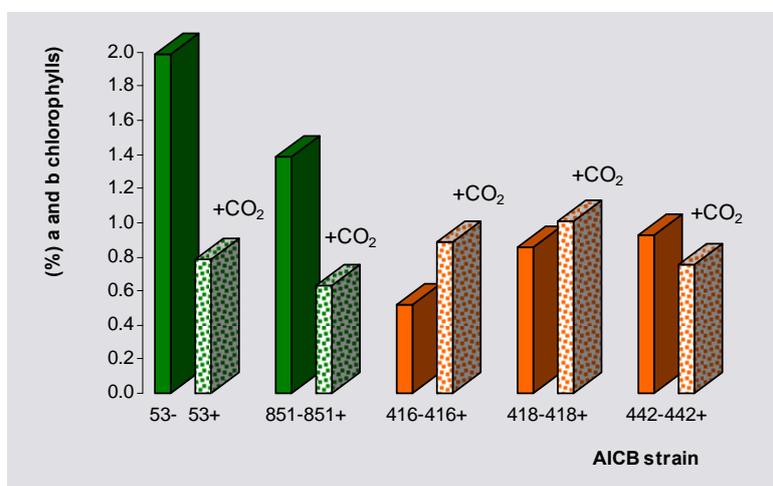
Fig. 6. The *a* and *b* chlorophyll content in AICB strains, belonging to both A and B chemical races, cultivated with or without CO_2 (l/min).

4.2 Morphological and ultrastructural characterization of AICB strains

The light microscopy investigations showed some distinctive features of both chemical races strains. These features were elaborated in the explanations beneath the 3 plates (Pl. 1-3). The morphologic characteristics of the colonies obtained from air-lift (CO_2) cultures showed no significant differences with the exception of extracellular matrix, which was larger due to the intensified hydrocarbon synthesis.

The ultrastructural approach (TEM) in characterization of AICB strains did not showed significant differences between the strains from A and B chemical races.

In the A strains (AICB 851, 53), the hydrocarbon were localized mostly within the cell, disposed as lipid bodies. In the B race strains (AICB 440), these were arranged outside the cell, between the plasmalema and the cell wall (TLS), forming an abundant extracellular matrix with the successive layers of TLS. The lipid bodies were surrounded by tubular structures (probably SER) and numerous polyribosomes. The parietal chloroplast was localised near the plasmalema. The pyrenoid could be observed in AICB 440 strains, together with the numerous starch granules, meanwhile the AICB 851 strain lack this structure. RER and Golgi apparatus were observed in the cell apical region, below the hydrocarbon cup.
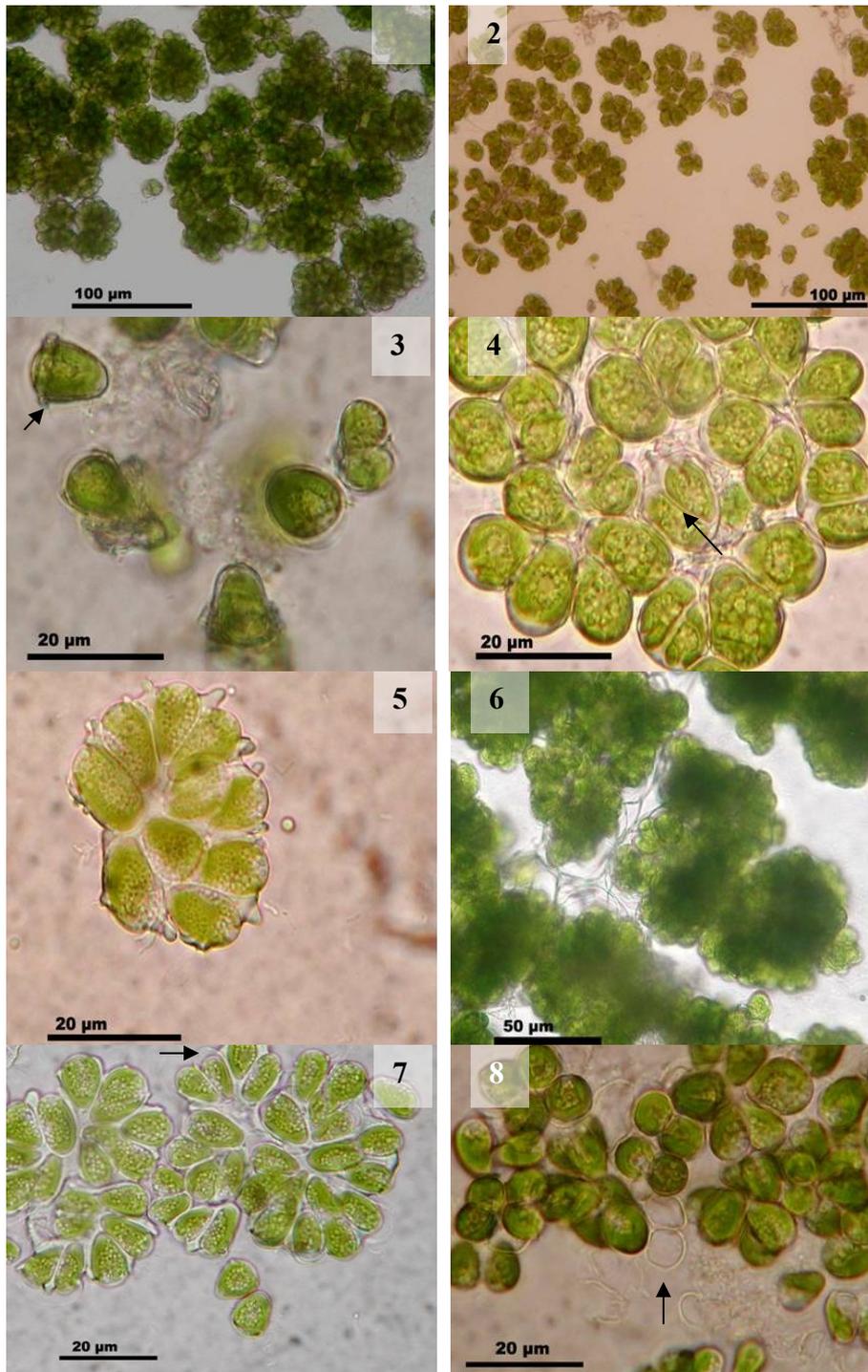
Pl. I (fig. 1-8). *Botryococcus braunii,* A race – light microscopy. Colonies differ by consistency and cell number; compact colonies with higher number of cells in AICB 859 strain (fig. 1) and lax colonies with smaller number of cells in AICB 441 (fig. 2) were observed. Singular cells with a "collar" structure in the upper part of the cup were observed also (showed by arrow) (fig. 3 – AICB 861). In some strains (AICB 53) the cells did not present typical shapes (fig. 4) and the cell division had a longitudinal orientation (showed by arrow). By mechanical pressing, the hydrocarbons were released as drops, outside the colony (fig. 5 – AICB 857). Fibrillar structural elements were observed in some algal cultures (fig. 6 – AICB 415). Sometimes, the cells presented a cap which covered the apical region (showed by arrow) (fig. 7 – AICB 857). When, the cups released the cells, they preserved their shapes (fig. 8 – AICB 475).
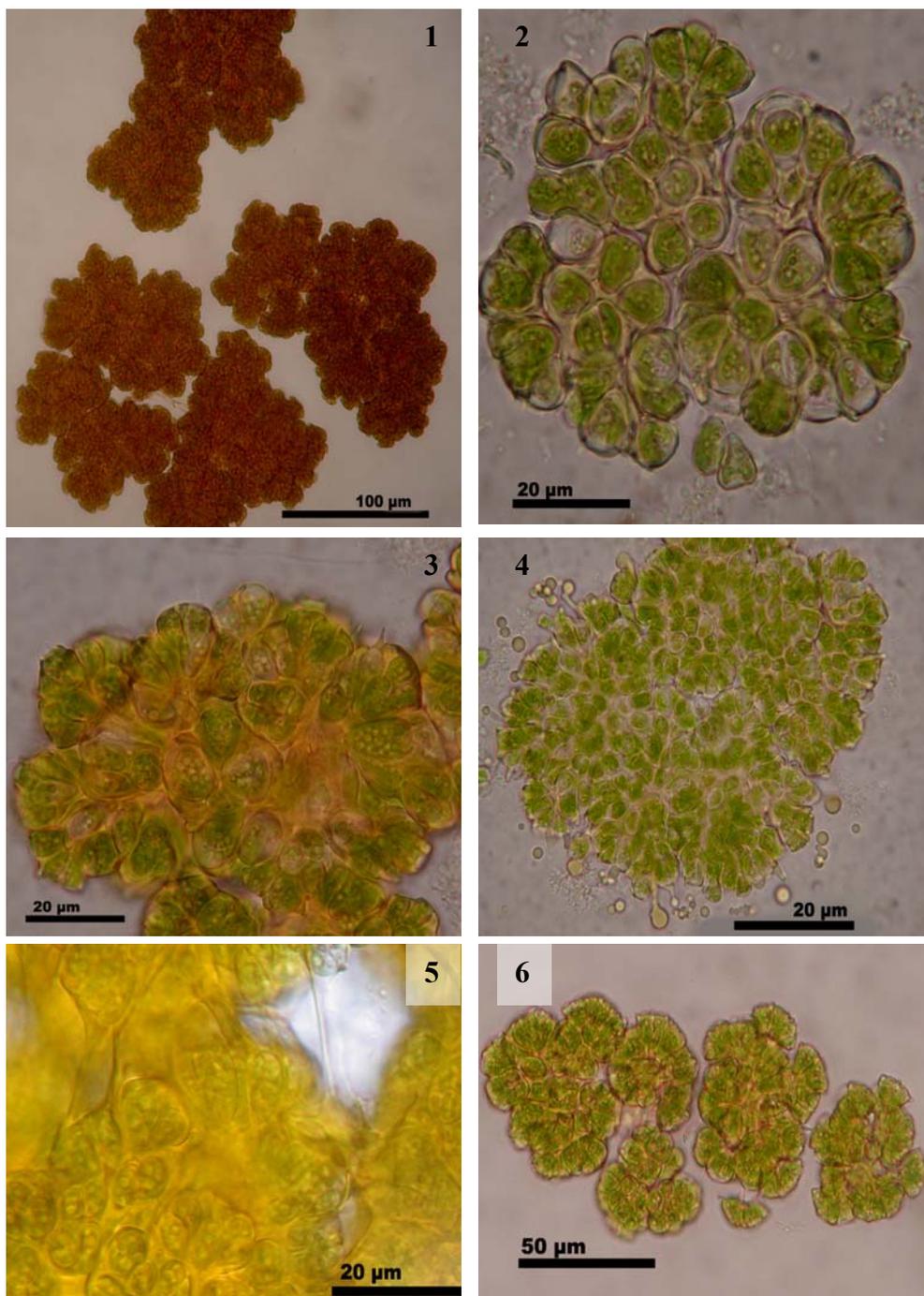
Pl. II (fig. 1-6). *Botryococcus braunii,* B race – light microscopy. The colonies are dense, with higher number of cells (fig. 1 – AICB 442), completely immersed in extracellular matrix (fig. 3 – AICB 872). The cells are typically pyriform and could be observed just after the mechanical pressing of the sample (fig. 2, fig. 4 – AICB 872). The fibrillar structures which connect the colonies were observed in all AICB strains that were analysed (fig. 5 – AICB 413). Some colonies may be small (fig. 6 – AICB 872), but the botryoid pattern is kept in this case also.
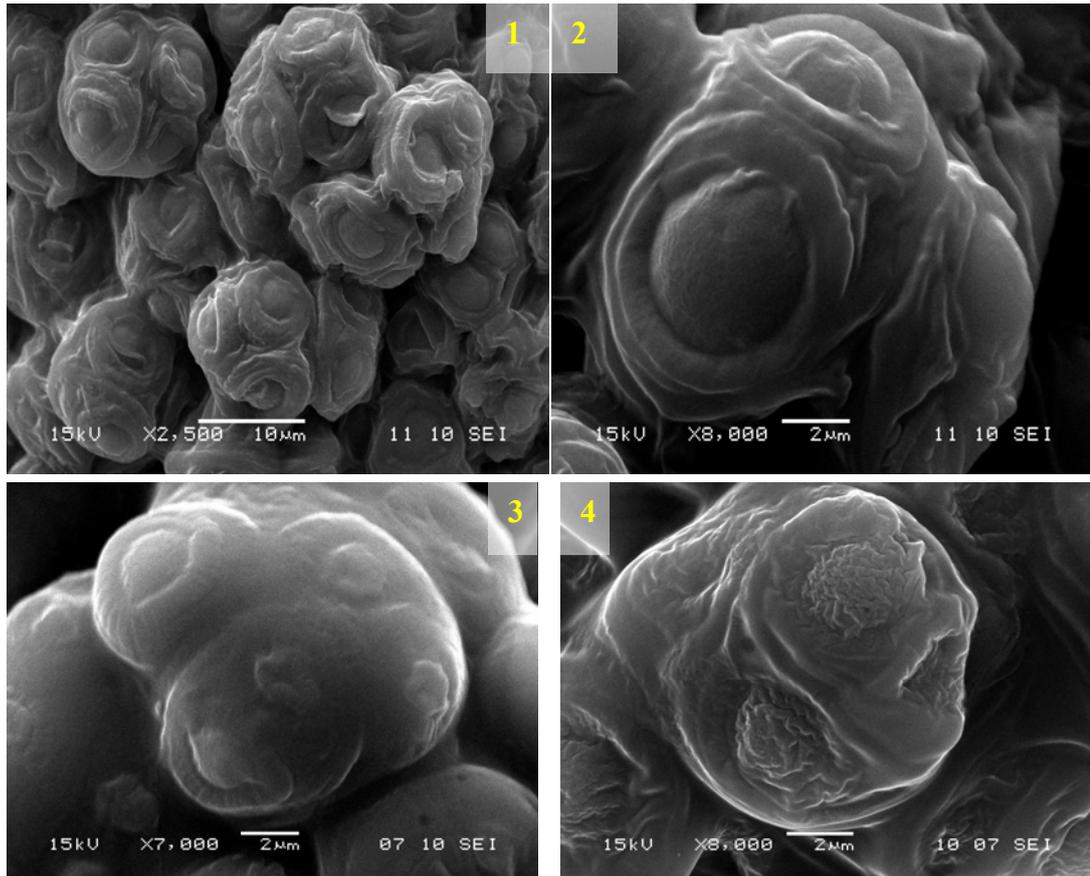
Pl. III (fig. 1,2). *Botryococcus braunii,* A race – SEM. The cells are 2/3 covered by the polyglucidic cup impregnate with hydrocarbons, the cellular apex being exposed (fig. 1,2 – AICB 415); **(fig. 3,4)**. *Botryococcus braunii,* B race – SEM. The colonies had a dense aspect, with a very large extracellular matrix. The cup which is impregnated with hydrocarbons, totally covered entirely or almost entirely the cellular apex (fig. 3 – AICB 438).

