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Application of Response Surface Methodology (RSM) for Culture Conditions and Biomass Production of Psychrophilic Microalgae Isolated from High Mountains Lake During the ice-free Season

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ABSTRACT: Many studies on cold environments have been developed over the past two decades. High mountain freshwater presents high variability of nutrients and chemico-physical parameters, showing variations of pH, oxygen concentration, metals and temperature throughout the year. National Park of *Sierra Nevada* (Granada, Spain) (37°032 N 03°182 W), has almost 40 lakes that are reported to be both endemic and oligotrophic. However, very little information about their microbial diversity can be found in literature. In this work, a Response Surface Methodology (RSM) was used to find best nutritional conditions for the isolation of psychrophilic microalgae from *La Caldera* Lake. The results showed that best culture medium, was the Rodriguez-Lopez medium (RL); data were adjusted to a quadratic prediction model reporting a biomass concentration over 600 mg/L at 10 and 20ºC. In this paper, the diversity of culturable freshwater microalgae in the *La Caldera* Lake was observed by PCR using specific primers for eukaryotic 18S rRNA genes. Samples were taken in early July and late Agust, 2011. In July presence of strains belonging to the *Eustigmatophyceae*, *Bacillariophyceae*, *Trebouxiophyceae*, *Chlorophyceae* and *Scenedesmaceae* families were found. In August, only microalgae from the *Eustigmatophyceae, Trebouxiophyceae, Chlorophyceae and Scenedesmaceae* families were found. An individual culture of each isolated strain was carried out. Microalgae S21 had phylogenetic similitude with *Chlorophyceae*, and showed best growth being biomass concentration in RL 393.73 mg/L and 128.52 mg/L at 20ºC and 10ºC, respectively. Moreover, specific growth rates (μ_{max}), 0.25/h and 0.13/h at 20ºC and 10ºC, respectively, were detected for strain S21.

Key words: Biomass, Micro-Algae, Lake, Environment

INTRODUCTION

The National Park of *Sierra Nevada* (Spain) is located in the Iberian Peninsula (37°032 N 03°182 W) and is the most meridional mountain system of Europe. This range is characteristic for its filling and elongated morphology which includes more than 20 peaks at 3000 metres a.s.l., with Mulhacen being the highest peak of the Iberian Peninsula. There are many small lakes (lagoons) in the whole area of the range, at different heights. The lagoons in the *Sierra Nevada* National Park (Spain) are the result of quaternary glaciations. The majority of studies based on aquatic ecosystems in the *Sierra Nevada* Lakes have used an ecological approach, considering algae as primary producers as a whole (Fanés *et al*., 2009). Several microalgae have been described in numerous aquatic matrices of the Natural Park of *Sierra Nevada*. Thus, in *La Caldera* Lake*,* studies have been performed on microalgal diversity, including those made by Martínez-Silvestre (1977) identifying species such as *Scenedesmus ecornis* and *Tetrahedron minimum;* this last microalga also has been studied by Sánchez-Castillo (1986), who also identified other species of microalgae such as *Oocystislacustris*, *Scotiella tuberculata* (Sánchez

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Castillo, 1986) and *Scenedesmus armatus* (Sánchez Castillo, 1988). Most of these lakes are oligotrophic and endemic systems (Gibson *et al.*, 1995). However, there are some physical differences among them, such as size, depth, and location in the range. In this context, *La Caldera* Lake is the deepest and biggest, followed by *Las Yeguas* Lake. However, little information about the communities and microalgae diversity in these lake systems is available in the scientific literature (Castillo *et al*., 2005; Reboleiro-Rivas *et al*., 2013). Many microalgae have been reported as organisms that showing high ability to adapt to extreme environments (Takeuchi *and* Kohshima, 2004). One example is *Chlamydomonas nivalis* (Stibal *et al*., 2007); this microalga (Phylum Chlorophyta) is able to grow over the snow, giving a pink colour to the surface in high mountains. Other microalgae with this ability are *Ancylonema nordenskioeldii* and *Mesotaenium berggrenii,* both from the phylum Charophyta; these were found in almost all studied glaciers by Takeuchi *and* Kohshima (2004).

One of the most influential factors over the microbial biodiversity in natural system is seasonal rotation. Several authors have shown the influence of weather and season on population dynamics (Yannarell *et al*.; 2003; Pesciaroli *et al*.; 2012; Reboleiro-Rivas *et al*., 2013). The same occurs in high mountain freshwater systems (Pernthaler *et al.,* 1998), where changes in temperature and therefore the concentration of nutrients are factors that are correlated with seasonal weather changes (Liu *et al.,* 2013). These authors describe the effect of temperature and concentration of nutrients in the phytoplanktonic community in the oligotrophic lake Namco (Tibet), concluding that there are three predominant bacterial groups that have dominant peak abundance, such as Actinobacteria in January, Cyanobacteria in May and Beta-proteobacteria in June. Similar results were obtained by Piwosz *and* Pernthaler (2010), who showed that Cyanobacteria and Diatoms are dominant in spring and summer, decreasing as summer progresses. On the other hand, same authors showed that microalgae from the group Cryptophyceae appear in late summer only. The same occurs with the group Chlorophyta, which are abundant in later summer (Ortega-Mayagoitia *and* Rojo, 2000). Psychrophilic and oligotrophic environments, such as those of high mountain lakes, are definitely special habitats, where organisms must have the ability to thrive at very low concentrations of nutrients and their metabolism must function at low temperatures. Thus, isolation of these organisms must involve reproduction of same, or at least similar, conditions of growth. In this context, evaluation of different media is necessary to find best growth conditions (Lananan *et al*., 2013). Many researchers have compared the effects of different culture media on microalgae growth and optimal media were reported according to the objectives (Berges *et al*., 2001; Barsanti *and* Gualtieri, 2006; Martínez-Córdova *et al*., 2012). Growth optimization must be carried out at optimal temperatures; thus, temperature growth limits of psychrophilic and/or psychrotrophic organisms must be established (Pesciaroli *et al*., 2012).

Main goal of this paper was defining best culture conditions for the growth of psycrophylic microalgae isolated from a Sierra Nevada Lake (*La Caldera*) during seasonal rotation, by RSM. Moreover, taxonomic identification of cultivable microalgae, that can thrive in that oligotrophic habitat, was performed and phylogenetic relationships among the various isolates were established.

