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Modeling biochemical composition of thermo tolerant *Cosmarium* strain isolated from Tunisian Geothermal water, as function of temperature and light intensity.

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Abstract

A thermotolerant Desmid was isolated from Tunisian geothermal spring (Ain-Echfa) in the region of Korbous northeast of Tunisia. Under light microscope, this alga was identified as belonging to the genus *Cosmarium*. Combined effect of light intensity and temperature on biochemical composition (carbohydrate, lipid and protein) of this strain was evaluated using a 3^2 factorial design. This design was carried out with light levels of 20, 70, and 120 µmol photons m^{-2} s^{-1} and temperature of 15, 30 and 45 °C. Our findings revealed that, at the stationary stage, the major compound of *Cosmarium sp* was the carbohydrate with a maximum of 81.8%. Statistical analysis demonstrated that biochemical composition of this strain was mainly influenced by light intensity. The experimental design showed that, the highest levels of protein, carbohydrate and lipid were obtained at the lowest light intensity (20 µmol photons m^{-2} s^{-1}).
Keywords: *Cosmarium;* light; temperature; biochemical composition; modeling.

Introduction

In recent years, microalgae are found to contain large quantities of valuable products, including chemicals, pharmaceuticals and nutraceuticals. Much attention has been attracted to microalgae living in extreme environments (habitats characterized by extreme levels of temperature, salinity, pH or mineral deficiency or excess). Thermophilic microalgae are of particular interest to biologists for both biochemical and physiological characteristics as they are able to produce a wide range of active substances in response to the ecological pressure **(Antonio Flores-Moya et al. 2004)**.

The genus *Cosmarium* is unicellular freshwater algae belonging to Placoderm desmids. **(Felisberto and Rodrigues 2004)**.Desmids represent a group of advanced green algae that are commonly found in biofilm communities of freshwater wetlands. They are coccoid and have a striking morphology characterized by two symmetrical halves (semi cells). They comprise both solitary and colonial taxa **(Coesel and Krienitz 2008)**. Desmids are now gaining importance because of their use as tool of bio-indicators and bioremediation by decolorizing a wide range of dyes **(Coesel 1983, 2001; Ngearapat and Peerapornpis 2007; Krasznai et al. 2008)**. As a member of the desmids, the *Cosmarium* species were investigated as a viable biomaterial for biological treatment of triphenylmethane dye and Malachite Green (MG) **(Daneshvar et al. 2006)**.

Other works have demonstrated the ability of *Cosmarium* species to produce significant amounts of extracellular polymeric substances (EPS) that form an extensive sheath on the external cell wall; and function in adhesion and ensheathment within the biofilm complex. EPS desmids contained significant uronic acid (3 to 29%), protein content (2 to 10%) and polysaccharides sulfated to various degrees **(Kiemle et al. 2007; Challouf et al. 2012)**.

Cosmarium species were found inhabit environment with high temperature, in Tunisian geothermal sources. There are more than 70 hot springs across the country, mainly in the north-east (N-E), north-west (N-W) and south **(Ben Dhia and Meddeb 1990).** Ain-echfa Spa (Korbous) was known as thermal spring in Tunisia used for curative purposes, mainly as a remedy for skin conditions and rheumatism. It was characterized by sodic chloride hyperthermal waters which host a planktonic community, mainly composed of cyanobacteria and microalgae **(Ghozzi et al. 2013).** The survival of eukaryotic algae such as Desmids in Ain-echfa Spa might be due to development of adaptive mechanisms conferring tolerance to such adverse conditions.

Very little is known about desmids living in such environment, and especially about their ecophysiological constants and their ability to produce biocompounds. Taking in consideration the lack of studies dealing with biochemical composition of thermophilic desmids, a first investigation of *Cosmarium* strain isolated from Tunisian geothermal waters was realized. The objective of this investigation after isolation of *Cosmarium* strain was to evaluate the combined effects of light intensity and temperature on the biochemical composition (carbohydrate, lipids and proteins) of this strain in order to understand the adaptation of *Cosmarium* to warm temperatures and for further possibility for economic usage.