4.3 The identification and quantification of hydrocarbons in AICB strains

The qualitative and quantitative synthesized hydrocarbons were investigated in 30 *Botryococcus braunii* AICB strains, 10 strains from B race and 20 strains from A race. In the 20 strains, belonging to both races we did 2-3 extract/strains derived form different growth experiments. Five AICB strains (2 from A race and 3 from B race) were cultivated in condition of CO_2 administration.

The AICB strains belonging to the B race synthesized higher hydrocarbon content (16.77%), comparative with the A race strains (5.26%) (fig. 7).

Diolefins (alkadienes and alkatrienes) and hexaolefins (hexadienes) also were identified in all lipid extracts obtained from AICB strains, regardless of the race. So, the first category prevailed in the A race strains (4.29%) and it was less representative in the B race strains (2.74%). The hexadienes represented the major fraction of the B race strains (14.41%) and were presented in a insignificant fraction in the A race (0.39%) (fig. 7, 8).
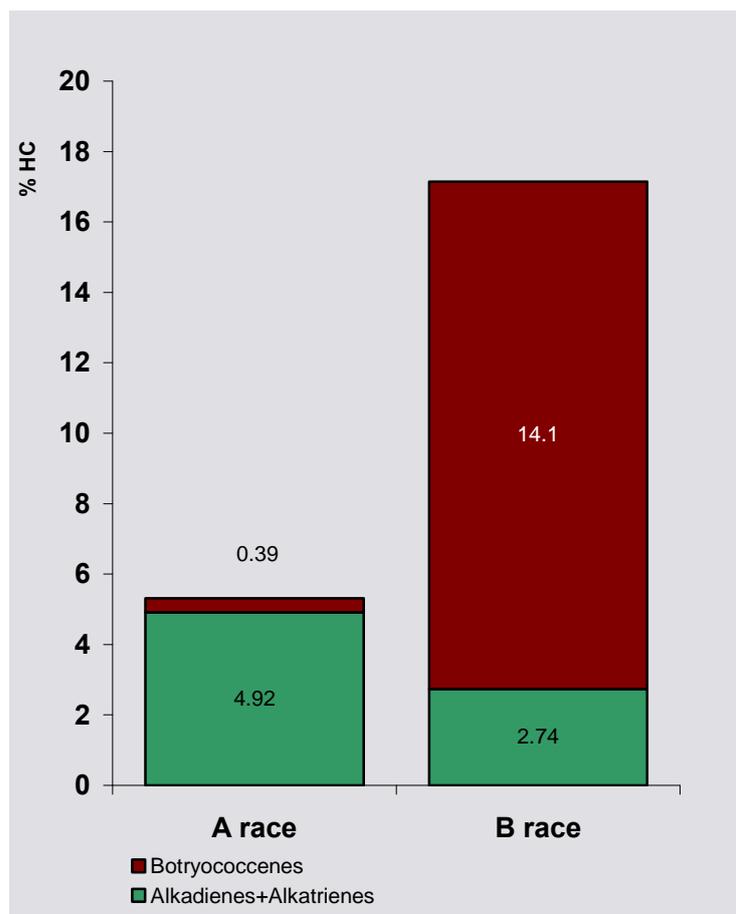
Fig. 7. The percentages of alkadienes and alkatrienes (green) and hexadienes (brown) in AICB strains belonging to both chemical races.