MATERIALS & METHODS

Water samples were taken from *La Caldera* Lake during the ice-free periods at July 2 and August 29, 2011. *La Caldera* Lake (37° 3' 16.17" N3° 19' 54.08" W) is located at 3050 m a.s.l. in the National Park of *Sierra Nevada* (Granada, Spain). *La Caldera* has an extension of 30,000 square metres. Two sampling points were selected; one located in the central area of the lake and another five metres offshore. Surface water samples were collected in a sterile drum and the cold chain was interrupted only at the time of analysis at the laboratory; physical-chemical parameters were measured *in-situ* (Reboleriro-Rivas *et al.*, 2013). Two microalgae strains were used as controls to evaluate the effect of the culture medium on growth and biomass production. The psychrophylic microalgae, strain SX1 previously isolated from olive oil washing water (unpublished data)has an optimum growth at10±2ºC and presented phylogenetic similarity of 99% with *Chlorella vulgaris*, according to the National Center for Biotechnology Information Database. *Scenedesmus obliquus* strain CCAP 276/3A, obtained from Culture Collection of Algae and Protozoa (Oban, UK), was used as a mesophilic control, and presented an optimum growth of 25±2ºC. Two culture media for microalgae were selected and used in our study: RL medium described by Rodríguez-López (1964) and F1 medium described by Guillard *and* Ryther (1962), later modified by Stein (1973), and Guillard (1975) (Table 1). Solid media were obtained by the addition of 2% of Bacto-Agar and pH was adjusted to 7.2 with 0.1M KOH. Growth rate and biomass production was determined in liquid medium by spectrophotometric measurements at 560 nm every 12 h, according to Martínez *et al*. (2000). The specific growth rate was calculated by the equation proposed by Thompson *et al*. (1989).

$\mu_{max} = Ln\ (N_1 - N_0) / T_1 - T_0$

Table 1. Nutrient concentration (mg/L) of tested media culture

Nutrient	Rodriguez-Lopez (RL)	F-Guillard (F1)
Macro-Nutrients		
KNO_3	10.111	-
$NaNO_3$	-	85.01
$NaH_2PO_4·2H_2O$	0.780	-
$Na_2HPO_4·12H_2O$	16.310	-
K_2HPO_4	-	8.71
$MgSO_4·7H_2O$	24.65	36.97
$NaHCO_3$	-	12.60
$CaCl_2·2H_2O$	147	-
$CaCl_2·H_2O$	-	36.76
$Na_2SiO_3*9H_2O$	-	28.42
Micro-Nutrients		
$FeCl_3*6H_2O$	-	3.15
$FeSO_4·7H_2O$	7	-
Na_2EDTA	-	4.36
EDTA	9.3	-
$MnSO_4·H_2O$	0.170	-
$MnCl_2*4H_2O$	-	0.18
$ZnSO_4·7H_2O$	0.29	0.022
$CuSO_4·5H_2O$	0.25	0.01
$CoCl_2*6H_2O$	-	0.01
$Na_2MoO_4*2H_2O$	-	0.006
Thiamin	-	0.1
Biotin	-	0.5
Cyanocobalamin	-	0.5

Where,
N_1,N_0 = Biomass (final and initial)
T_1, T_0= Time (final and initial)

Microalgae growth studies for biomass production at 10±2ºC and 15±2 °C were performed in a cold Zanotti Electronic chamber without agitation. The 20±2°C assays were performed on a photosynthetic adapted laboratory with an air conditioning system that maintained constant temperature during the assay. Cultures were performed in the absence of agitation, and each Erlenmeyer flask containing microalgae growth medium was coupled to an aeration system for constant CO_2 supply (12.56 mg/L). As above mentioned, a lighting system (758.8μmol) was used for cycles of night/day with intervals of 12/12 h. One litre Erlenmeyer flasks containing 500 mL of sample were supplemented with nutrients according to Table 1 and used at 50% dilutions. Samples were incubated for 3 months at 10, 15 and 20ºC as described above. Every 7 days, 1 ml samples were serially diluted (1/10, 1/100 and 1/1000) and spread on solid media at a 50% dilution of nutrient concentrations, and then incubated on a white surface upon which light was constantly irradiated with a power of 758.8mol for 7 days at 10, 15 and 20ºC; this was continued until individual colonies were identified as single, morphologically well-formed colonies. Isolated representatives of the dominant colonies were spread on plates containing RL and F1 medium at a 100% nutrient concentration. All of the selected colonies were purified by restreaking methods. All experiments were carried out in triplicate.

For the extraction of DNA, microalgae cells grown in solid medium were washed 3 times in sterile saline solution (3% NaCl) to remove polysaccharides that may interfere with the extraction process. Approximately 0.5 mg of washed cells were resuspended in 1.5 ml of DNA extraction buffer (PVPP 10% y proteinase K0.5 mg/mL); the samples were incubated at 56ºC in a hotplate BIOER Mixing MB-102 for 4 hours. Following centrifugation, 2 layers were separated and the aqueous phase was pipetted off into new, labelled Eppendorf tubes. Ice-cold isopropanol was added. The samples were incubated for 15 min at -20°C and centrifuged for 10 min. The liquid phase was discarded. Cold diluted Ethanol (70%) was added and centrifuged for 10 min. The liquid phase was pipetted off and the pellet was dried. The samples were resuspended with 50μL of TE buffer (10 mMTris, 0.1 mM EDTA) and kept in a refrigerator. Reactions were run using 5 μL of the DNA at concentration of 0.1%. The nuclear-encoded 18S rRNA genes were amplified by PCR by using a set of primers specific for eukaryotic 18SrRNAgenes,EukA (AACCTGGTTGATCCTGCCAGT) and EukB (TGATCCTTCTGCAGGTTCACCTAC)

(Sigma-Genosys, UK) (Díez *et al*., 2001). The nucleotide sequence of the purified bands was determined by the dideoxy chain terminator method, using Kit ABI-PRISM Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Germany) and an Automatic Sequenciator 3100 Avant Genetic Analyser (Applied Biosystems, Germany). The information obtained by the sequencing was analysed by Chromas v.1.51 and using the database from the European Bioinformatics Institute (http://www.ebi.ac.uk). The obtained sequence results were compared with the information in the EMBL and GenBank databases.