Materials and methods

Microalgae and growth conditions

Cosmarium strain was isolated from geothermal source Ain Echfa localized in the region of korbous, N-E of Tunisia (36° 49' N, 10° 34' E). A purified strain was identified as *Cosmarium sp*., based on microscopic morphological traits (**Prescott et al. 1978, Croasdale and Flint 1988, Brock 2002, Felisberto and Rodrigues 2004).** *Cosmarium* was grown in standard flasks containing a Bold's growth medium **(Bischoff and Bold 1963).** Cultivation was carried out in sterilized photo-bioreactors (5 l) and equipped with a device for aseptic removal of samples. Illumination was provided by fluorescent tubes to give constant light intensities of 20, 70, or 120 µmol photons m^{-2} s^{-1}. Each culture was inoculated with an initial biomass concentration of 0.15 mg ml^{-1}. Cultures were stirred continuously with air at a constant flow rate (0.1 v/v/min).

Biochemical characterization

The biochemical composition was carried out using freeze-dried powder of *Cosmarium* sp The microalgae biomass was harvested at the end of stationary growth stage (21 days) for different conditions of temperature and light intensity as described in table 1.

Table 1. Protein, Carbohydrate and lipid percentage of *Cosmarium sp* for various culture conditions of temperature and light intensity.

Experiments	Factors		Responses		
	Temperature °C	Light intensity (µmol photons m^{-2} s^{-1})	Protein % dry weight	Carbohydrate % dry weight	Lipid % dry weight
1	15	20	20.53	72.12	16.2
2	30	20	19.8	84.21	18.1
3	45	20	19.16	69.4	19.16
4	15	70	11.46	41.48	8.2
5	30	70	13.9	50.39	10.6
6	45	70	8.79	40	9.6
7	15	120	3.92	68.79	7.6
8	30	120	9.32	69.7	9.4
9	45	120	8.00	56.55	12.5
10	**30**	**70**	**15.7**	**45.15**	**5.79**
11	**30**	**70**	**17.70**	**44.84**	**7.32**

Bold values correspond to the central point repeated two times

Total carbohydrate analysis

Total carbohydrate content of freeze-dried *Cosmarium sp* was determined spectrophotometrically using a phenol–sulfuric acid method **(Dubois et al. 1956)**, and standardized with glucose. After color development, the absorbance read immediately at 485 nm with a spectrophotometer (Beckman Coulter DU.640B).

Protein analysis

The Total protein content of 5 mg of freeze-dried algae was determined spectrophotometrically using a Pierce test kit Bicinchoninic Acid (BCA1 and B 9643) based on the procedure of Lowry et al. **(1951)** and Kreeger et al. **(1997)** and standardized with bovine serum albumin (Sigma, USA). The protein extraction was realized using the Rausch method **(Rausch 1981).**

Lipid analysis

The lipid content was estimated using chloroform/methanol mixture as described by **Rezanka et al. (2003)**; briefly: to 130 mg of sample, 50 mL of MeOH-H_2O-HCl (30:3:1, V/V/W) mixture was added and then incubated at 55 °C for 6 h. After cooling to 10 °C, 150 mL of cold pentane-H_2O (1: 2, V/V) mixture was added. The mixture was stirred for 1 h and then filtered. The chloroform-methanol mixture was evaporated using a rotavapor and then the glass tube containing lipids were weighed to calculate the weight of the lipid present in the sample. The rotavapor was used to evaporate the solvent from the lipid mixture. The glass tube and lipid were then weighed and the amount of lipid was calculated. Biochemical composition was calculated as a percentage of total algal dry weight from the absolute concentrations for each biochemical component

Experimental design

Two factors were examined (light intensity and Temperature) at three levels as listed in Table 1. The levels of each factor were chosen based on the available literature. To evaluate the effect of the two factors on biochemical composition (Protein, Lipid and carbohydrate), a quadratic full factorial design was used. A total of 11 experiments (nine points of the factorial design and two center points to establish the experimental errors) were carried out in randomized run order. Using this design, both factors were tested at three different levels: light intensity at 20 µmol photons m^{-2} s^{-1} (level -1), 70 µmol photons m^{-2} s^{-1} (level 0), and 120 µmol photons m^{-2} s^{-1} (level 1) and temperature at 15 °C (level -1), 30 °C (level 0), and 45 °C (level 1). The response variables selected were the protein, lipid and carbohydrate concentrations. The parameters of the model were estimated by multiple linear regression using program MODDE 7.0, a software for experimental design and optimization (Umetrics AB,Umeå, Sweden). The accuracy of the model fit was evaluated by the explained variation (R^2 adjusted) and the model validity (lack of fit).