Regardless of chemical race, the C_{29} alkadiene was the major fraction in the AICB strains and the C_{27} alkadiene lacked in the majority of lipid extracts. Among the hexadienes, the C_{30} compound (most probably squalene) prevailed in the strains of the A race. In the B strains, the higher homologues of hexadienes C_{31} and C_{32} (most probably botryococcenes) were dominant.

Administration of 0.02 l/min CO_2 concentration contributed in increasing the hydrocarbon content in three strains (AICB 53, 416, 418), meanwhile in the two strains a hydrocarbon decreasing was observed (AICB 851, 442) (fig. 10). These results were independent of chemical race. The hydrocarbon content increased due to the C_{29} compound in AICB 53 (A race) and to the C_{31} and C_{32} hydrocarbons in the B race (AICB 416, 418) (fig. 11). The decreasing of hydrocarbon content was observed in the absence of C_{29} alkadiene in the AICB 442 (B race) and 851 (A race) strains (fig. 11). In this last case, we observed the raising of the C_{31} alkadiene quantity.
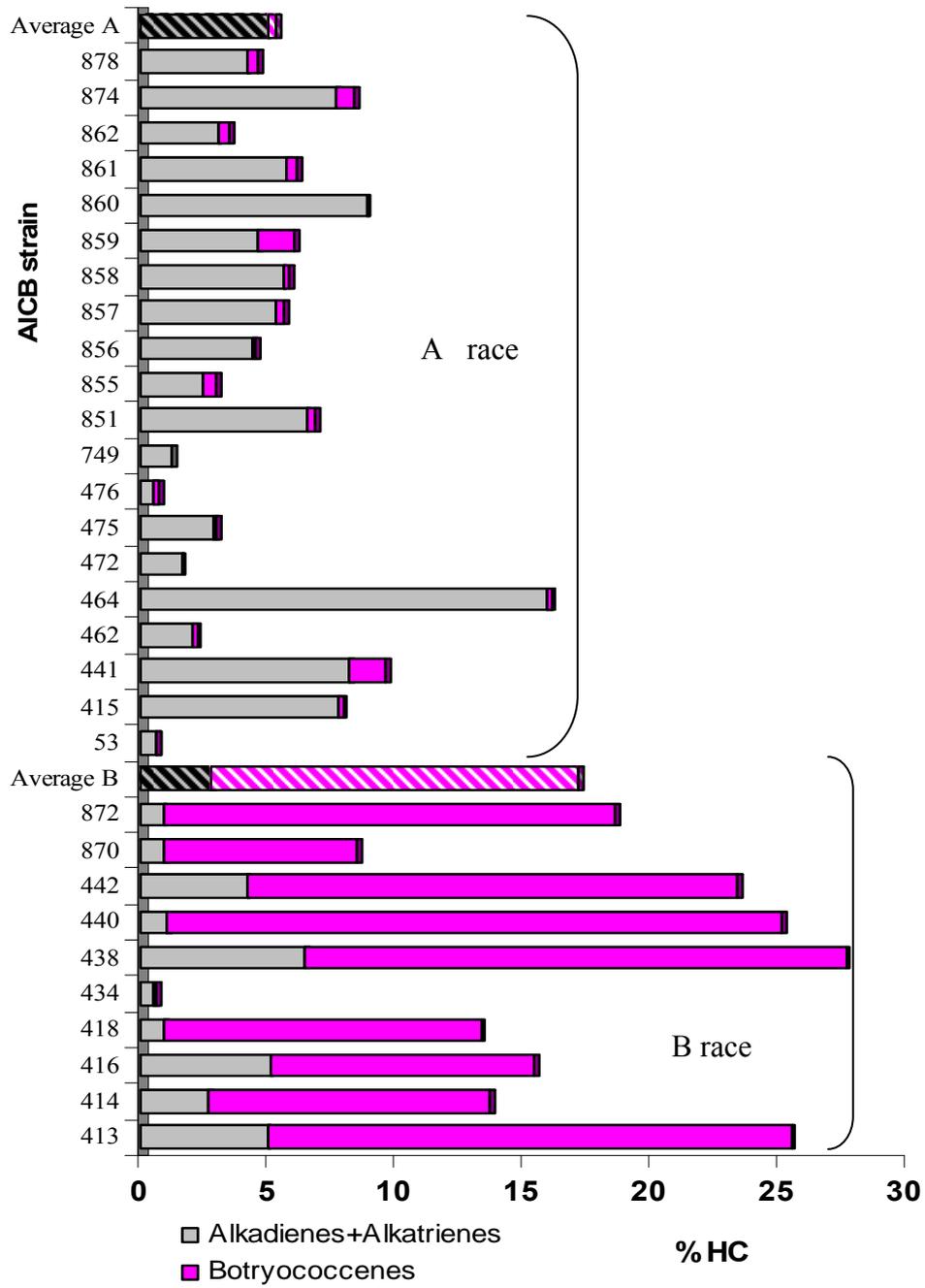
Fig. 8. The percentages of the alkadienes, alkatrienes (grey) and hexadienes (magenta) in the AICB strains.
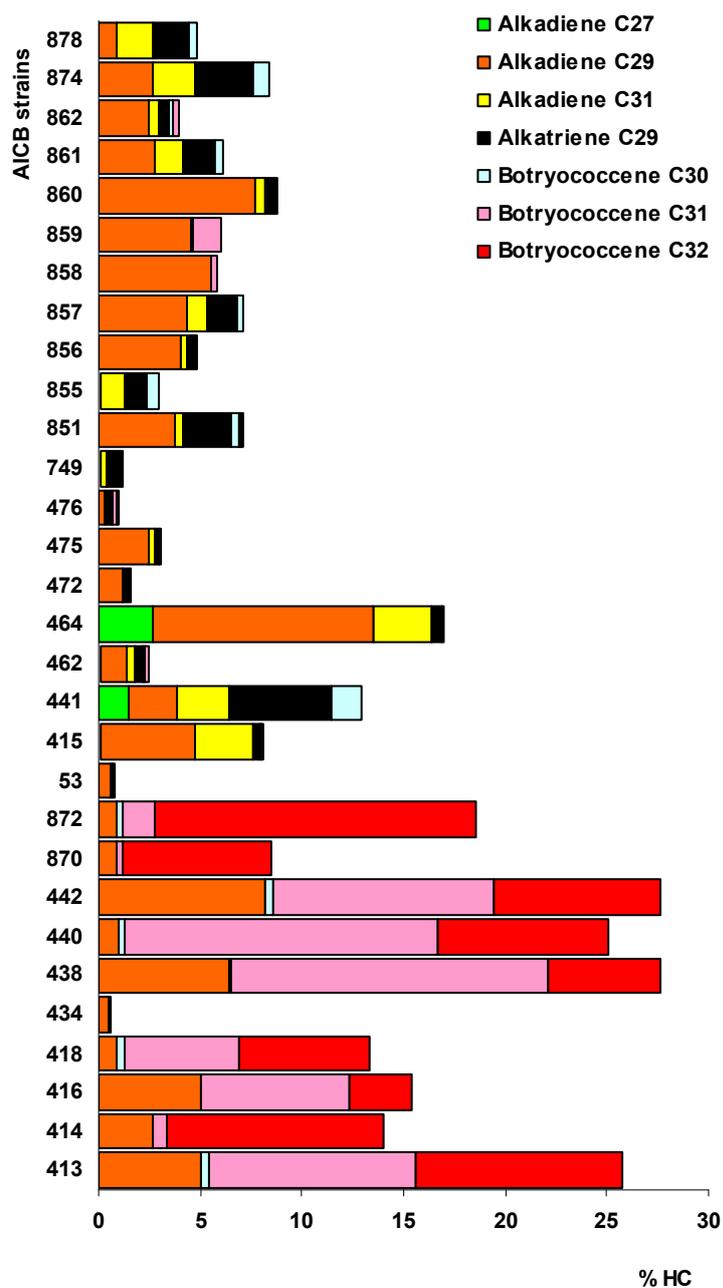
Fig. 9. The different types of synthesized hydrocarbons in the AICB strains belonging to the *B. braunii* alga, belonging to both chemical races.
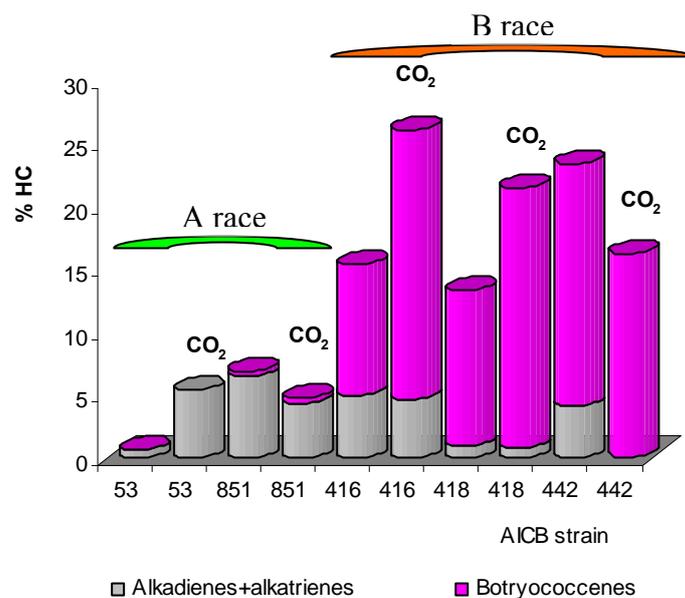
Fig. 10. The content of AICB strains from A race (53, 851) and B race (416, 418, 442), cultivated in the CO_2 presence or absence.
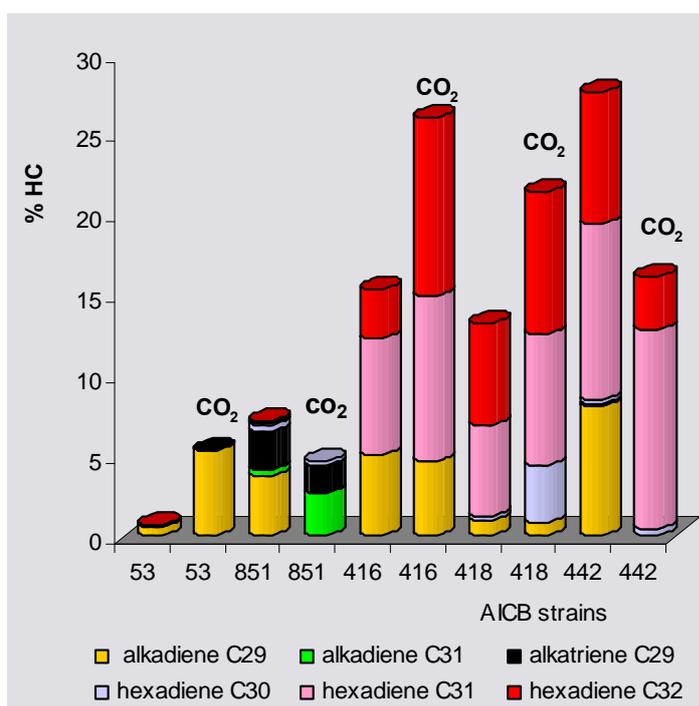
Fig. 11. The content of different hydrocarbon in AICB strains form A race (53, 851) and B race (416, 418, 442), cultivated in the CO_2 presence or absence.