Pure cultures of each isolated microalgae were carried out in RL liquid medium in order to obtain their specific growth rates and optimal conditions of growth at different temperatures (10ºC and 20ºC). In this case, each microalgae strain was inoculated in Erlenmeyer flask containing diluted (50%) RL medium and incubated for 15 d as described above. In order to obtain the best conditions of culture using the RL medium by the microalgae, assays were designed by statistical program v8.0.7.1 Design-Expert using as independent parameter temperature and percentage of dilution. The biomass obtained was the dependent parameter (Table 3).

RESULTS & DISCUSSION

According to the results presented in Table 2, the highest biomass values †were obtained in the experiments carried out with the microalgae *C. vulgaris* grown in diluted RL medium (50% (p/v) of nutrient concentration), with a larger amount of biomass obtained when the microorganism was cultured at 20°C (753±5.8 mg/L). However, when *C. vulgaris* was cultured in -diluted RL medium at 10°C, a lower amount of biomass was reported (691.4±3.8 mg/L). Also, lower amounts of *C. vulgaris* biomass was obtained in 1:5 dilution (20% nutrient concentration) F1 medium incubated at 10°C (331.2±11.9 mg/L) and undilutedF1 medium (364.7±54.7mg/L). Finally, it is interesting to note that the amounts of biomass obtained using the F1medium at 50% nutrient concentration and incubated at 10ºC and 20ºC were higher (422.9±67.2 and 532±9.8 mg/L) than those obtained at 20% and 100% nutrient concentrations at the same temperature (10ºC and 20ºC). When *Scenedesmus obliquus* was grown in 1/5 diluted RL and F1 media, similar biomass production was observed in both culture media. However, when the concentration of nutrients was increased in both culture media, a significant increase of biomass was detected. In this context, the highest amount of biomass was detected for *S. obliquus* grown in undiluted F1 medium incubated at 20ºC (638.5±4.2 mg/L). On the other hand, similar biomass production was also shown (566.5±3.6 mg/L) by *S. obliquus* when the microalgae was grown in medium F1 medium at 20°C. Finally, a slightly lower amount of biomass in undiluted RL medium at 10°C was produced by *S. obliquus* (561.7±5.2 mg/L). According to these results, it could be suggested that biomass production was different for the two strains and that this performance was closely related to culture conditions (nutritional composition, dilution of the culture and temperature of incubation).

Many authors have performed assays of microalgal growth at different nutrient concentrations (Illman *et al*., 2000; Hsieh *and* Wu, 2009; Wang *et al*., 2012), suggesting that under certain culture conditions the nutrient concentration drastically affects biomass production of microalgae such as *Chlorella* sp.(Hadj-Romdhane *et al*., 2012; Tang *et al*., 2012; San Pedro *et al*., 2013). In our study, it was observed that the dilution medium of the RL and F1 media increased the biomass production of *C. vulgaris* incubated at 10ºC or 20ºC (Table 2). As evidenced by Aguirre *and* Bassi (2013), a correlation exists between the production of biomass and the nitrate concentration in the culture medium; in our case, the use of two culture media with different concentrations of nitrates and their dilution rates affected biomass production. This difference could be explained due to the fact that, even though each culture medium as a specific phosphorus and nitrogen concentration, RL medium contains KNO_3 and F1 medium contains $NaNO_3$ as the nitrogen source. In this sense, it could be suggested that KNO_3 increases the biomass production of *C. vulgaris* as Talukdar *et al.* (2012) proposed for other microalgae. According to these authors, the use of nitrogen by some Chlorophyceae microalgae such as *Ankistrodesmus falcatus* follows the following order of preference: $KNO_3 > NaNO_3 > NH_4NO_3 > (NH_4)_2HPO_4 > (NH_4)_2SO_4$. Similar results have also been reported by Smith *and* Thompson (1971). RL medium is a culture medium with less nutrients than F1 medium (Table 1), including the amount of nitrate added. Shih-Hs in Ho *et al*. (2013) reported that nitrogen-deficient media such as RL medium can affect the metabolism of *Chlorella vulgaris* and its growth rate. Consequently, the biomass production of *C. vulgaris* in RL medium could be influenced by the nitrate concentration present in the culture medium. Moreover, this effect was confirmed in experiments in diluted RL medium. Thus, while F1 medium contains an excess of nitrogen and phosphorus, RL medium contains approximately 80% less nitrogen, meaning that nitrogen deficiency could be expected. Several authors have demonstrated that *Chlorella vulgaris* has a high degree of inhibition at high nutrient concentrations (Tam *and* Wong, 1996), decreasing the assimilation of nitrogen when it was at concentrations higher than 80 mg/L.On the other hand, Shukla *et al.* (2011) reported several microalgae, including *Chlorella vulgaris*, requiring low amounts

Table 2. SX1 and Scendesmus obliquus (S.O) biomass (mg*L^{-1}) after 168h incubation in different concentration media (RL and F1) and at different temperatures (10ºC and 20ºC)

SC	MC	Temperature culture (ºC) 10			20		
		Concentration media, p/v (%)					
		20	50	100 (non dilution)	20	50	100 (non dilution)
SX1	RL*	513.6± 9.8^{C}	691.4± 3.8^{C}	585.7± 27.6^{AB}	311.9± 3.9^{C}	753± 5.8^{AB}	489.6± 15.7^{A}
	F1*	331.2± 11.9^{C}	422.9± 67.2^{A}	364.7± 54.7^{A}	480.3± 8.9^{A}	532± 9.8^{A}	470.4± 9.4^{A}
S.O	RL*	427.2 ±4.5^{A}	432 ±4.8^{A}	460.9 ±3.9^{A}	427 ±4.7^{A}	561.7±5.2^{AB}	513.7 ±5.1^{C}
	F1*	383.9 ±2.8^{A}	465.6 ±4.8^{A}	379.2 ±2.9^{A}	566.5 ±3.6^{C}	417.6 ±3.8^{A}	638.5 ±4.2^{C}