Results

In this work, the effects of light intensity and temperature variation on carbohydrates, lipids and proteins levels were investigated using response surface methodology (RSM). Results are summarized in Table 1. Analysis of the results was carried out using MODDE. 7.0. The effect of each factor and their interaction was obtained by ANOVA with a confidence interval of 90%.

Results of the ANOVA are reported in Table 2 and indicated that the models developed for carbohydrates and lipids percentages had a good R^2 (>0.85). Moreover the lack-of-fit of each model was positive and higher than 0.05. This indicated that the models had good predictive ability. To interpret these models, we had to display the scaled and centered coefficients and the factor effect plots. For the carbohydrate model the P-values indicated that except for linear term of temperature and interaction term of light and temperature all other terms had significant effects ($P < 0.1$). Concerning the lipids model the temperature has only a significant linear effect; however, light has significant linear and quadratic effects. The interaction term was not significant. For proteins responses, ANOVA results indicated that polynomial model appeared adequate without any lack of fit and had an acceptable R^2 (0.82). The P-values showed that only linear effect of light and quadratic term of temperature were significant while the temperature linear term, interaction term (Temperature*light) and the light quadratic term were insignificant.

Table 2. R^2(adjusted) and lack of fit of the polynomial models and coefficients (scaled and centered) and P values of both linear and quadratic effects of light intensity and temperature and their interaction for carbohydrate, protein and lipid

	Carbohydrate (%)		**Protein (%)**		**Lipid (%)**	
R^2 adj	0.866		0.828		0.924	
Lack of fit	0.915		0.378		0.629	
	Coeff. SC	**P-value**	**Coeff. SC**	**P-value**	**Coeff. SC**	**P-value**
Constant	49.863	9.82E-06	14.838	5.753E-05	7.609	8.358E-05
Light	-4.224	0.0566	-4.975	0.0010	-3.162	0.0005
Temperature	-2.263	0.2431	0.005	0.9945	1.222	0.0293
Light*light	15.879	0.0005	0.495	0.5936	3.288	0.0010
Temp*Temp	-5.676	0.0387	-1.990	0.0709	0.587	0.277
Light*Temp	-1.523	0.3911	0.823	0.2878	0.297	0.4731

Underlined terms are not significant

Analysis of the coefficients list (table 2) of the models indicated that the quadratic term of light was the main effect and influence positively the carbohydrate levels. Nevertheless, all other terms had a slight negative influence. For lipids, the linear term of light had a negative effect while quadratic term of light influence positively the model. As for the protein the linear term of light intensity had a negative effect. The quadratic term of temperature slight influenced negatively the proteins.

Analysis of the predicted data by response surface plots revealed that the maximum carbohydrate percentage (81.8%) was achieved at the lowest light level (20 µmol photons m^{-2} s^{-1}) and a temperature of 30 °C (Figure. 1). As shown in figure 2 the maximum protein level was observed at the lowest light intensity (20 µmol photons m^{-2} s^{-1}) and a temperature of 26 °C. The maximum lipid level (19 %) was achieved at the highest temperature (45 °C) and lowest light intensity (20 µmol photons m^{-2} s^{-1}) as observed in figure 3.