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4.4 The identification and quantification of fatty acids in AICB strains

The results obtained for the 30 AICB strains showed the dominance of unsaturated fatty acids (73.70%) against the saturated fatty acids (26.29%) (fig. 12).
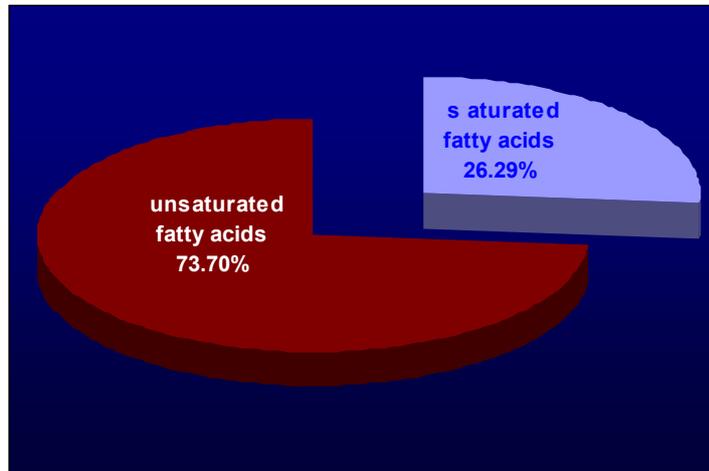
Fig. 12. The percentages of saturated and unsaturated fatty acids (FA) in *B. braunii* AICB strains.
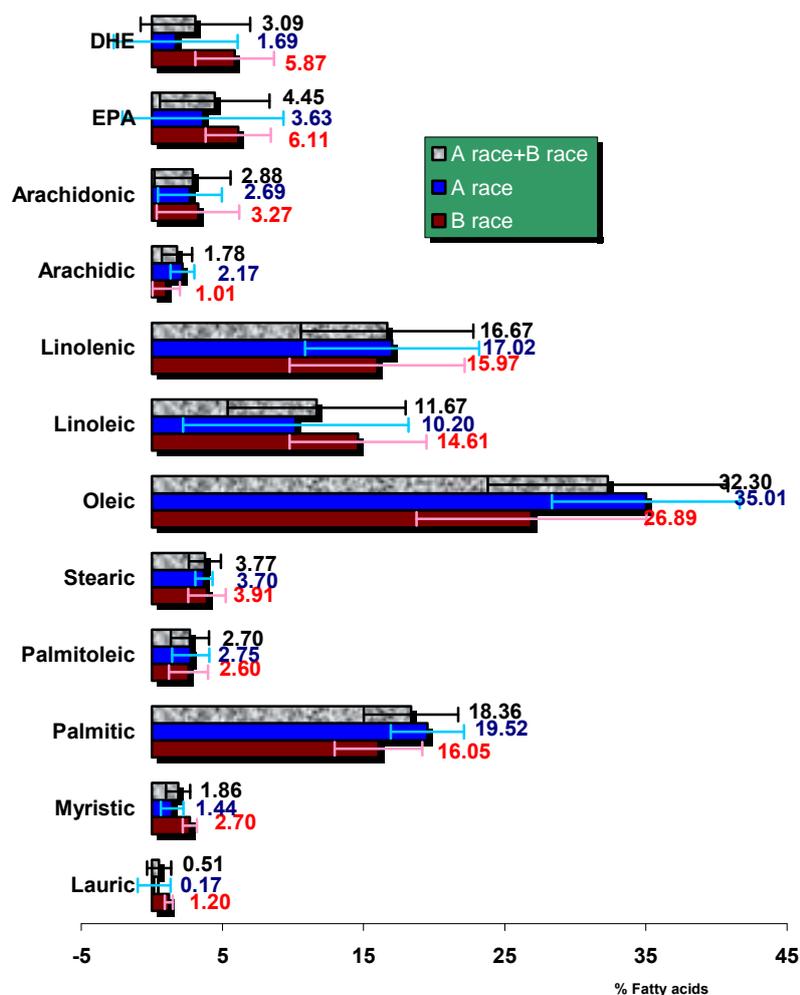
Fig. 13. The average percentages of fatty acids (FA) for all AICB strains, and separately for the two chemical races (A and B).

Five types of saturated fatty acids (26.29%) and 7 types of unsaturated fatty acids (73.7%) with various degree of unsaturation were present in the majority of lipid extracts. With the exception of lauric, arachidic, arachidonic, EPA and DHE, the majority of fatty acids were present in all extracts.