RL*: Culture Rodriguez-Lopez Medium (Rodríguez-López, 1964); **F1***: Culture F-Guillard (Stein. 1973; Guillard, 1975). Significant different at 10 and 20ºC with RL medium. ($F<0.001$). Line means followed by the same superscrip letter were not significantly different ($P<0.01$) as determined by the Tukey test. **SC**: strain control. **MC**: media control

Table 3. Experiental design used for RSM for the strain SX1 in RL medium

Run	Assay ID	Temperatureº C	Dilution% p/v	Biomass mg*L^{-1}
7	1	15	50	510
8	2	15	50	520
11	3	20	100	495
6	4	15	50	509
2	5	10	20	514
1	6	10	20	520
9	7	15	50	519
4	8	10	50	695
3	9	20	20	310
10	10	10	100	580
5	11	10	50	700

of light, temperature and nutrients. Therefore, the dilution factor in our assays seems to be the best nutritional condition for obtaining biomass concentrations of around 700 mg/L(with the strain SX1 at 50% at 10ºC and 20ºC).

Experiments with *Scenedesmus obliquus* howed that it produced larger quantity of biomass in F1 (at 20°C) than that obtained in RL. As indicated above, F1 contained higher concentration of nitrogen and lower of phosphorus than RL; consequently the N/P ratio found in F1appeared to better support the microalga growth. Voltolina *et al*. (2005) reported that this microalga showed a decrease in the efficiency of nitrogen assimilation when the concentration of nitrate was reduced in the culture media. Similar results have been found in our study using both diluted F1 and RL media.The application of Response Surface Methodologies (RSM) for optimisation of biotechnological assays has been demonstrated by numerous authors (Xie *et al*., 2012, Barghini *et al*., 2013; Cheng *et al*., 2013, Silvi *et al*., 2013). In this context, Cheng *et al.* (2013) reported significant increases in biomass and lipid production when growing *Chlorella protothecoides* in different culture media according to RSM. Similar results were obtained with *Chlorella vulgaris* by Mallick *et al.*(2012). The chlorophyte *Chlorella vulgaris* is a microalga that has been the subject to numerous biotechnological studies using RSM (Kong *et al*., 2012; Kousha *et al*., 2013). Ho *et al.* (2013) showed that cell size and light intensity could affect the production of carbohydrates with potential biotechnological interest. On the other hand, García-Sánchez *et al*. (1996) studied the interaction of temperature and lighting on *Chlorella* sp. cultures obtaining high values of specific growth rate (μ =0.125/h, at 35°C and 2,200 µmol/m^{2}s), concluding that temperature and nutrient concentrations are the most influential factors with regard to growth rate.

According to the biomass obtained from several proposed assays by Design-Expert, after this statistical analysis, a surface response was obtained where biomass production (Y) was dependent on two factors: temperature (X_1) and nutrient dilution (X_2),which are based on the following empirical equation: Eq. 2:

Eq. 2: $Y (mg/L) = 1327.9 - 142.6*X_1 + 11.6*X_2 + 0.14*(X_1*X_2) + 3.97*X_1^2 - 0.1*X_2^2$

Table 4 shows the analysis of variance (ANOVA) of the mathematical model of the response surface. Fig. 1 shows the response surface predictions and

confirms that the RL medium is appropriate for the cultivation of *C. vulgaris* (control strain). Moreover, the mathematical model can predict the interaction between temperature and nutrient concentration of the culture medium in relation to the production of biomass. According to this model, for a biomass production of 600mg/L, RL medium must be used at a nutrient concentration of 50% and incubated at 10°C. However, similar biomass production can be obtained in RL medium containing 99.80% nutrient concentration and at an incubation temperature of 14.23°C (Fig. 1-A). This result suggests a significant effect of both parameters (temperature and nutrient concentration) on the biomass production of the strain SX1 (*C. vulgaris*). The mathematical model (Fig. 1) also showed that a nutrient concentration in the RL medium below 50% decreases the biomass production of *C. vulgaris*. However, as expected, incubation temperature also affected biomass production under the mentioned nutritional conditions. These predicted results are correlated with experimental results obtained in our assays (Table 2). Consequently, our data suggest that diluted RL medium (50% of nutrient concentration) could be considered the best culture conditions for growth studies of psycrophilic microalgae. Different microalgae strains (Fig. 2 and 3) were isolated and classified during the ice-free period at beginning of July and end of August. Microalgae strains isolated in July were classified as members of the following taxonomic families: *Eustigmatophyceae*, *Bacillariophyceae*, *Trebouxiophyceae* and *Chlorophyceae*; however, all microalgae isolated in August were identified as members of *Trebouxiophyceae, Estigmatophyceae* and *Chlorophyceae*. Generally, *Chlorophyceae* were present during the whole summer, as found in our studies. However, it must be taken into account that, as for other microorganisms, not all microalgae are cultivable strains. The dynamics of photosynthetic communities in high mountain lakes is particularly dependent upon the available nutrients and temperature along the ice-free season. Several autors (Garnier *et al*., 1995; Maurin *et al*.,1997 and Naz *et al*., 2012) showed that in late summer in high mountain lakes, the microalgae community is dominated by *Chlorophyceae*. This could be due to a peculiar temperature dynamic, resulting in increased amounts of available N and soluble P, as demonstrated by Garnier *et al*. (1995).

In our study, 27 strains of microalgae were isolated from water sampled in July. Most of the strains were related to the Phylum *Chlorophyta* and *Ocrophytas*. Similar results were reported by Sánchez-Castillo *et al.*(1988), although these authors described that in *La Caldera* others Phylum such as *Heterocontophytes, Chrysophytes* and *Cyanobacteria* were also present. Specifically in our case, 17 strains were included in the Phylum *Chlorophyta* and 10 strains were related with the Phylum *Ocrophytas.* Our data show that in early summer in *La Caldera* Lake, 3 culturable microalgae related to the class *Trebouxiophyceae* and 4 related to the class *Chlorophyceae* were found, both belonging to the phylum *Chlorophyta*. On the other hand, our study showed the presence of two microalgae related to the class *Eustigmatophyceae* and only one related to *Bacillariophyceae,* both belonging to the Phylum *Ocrophytas* (Fig. 2). It should be noted that in early summer only two microalgae related to *Eustigmatophyceae* class were identified; however, in late summer, 7 strains of the same class were isolated: S50, S55, S61, S62, S64, S70 and S77. It is also note worthy that no culturable microalgae of the class *Bacillariophyceae* were found. The studies performed in August in *La Caldera* Lake showed that 17 microalgae were able to grow in RL medium (Fig. 3). When the selected strains were taxonomically identified, 10 were related to *Chlorophyta*, 3 to *Trebouxiophyceae* and 7 to *Chlorophyceae*. These results suggest that the culturable microalgae community in *La Caldera* Lake is relatively stable through the summer season, although this could be different in other periods of the year. In this context, several authors (Robinson *et al.,*1998; Hoham *and* Duval, 2001, and Villar-Argaiz *et al.,* 2001) showed inter- and intra-annual changes that occurred in the phytoplankton community in *La Caldera* Lake, for a period of three years, taking into