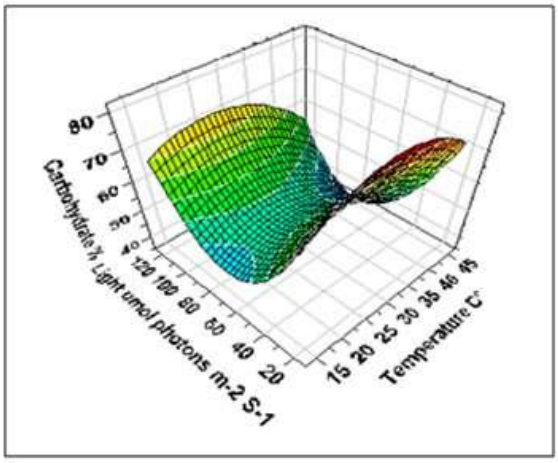

Figure 1. Response surface plot vs light intensity and temperature for carbohydrates

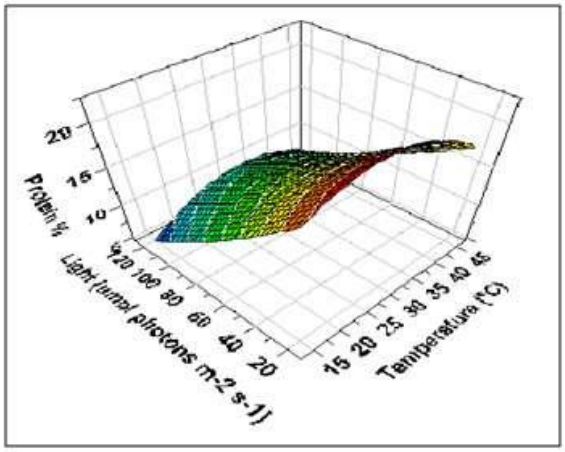

Figure 2. Response surface plot vs light intensity and temperature for proteins

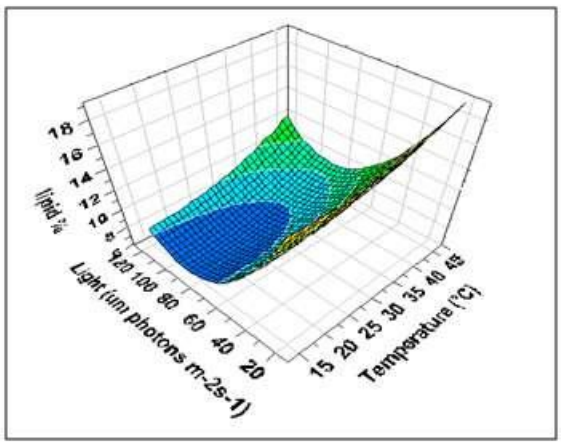

Figure 3. Response surface plot vs light intensity and temperature for lipids

Discussion

The survival of *Cosmarium sp in* the hyperthermal waters from Ain Echfa Spa might be attributed to the resistance of this specie to the elevated temperature. According to **Ben Dhiab et al. (2012)**, the maximal growth and photosynthetic activity of this specie were maintained at 20 °C. Nevertheless, it was acclimated and withstood under 60 °C by down-regulating electron transport at both donor and acceptor sides of PSII. Otherwise, the conversion of some active reaction centers of PSII into inactive form provides a protective mechanism for quenching excessive energy. To withstand and grow under harsh environments or extreme conditions, several adaptive mechanisms might be developed. These mechanisms were accompanied with their ability to adjust their photosynthetic apparatus which affect their biochemical composition in order to acclimate to the prevailing environmental conditions.

Lipids, proteins and carbohydrate are the most important biochemical components in algal biomass **(Boechat and Giani 1999).** Nevertheless, biochemical composition in alga can change with light, temperature, and growth stage. Variation in biochemical composition due to growth stage is frequently related to culture age and nutrient depletion, particularly if an organism is grown in batch culture **(Harrison et al. 1977; Morris et al. 1983).** The biochemical composition of *Cosmarium sp* strain in stationary stage, under different conditions of temperature and light intensity showed that the carbohydrate was the major compound. The increased amount of carbohydrates may also appear as a consequence of increased mucilaginous sheaths around cells in the stationary phase. Desmids may develop large sheaths in oligotrophic environments which may enable them to accumulate nutrients **(Coesel and Wardenaar 1990, 1994)**; the same happens with desmids in the stationary phase when they are depleted of nutrients (**Stamenkovic and Hanelt 2011**).