With the exception of oleic and palmitic acids, the content of fatty acids was higher in the B strains. The prevalence of palmitic and oleic acids in the A strains is corroborated with the ability of these strains to synthesize higher amounts of diolefines (4.92%), against the B race (2.79%) (fig. 13).
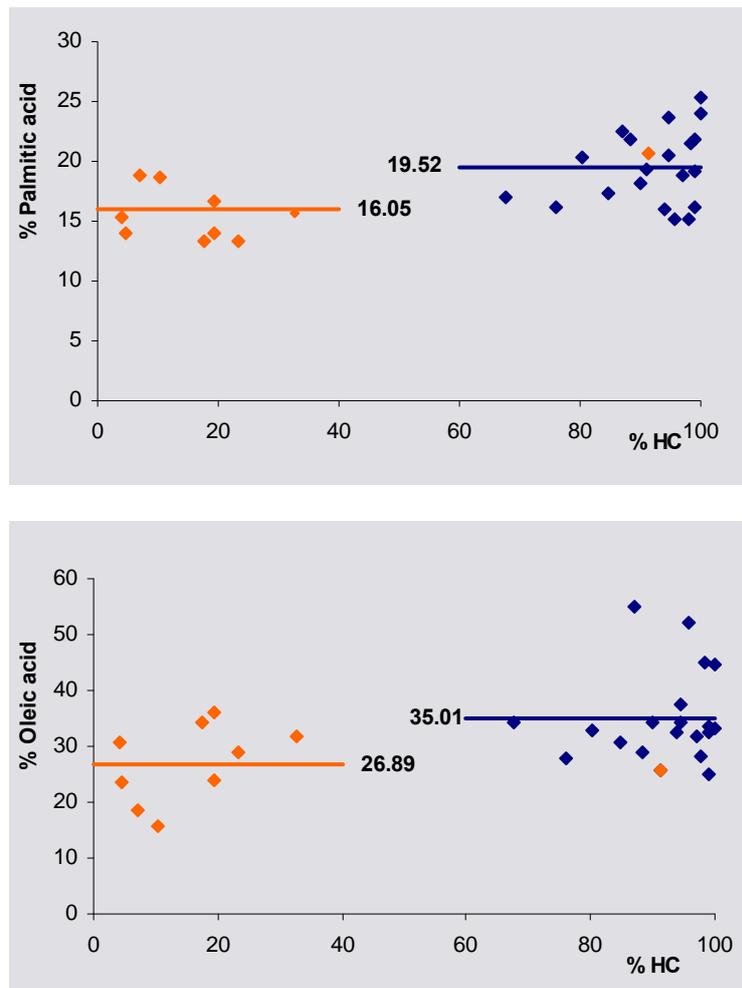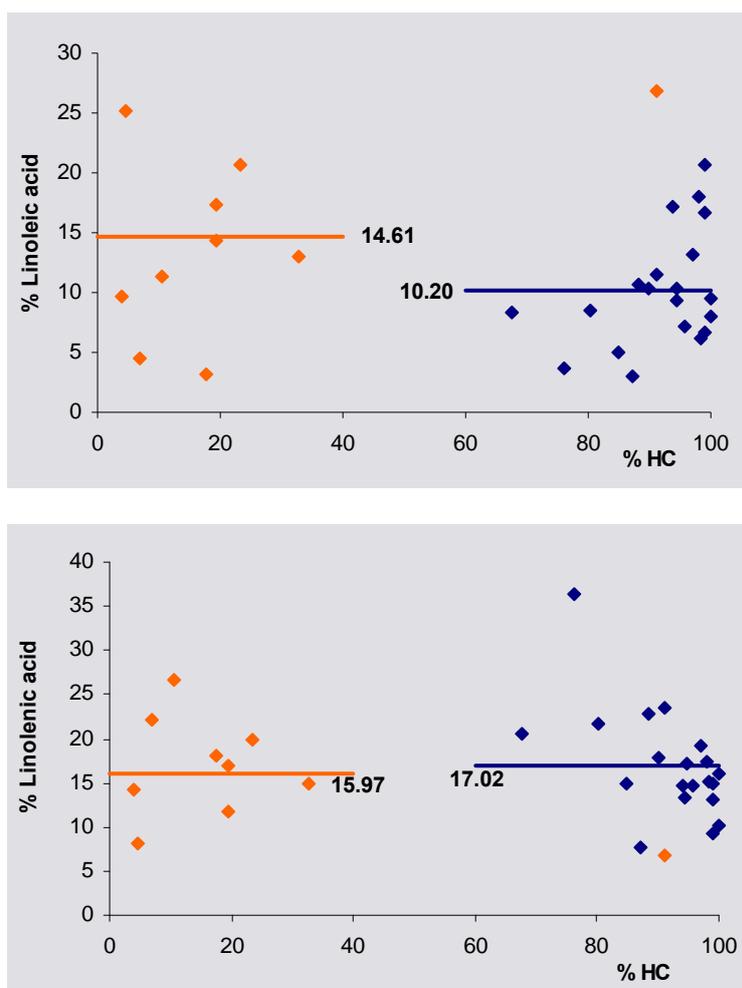
Fig. 14. The HC (alkadienes and alkatrienes hydrocarbons) and oleic, palmitic, linoleic and linolenic contents in AICB strains, belonging to both chemical races, A (blue) and B (orange). The values represent the relative content of fatty acid and the absolute content of hydrocarbon (g/100 g dry biomass) respectively. The averages specific to each race is shown by lines.

The oleic (31%), palmitic (19%), linoleic (17%) and linolenic (12%) acids were dominant in the total amount of fatty acids (fig. 13). The higher values of palmitic and oleic acids are corroborated with the use of these acids as precursors in the synthesis pathway of diolefines.

The increasing tendency of the absolute and relative content of diolefines was not clearly related with a certain tendency of the dominant fatty acids found in the majority of lipid extracts, between the strains of the same race. This fact suggests a high degree of variability within each race, but these relations are valid for each strain.

The lack of a similar relation between the data obtained from lipid extracts of the same strain was observed in the strains from both races. Although, in 11 strains form 19 analysed, a decreasing of the oleic and palmitic contents and also of the absolute content of hydrocarbon

were observed (fig. 14). Most probably, the synthesis of those is corroborated with the consummation of the precursor's fatty acids.

The relative content of fatty acids varied between the 5 strains cultivated in CO_2 presence or absence, with the exception of stearic acid, regardless of chemical race. This last compound decreased in the extracts of the most algal air-lift cultures (fig. 15, 16).

The increasing of hydrocarbon content was corroborated with the decreasing of oleic and palmitic percentages. The relative content of the four fatty acids with higher content in the lipid extracts raised in the biomass derived from air-lift cultures, regardless of chemical race or the diolefines content.
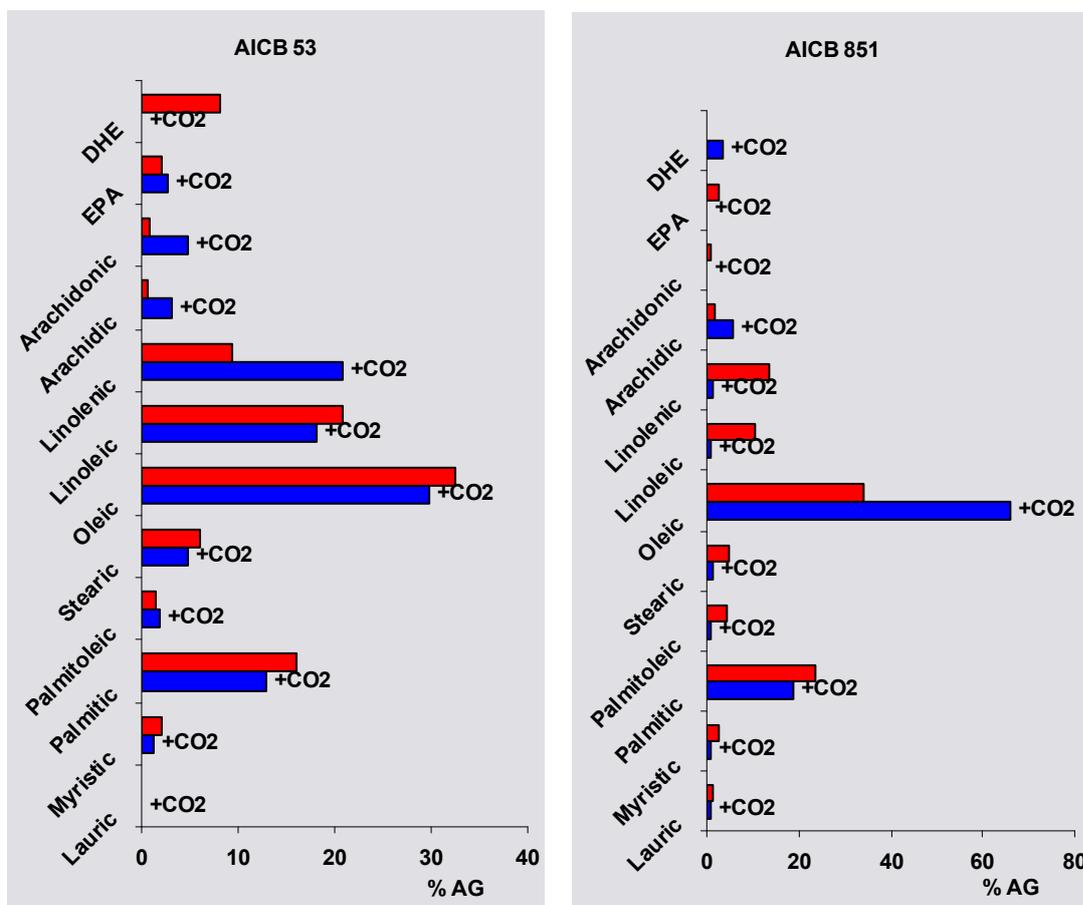
Fig. 15. The relative content of fatty acids in the AICB strains (A race) cultivated in CO_2 presence or absence.
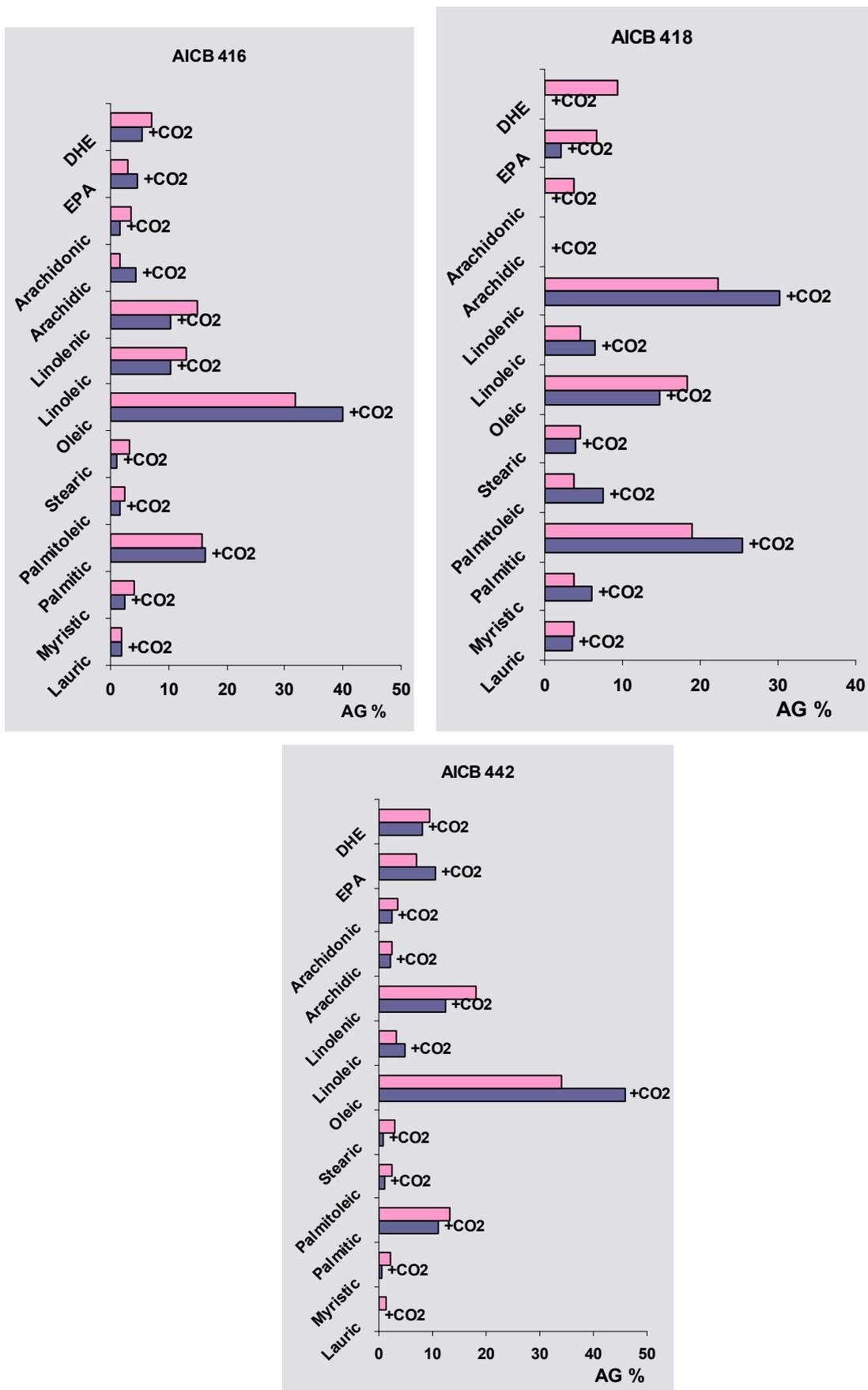
Fig. 16. The relative content of fatty acids in the AICB strains (B race) cultivated in CO_2 presence or absence.