Table 4. ANOVA of Equation Nº 2

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F
Model	108357.5	5	21671.5	646.4	< 0.0001
A-Temperature	26187.9	1	26187.9	781.1	< 0.0001
B-Dilution	17269.2	1	17269.2	515.1	< 0.0001
AB	3479.2	1	3479.2	103.8	0.0002
A^2	10212.6	1	10212.6	304.6	< 0.0001
B^2	27545.3	1	27545.3	821.6	< 0.0001
Pure Error	167.6	5	33.5		
Cor Total	108525.2	10			

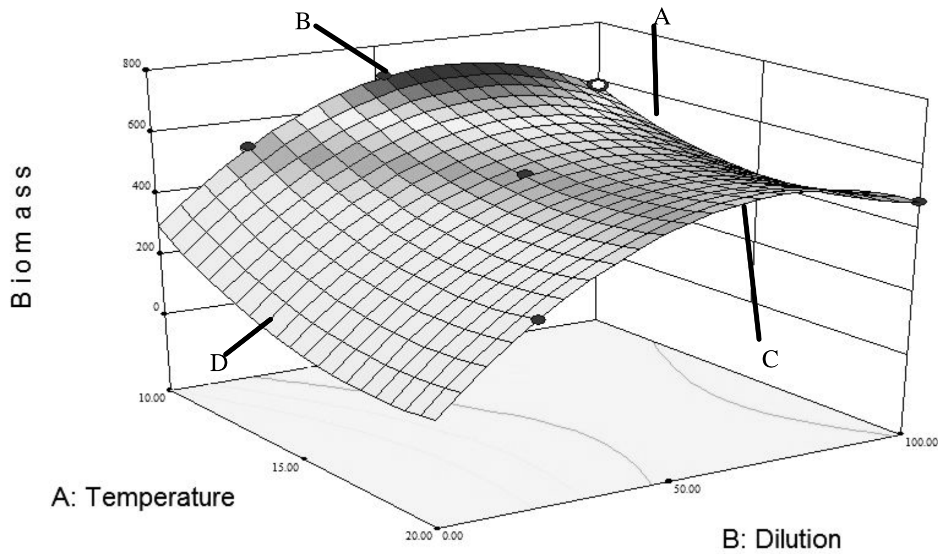

Fig. 1. Surface response of the optimal growth of the strain SX1 with RL medium for the production of biomass in function of temperature and grade of dilution. A: 14.23ºC, 99.80%, 600mg/L. B: 10ºC, 44.90%, 600 mg/L. C:20ºC, 46.77%, 450 mg/L.D: 17.58ºC, 20%, 450mg/L

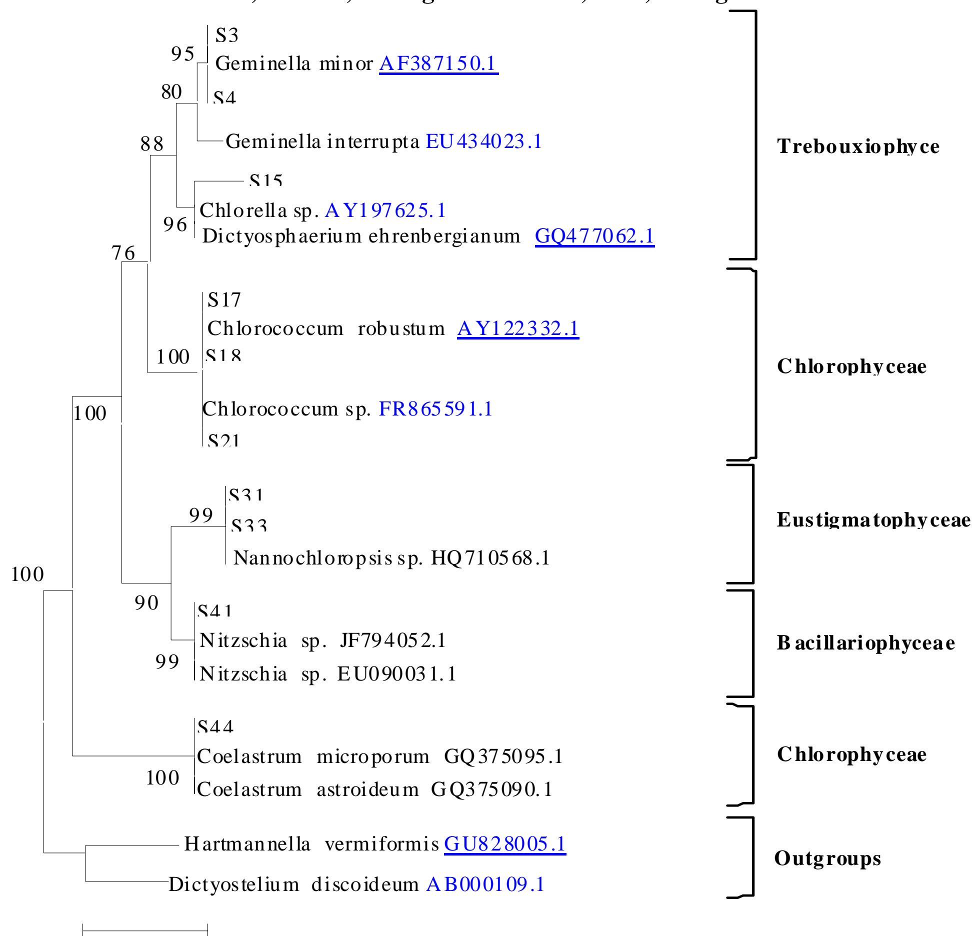

Fig. 2. Phylogenetic tree based in the partial sequence of 18S rDNA, showing the position and relationship of the isolated strains from the *La Caldera* Lake in July, 2011. Information was compared with the EMBL database. The number next to each branches show the Bootstrap values > 60 %