The carbohydrate content decreased when the light intensity was increased from 20 to 70 µmol photons m^{-2} s^{-1} then increase to reach 70 % at light intensity of 120 µmol photons m^{-2} s^{-1}. Similar results were observed for *Scenedesmus abundans* by **Tahiri et al. (2000).** Other results reported by **Shih-Hsin Ho et al. (2012)** showed that the amount of carbohydrate increased significantly from 53.44% to 73.12% as light intensity rose from 60 to 180 µmol photons m^{-2} s^{-1}. According to **Stamenkovic and Hanelt (2011)** the *Cosmarium* strains can be categorized as algae adapted to rather high light intensities.
The decreased amount of carbohydrates at elevated temperatures may be explained by the considerable increase of dark respiration and photorespiration. In addition, respiration is small in cool conditions and often large accumulations of carbohydrate result from continued photosynthesis in cold. With respect to protein, it is known that temperature influences growth of microalgae primarily via control of enzyme kinetics**.** Regarding irradiance, light limitation induces a deficiency in the carbon assimilated thus directing the assimilated carbon skeletons for pigment synthesis rather than for amino acid synthesis **(Ana et al. 2009).**

In our study, proteins were found to be minimum than the other biochemical parameters. The low proteins levels can be explained by the exhaustion of media nutrients in this stationary phase. This was confirmed by **Ogbonna and Tanaka (1996); Zhu et al. (1997); Lourenco et al. (1997)** typically, algal cultures become depleted in nutrients, as they enter stationary stages of growth. As known that the optimum temperature for majority of desmids is in the range 25-30 °C **(Coesel and Wardenaar 1990)**, which can explain the highest values of proteins within this range. Hence, from the combined effects of irradiance and temperature, the lowest amount of protein was obtained in the experiment performed at 15 °C and 120 µmol photons m^{-2} s^{-1}, which is contrasting with the results obtained for the microalgae *Pavlova lutheri* by Ana et al. **(2009).** Results reported by **Morris et al. (1974)** in a study on the marine diatom, *Phaeodactylum tricornutum*, showed that a low light (400 lux at the culture surface) led to an increase in the rate of protein synthesis.

Temperature is also reported to impact protein content in the algal cell. Rhee and Gotham **(1981)** observed an increase in protein concentration in *Scenedesmus sp*. with decreasing temperature. Similarly, **Morris et al. (1974)** reported a considerable increase in protein synthesis rates with lower the temperatures, for the marine diatom *Phaeodactylum tricornutum*. Therefore, we have to take into account that possible changes of assimilation and/or respiration rates during the temperature-irradiance experiments may influence the composition of proteins amounts.
Changes in lipids levels also have been observed as a function of temperature and light intensity. Lipids percentages present a maximum at highest temperature which was in concordance with the results obtained for *Scenedesmus abundans* by **Tahiri et al. (2000).** Increasing the growth temperature from 20 to 25 °C doubled the lipid content (from 7.90 % to 14.92 %) in *Nannochloropsis oculata*. However, the increase of temperature from 25 to 30 °C decreased the lipid content in *Chlorella vulgaris* from 14.71 % to 5.90 % **(Converti et al. 2009)**. According to **Ankita et al. (2013),** Light intensity also affects the cellular composition of algae. *Dunaliela tertiolecta* exhibits an increase in the lipid fraction with increasing light intensities up to saturation. A further increase in light intensity did not encourage the synthesis of lipids of our strain. This was in contrast with the results obtained by **Benjamas et al. (2012)** for different green algae. Absence of light was observed to increase the total lipid content of the *Dunaliela. virdis* but reduce triglycerides, free fatty acids, free alcohols and sterols.

Conclusion

The biochemical composition of the *Cosmarium sp* strain can be manipulated readily by changing the growth conditions**.** Our findings showed that the carbohydrate was the major compound that's explaining the orientation towards reserve synthesis. The current study provides the first assessment of an experimental design modeling the combined effect of light intensity and temperature on biochemical composition of *Cosmarium sp* strain. The results reports that biochemical composition (carbohydrate, lipid, protein) were mainly influenced by light intensity. The experimental design showed that, the maximum carbohydrates protein and lipids levels were obtained at low light intensity (20 µmol photons m^{-2} s^{-1}). Only lipids percentage was maximum at the highest temperature (45 °C), carbohydrates and protein reach their maximum at optimum temperature of 26 and 30 °C.

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