4.5 The identification and quantification of carotenoids in AICB strains
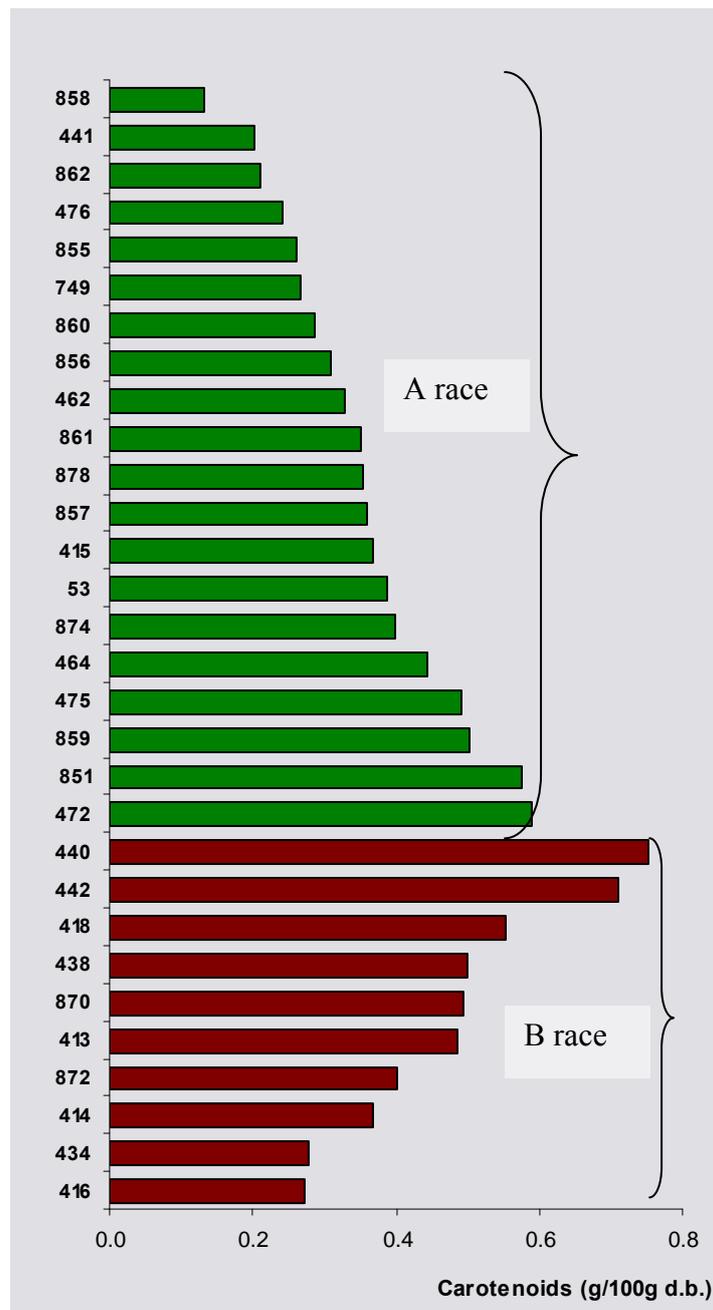
Fig. 17. The percentages (g/100g dray biomass) obtained form the carotenoids fractions extracted from the biomass of AICB strains belonging to A and B chemical races.

The *B. braunii* AICB algae produced various quantities of carotenoids depending of strains and chemical race (fig. 17).Generally, the biomass of the AICB strain form B race synthesized a higher percentage of carotenoids (0.48 g/100g dray biomass), against the A race (0.35%). This fact may be explained based on the terpenic structure, common to both carotenoids and botryococcenes, their metabolic pathways being interconnected (fig. 18).
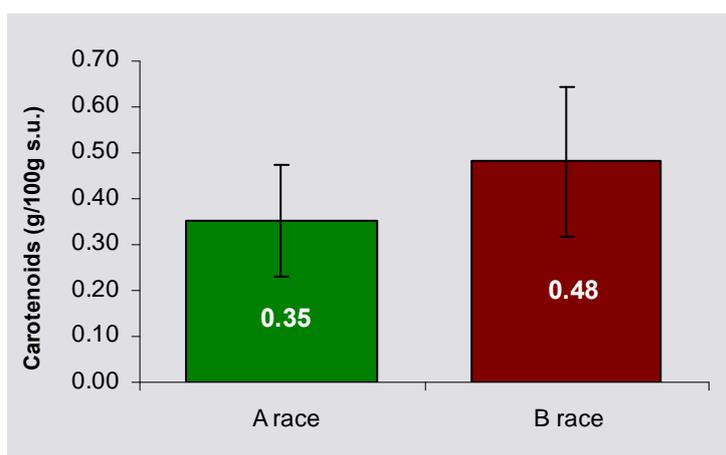
Fig. 18. The average percentages of the carotenoids of the AICB strains, belonging to A and B chemical races (P=0.022).