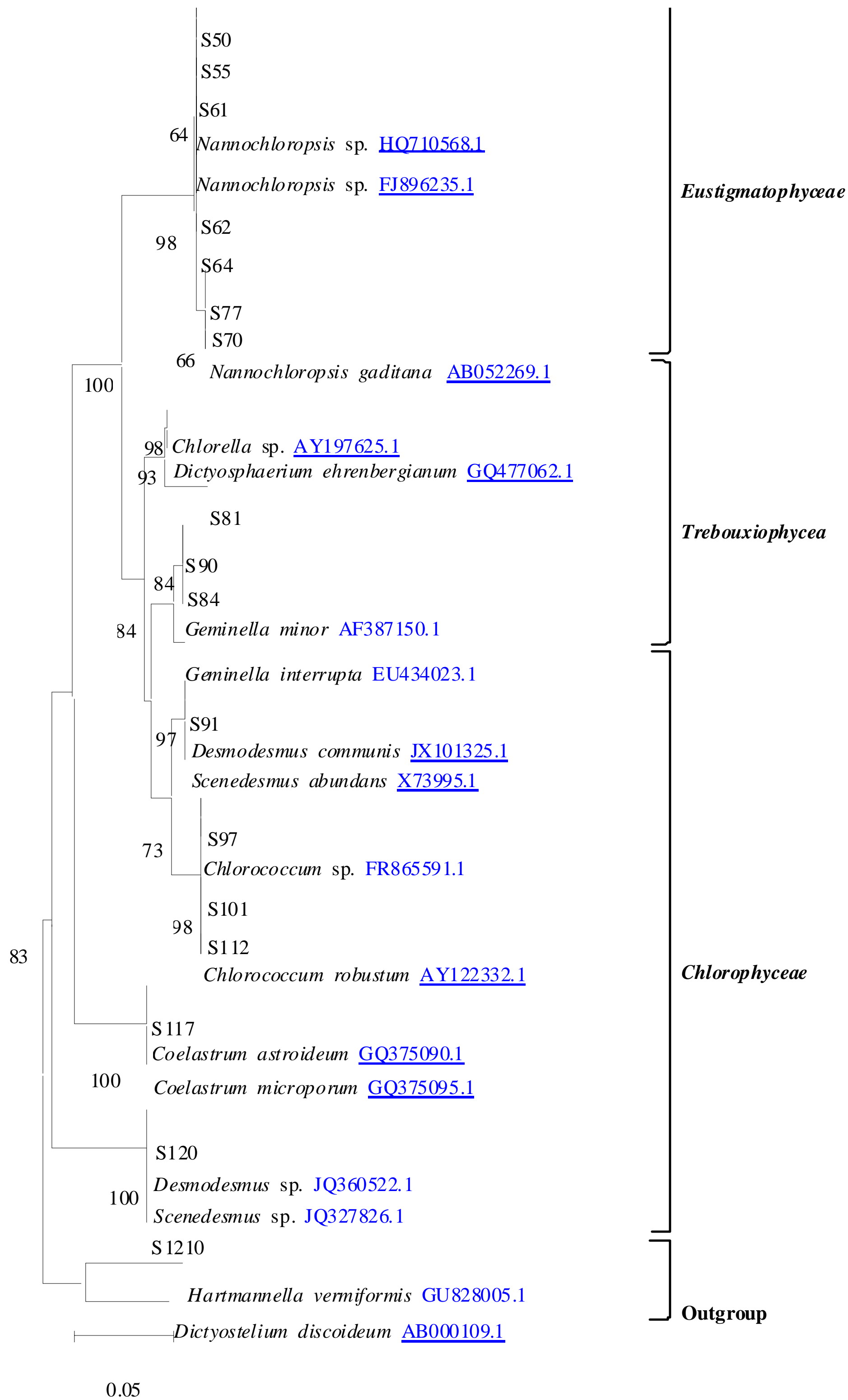

Fig. 3. Phylogenetic tree based in the partial sequence of 18S rDNA, showing the position and relationship of the isolated strains from the *La Caldera* Lake in August, 2011. Information was compared with the EMBL database. The number next to each branches show the Bootstrap values > 60 %

account the influence of external (atmospheric input) and internal (phosphorus supplied by zooplankton and phosphorus contribution by ice) phosphorus sources. These authors found that inter-annual differences in phytoplankton biomass were associated with temperature and dissolved phosphorus content, meaning that there was a positive relationship between phosphorus excretion by zooplankton and phytoplankton biomass. In the intra-annual period, variations in zooplankton were more pronounced than in the inter-annual period, and tended to be lower than the N:P ratio of zooplankton after thawing (when the zooplankton community was dominated by copepod *Nauplii*), and more than half that of late summer, which is dominated by other species. According to the composition of zooplankton, these authors explain the changes in the composition of phytoplankton. All microalgae isolated from *La Caldera* Lake have been previously described by other authors; however, strains S121 and S120, both related to the genus *Desmodesmus* from the family *Scenedesmaceae*, have not been found in *La Caldera* Lake so far, although others described presence of different species of *Scenedesmaceae* (Fanés *et al*., 2009). It was also observed that strains S50, S55, S62, S64, S77 and S70 were related to *Nannochloropsis* sp. (Fig. 2 and 3).