From qualitative point of view, 13 types of carotenoids were identified in the lipid extracts from 17 AICB strains. Among these, lutein was the best represented form quantitative point of view and the violaxanthin, antheraxanthin, α-caroten, β-caroten and lutein were the most frequent carotenoids in the majority of extracts (fig. 19).
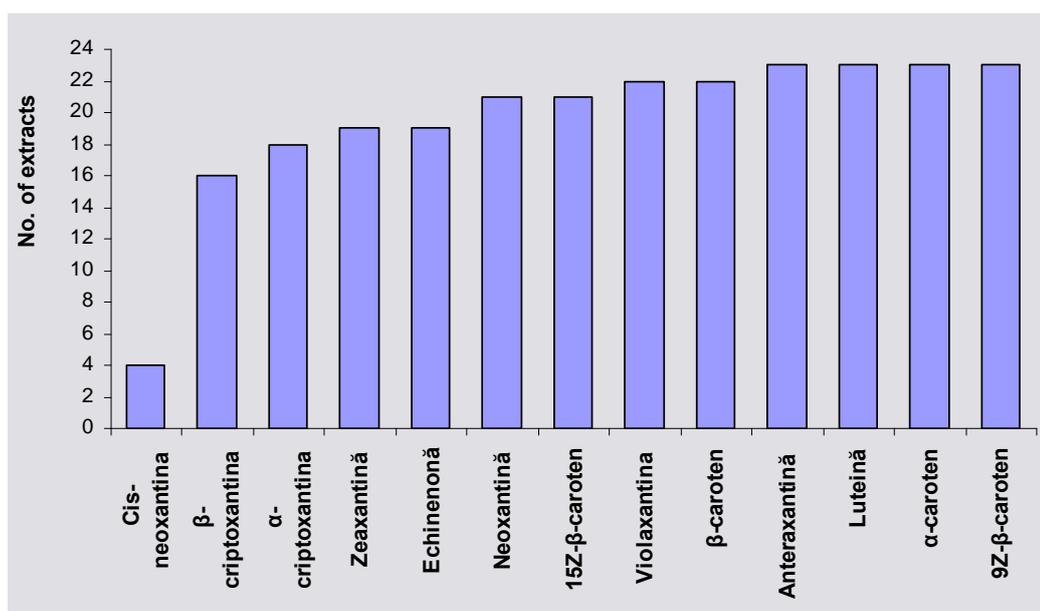
Fig. 19. The presence degree of different types of carotenoids in the 23 extracts derived from the 17 AICB strains, belonging to the A and B chemical races.

The quantitative results of carotenoids obtained from the algal suspensions cultivated in the air-lift conditions (0.02 l/min CO_2) showed a surprising aspect. Regardless of the increasing/decreasing of the produced hydrocarbons (both botryococcenes and alkadienes, alkatrienes), in the strains cultivated in air-lift conditions, the carotenoids percentage decreased against the same strains, cultivated in the CO_2 absence (fig. 20).
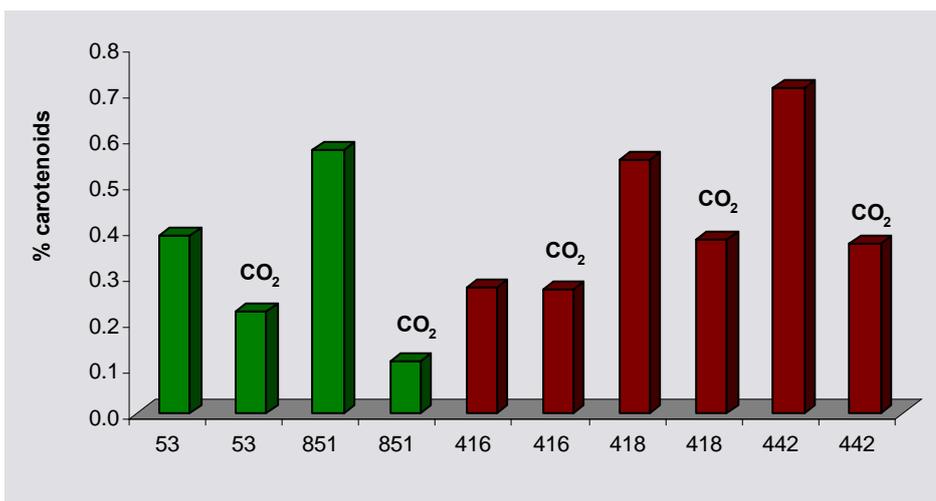
Fig. 20. The content of carotenoids in the AICB strains cultivated in the CO_2 (0.02 l/min) presence or absence. In order to differentiate between the strains of two chemical races, they were represented with distinct colours: green for the A race and red for the B race. The percentages are grams per 100 grams dray biomass.

The increasing tendencies observed in the case of carotenoids characterized the triterpenic hydrocarbons (hexadienes) also (fig. 21). In the same time, the content of long-chain odd-numbered hydrocarbons which are dominant in the A race showed a decreasing tendency (fig. 22).
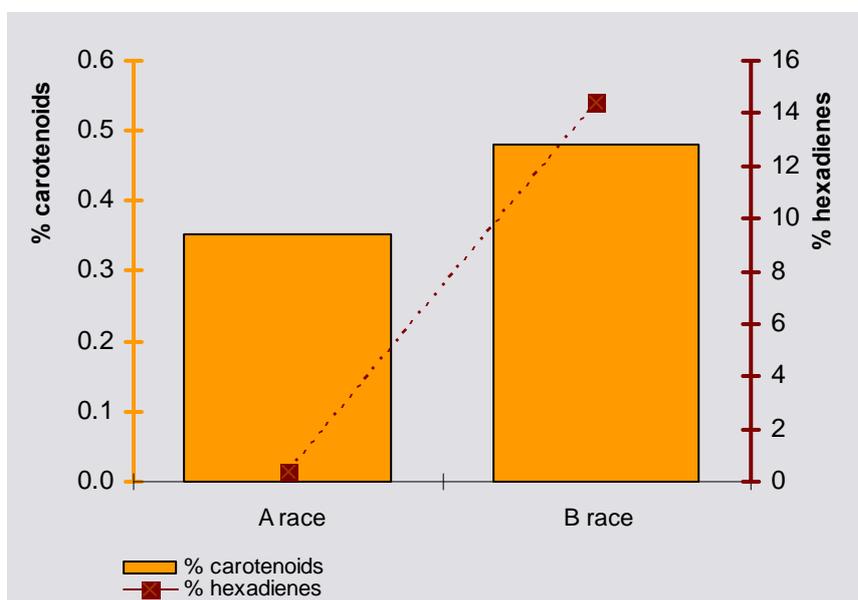
Fig. 21. The increasing tendencies based on the average percentages of the carotenoids and hexadienes fractions, identified in the lipid extracts of the AICB strains, belonging to the A and B chemical races. The percentages are grams per 100 grams dray biomass.
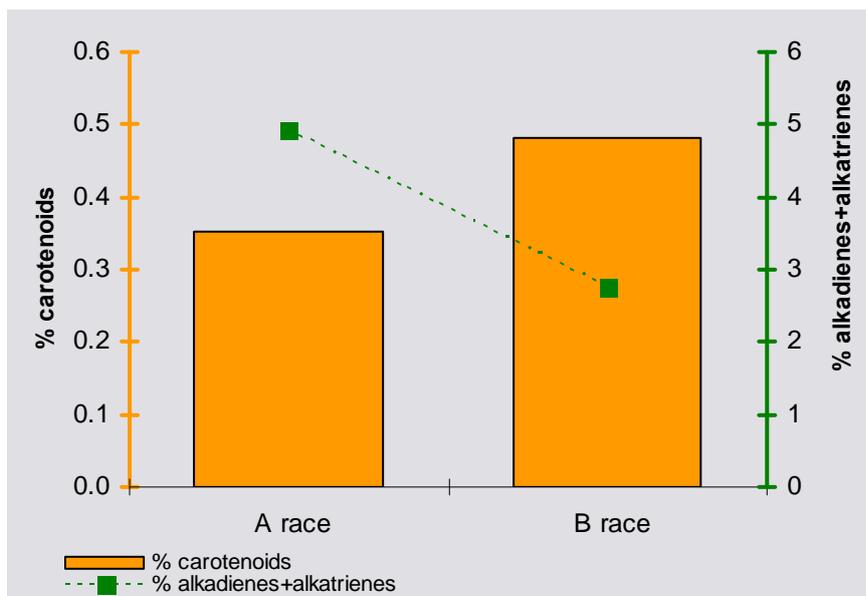
Fig. 22. The increasing tendencies based on the average percentages of the carotenoids and alkadienes and alkatrienes fractions, identified in the lipid extracts of the AICB strains, belonging to the A and B chemical races. The percentages are grams per 100 grams dray biomass.

4.6   Phylogenetic position of the AICB strains based on rRNA genes

17 strains belonging to AICB Culture Collection from A and B chemical races were investigated based on the sequence length of the 18S rRNA gene. Among these, 5 of them belonged to the B race and 12 of them to the A race.

Based on the sequence length, the strains separated into two categories. Thus, all the B strains and 4 strains from A race possessed an approximately 2000 bp fragment, meanwhile 8 B strains presented an approximately 2500 bp (fig. 23).
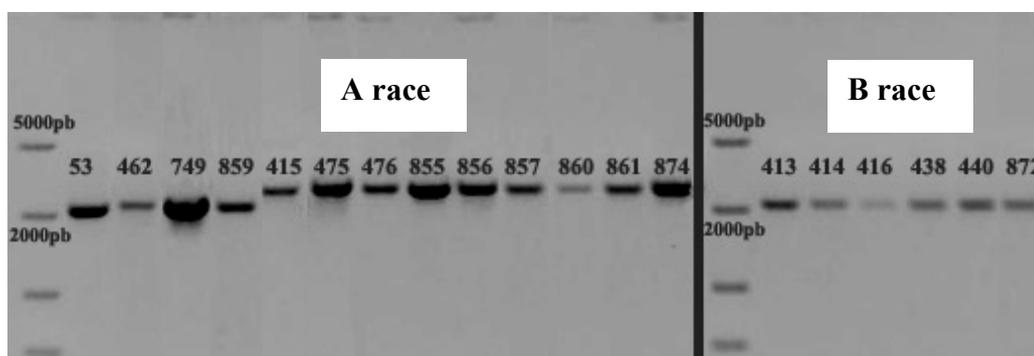
Fig. 23. The electrophoresis pattern of the 18S rRNA obtained by PCR, with the CV1, ITS2B pair primers.

The multiple sequence alignment of the sequences obtained in this study and those derived from data bases deposited in the public nucleotide data bases showed the presence of an intron sequence in 12 AICB strains. This region represented 457-461 bp (fig. 24). The sporadic presence of this region was found in other green algae and it was classified in the IC1 intron category.
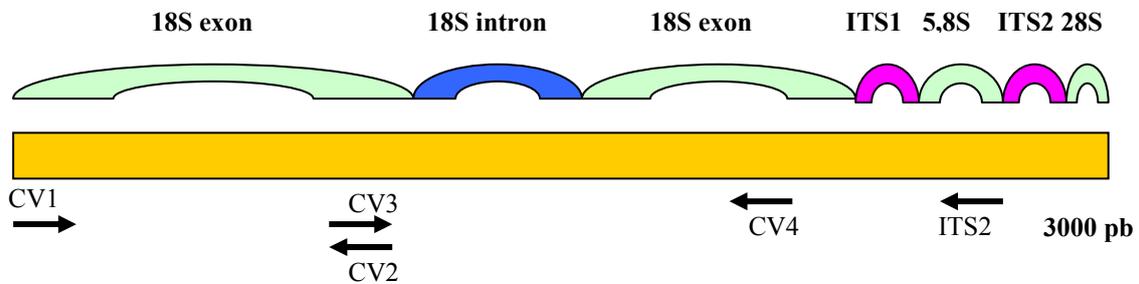
Fig. 24. The position of the five primers used for sequencing the 18S genes derived form the AICB strain, the genes succession, the exons and introns, also.

Regardless of the presence/absence of the intron region, the strains belonging to the A races gathered distinctively against the B race (meaningless of the Culture Collection), fact assured by a significant Boostrap value (fig. 25, 26). This fact is shown in all the constructed trees, regardless of the used algorithm. Based on this considerate we asserted the fact that the classification of the A and B strains is certified at a molecular level, based on the 18S rDNA marker.

In the obtained trees, the A strains grouped distinctively, depending on the intron presence or absence, with the exception of AICB 860 strain. Although, the differences between the 18S rDNA sequences derived from AICB A strains goes beyond the presence or the absence of this region. The removal of the intron fragment generated almost identical trees. Subsequently analyses, most probably sequencing, in the case of AICB 860 strain will certify the presumption that the two A strains cluster of sequences are in fact two different species or at least subspecies.
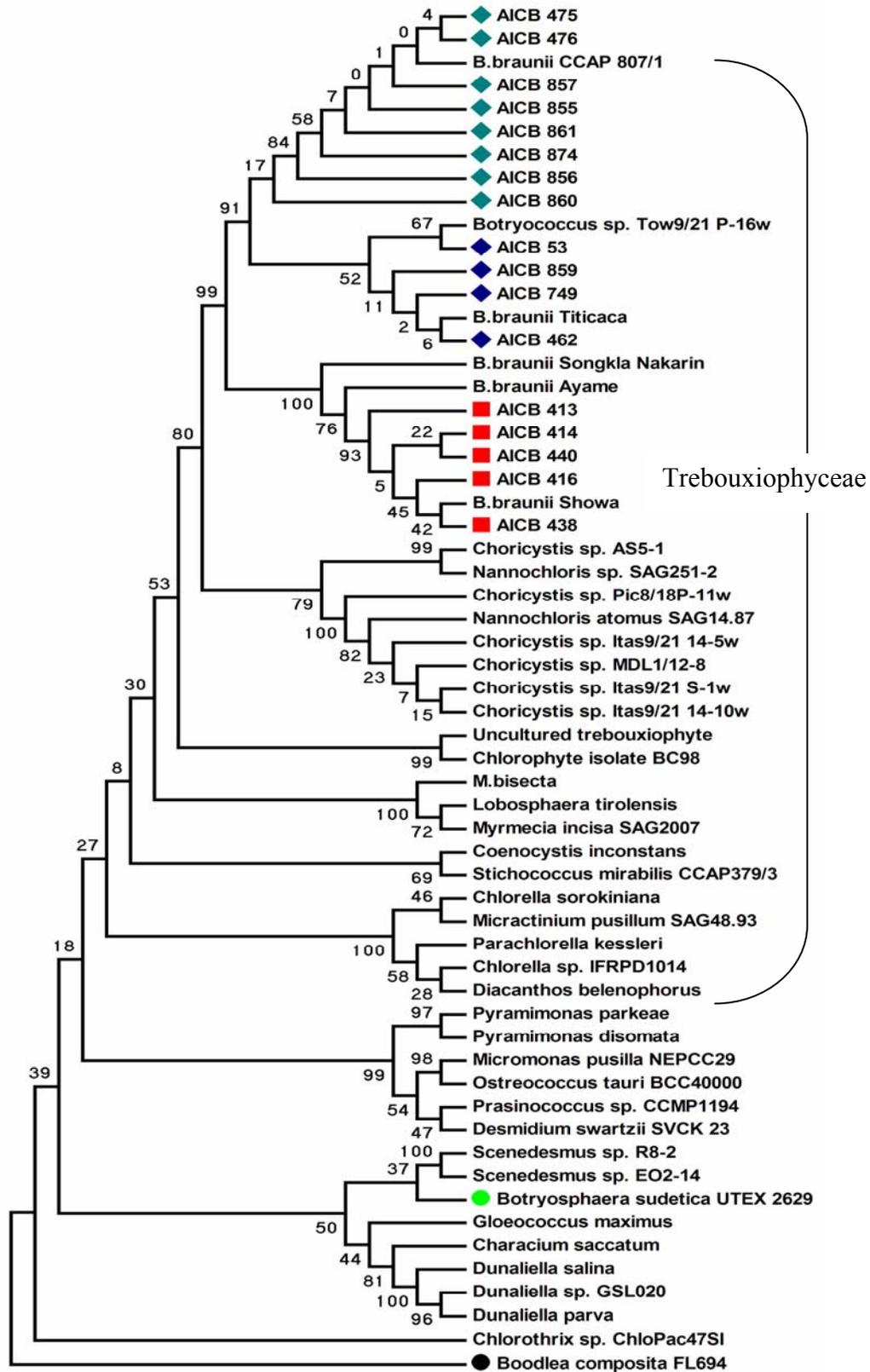
Fig. 25. The phylogenetic tree based on the 18S rDNA genes derived from the AICB strains and 42 sequences extracted from the GenBank (NCBI), constructed through the Maximum Parsimony (MP) algorithm. The values of the Boostrap significance test (100 replicates) are noted on the branches.

Fig. 26. The phylogenetic tree based on the 18S rDNA genes derived from the AICB strains and 42 sequences extracted from the GenBank (NCBI), constructed through the Minimum Evolution (ME) algorithm. The values of the Boostrap significance test (100 replicates) are noted on the branches.

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5. Final conclusions

✓ The investigations of morphological features of the 30 AICB strains identified some features (the aspect and colonies consistency, presence/absence of singular cells in cell suspensions, the degree of extracellular matrix development, etc.) which offer a morphological prove for classification of the *B. braunii* strains in the two chemical races.

✓ The biochemical analyses related to hydrocarbons, fatty acids and carotenoids identification and quantification showed major differences that serve as criteria for differentiating the A strains against highly hydrocarbon producing B strains. In the last ones, a major content of carotenoids was observed (responsible of brown-orange colour) and also of hydrocarbons and among those the dominant presence of botryococcenes. The capacity of the B strains to synthesize alkadienes and alkatrienes sustained the involvement of these categories of chemical compounds in the algaenan. The dominant presence of the oleic and palmitic fatty acids, especially in the extracts provided from the A strains, made them the most probably precursors of alkadienes and alkatrienes.

✓ The growth experiments corroborated with the hydrocarbon and fatty acid content showed a high degree of variability of the *B. braunii* strains, regardless of the chemical race. The CO_2 administration determined the increasing of the growth rate of the cell division and the reduction of the doubling time in almost all the AICB strains.

✓ In the phylogenetic trees based on the 18S rDNA the *B. braunii* strains, all the AICB sequences formed a tide cluster in the Trebouxiophyceae class. The classification of the strains in two different groups showed for the first time the existence of at least two different species, offering a genetic support in the chemical classification of the strains (A and B races).The presence of the intron sequence in the 18S rDNA gene in a significant number of A strains which could be sustained by adjacent molecular markers could certify a third *B. braunii* species.

✓ Among the investigated strains, the AICB 416 and 418, B race could represent a premise for future studies in the sense of development for new technologies for obtaining the hydrocarbons.

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