Five microalgae strains included in the Phylum *Chlorophyta* (Table 5) and one from the Phylum *Heterokontophyta* were selected for further analysis. Particularly, a study of the growth rate was performed for each strain in RL medium to evaluate the optimal conditions for growth and the production of substances with biotechnological interest. Individual cultures of microalgae S120, S91, S121, S21, S41 and S3, classified as *Scenedesmus* sp., *S. communis* sp., *Desmodesmus* sp., *Chlorococcum minutum* sp., *Nitszchia* sp., and *Geminella minor* (Table 5) grew in RL medium at 10°C and 20°C. However, S41 strain, classified to *Nitzschia* sp., grew in RL medium only at 20ºC. Even at this temperature, the growth of this strain was not satisfactory, probably due to the absence of silicate in the culture medium (Roleda *et al*., 2013). In the individual cultivation of S120 (Fig. 4e and 5e) maximum biomass concentrations of 159.28±21.79 mg/ Land 184.45±16.70 mg/L were obtained in the assays performed at 10°C and 20°C, respectively, after 15 days of culture. In relation to the biomass obtained at both culture temperatures, an initial lag phase of 4 days was observed, followed by an increase in biomass over time until the stationary phase was reached. However, it was evident that the biomass production of S120 strain was slightly higher (13.65%) when it was grown at 20ºC. When S91 was cultured in RL medium at 10°C (Fig. 4f), it was observed that values of μ_{max} were 0.09/ h after two days of growth, and production of biomass accumulated a maximum of 22.05±2.19 mg/L.This concentration of biomass was constant until the end of the experiment. Moreover, in the assays performed at 20°C (Fig. 5f) this strain showed a maximum biomass concentration after 12 days of culture, obtaining 70.38±5.18 mg/L of biomass with a μ_{max} of 0.08/h. However, μ_{max} was reduced to 0.05/h during the following 24 hours after culture (stationary growth phase) and then decreased until it reached zero.

The individual culture of S121 offered a similar growth in both temperatures tested (Fig. 4c and 5c), resulting in a maximum biomass concentration accumulated of 154.01±8.81 mg/L at 20ºC and 145.60±9.27 mg/L at 10ºC. In both assays performed, it was found that after 11 days of culture the μ_{max} values

Table 5. Numeric value of μ at different temperatures of the individual culture of isolated strains from *La Caldera* Lake

			Temperature			
			10		20	
Isolatedstrain	Genetic identification	Similitud (%)	μ^1	Accumulated biomass ^{2}	μ^1	Accumulated biomass ^{2}
S3	*Geminella* sp. / Chlorophyceae	99	0.11	40.99	0.11	83.27
S21	*Chlorococcumminutum*/ Chlorophyceae	99	0.13	128.52	0.25	393.73
S121	*Desmodesmus* sp. /Chlorophyceae	99	0.14	145.6	0.14	158.68
S41	Nitzschia sp. / Bacillariophyceae	98	0.01	6.10	0.12	125.54
S120	*Scenedesmus* sp./ Chlorophyceae	99	0.13	159.28	0.13	183.43
S91	*Desmodesmus communis*/ Chlorophyceae	99	0.09	21.59	0.08	75.21

μ^1: Growth rate, mg*L^{-1}*h^{-1} ; Accumulated biomass^2: mg*/L

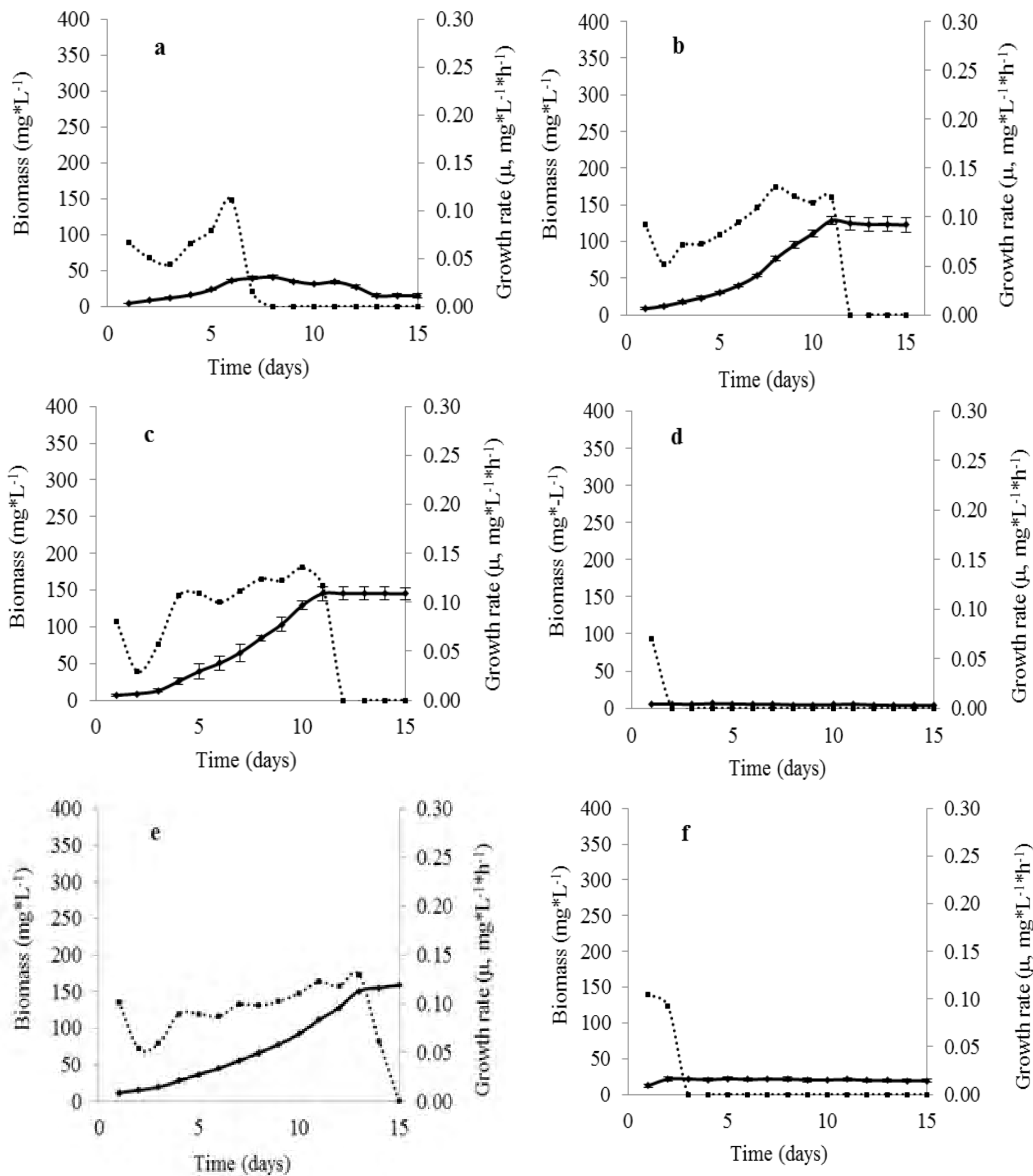

Fig. 4. Kinetic of growth of individual cultures of each selected strain at 10º C with RL medium, with total illumination of 180 µmol, without agitation and constant air supply. Continuous and dotted lines represent biomass (mg/L) and maximum growth rate µ (mg/L/h) for 15 culture days. a: S3; b: S21; c:S121; d: 41; e: S120; f: 91

were reduced close to 0, showing a stationary growth phase. Furthermore, it was observed that in assays at both 10ºC and 20ºC the μ_{max} was 0.14/h. When S21 was grown at 10°C (Fig. 4b), a lag phase of growth was shown after 3 days of cultivation. Later, the microalgae grew at μ_{max} of 0.13/h for 5 days and then progressively reduced its μ_{max} to close to 0/h, producing a stationary phase until the end of the experiment. Under this culture condition, the amount of biomass obtained was 128.52±5.69 mg/L. However, when the assay was carried out at 20°C (Fig. 5b) an increase of 306% in the accumulated biomass concentration (393.73±3.16 mg/L) was observed. This result also correlated with a significant increase in the μ_{max} of the S21 strain growing

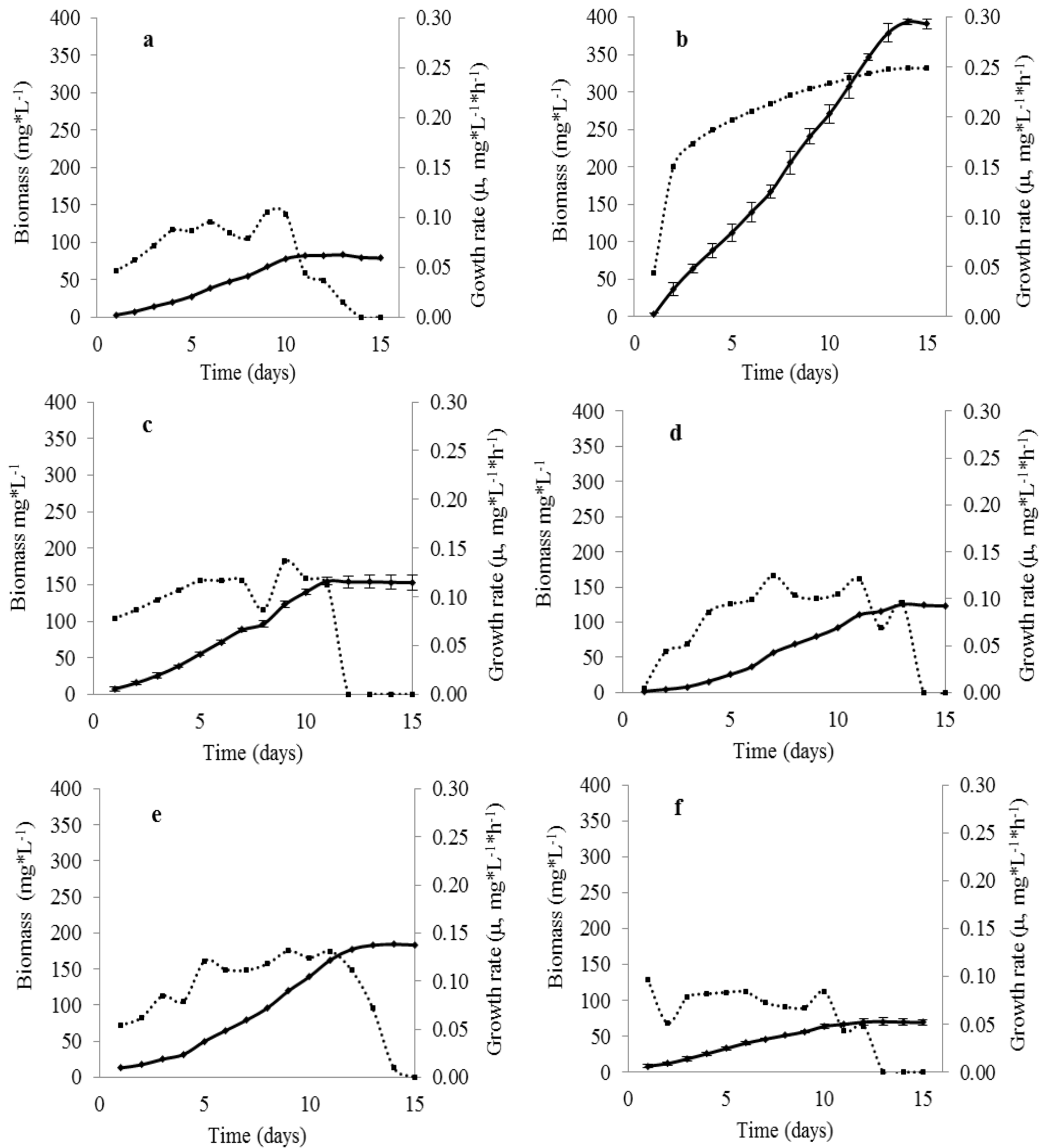

Fig. 5. Kinetic of growth of individual cultures of each selected strain at 20° C with RL medium, with total illumination of 180 µmol, without agitation and constant air supply. Continuous and dotted lines represent biomass (mg*L^{-1}) and maximum growth rate µm (mg*L^{-1}*h^{-1}) for 15 culture days. a: S3; b: S21; c:S121; d: 41; e: S120; f: 91

at 20ºC.Thus, while at 10ºC the μ_{max} value was 0.15 /h, but at 20ºC the μ_{max} value was 0.25/h. It was observed at 20ºC that this value increased by 92% (from 0.13 at 0.25/h). Strain S3 was also grown at 10°C and 20ºC. Thus, when the microalgae was grown at 10ºC (Fig. 4a), it showed a specific growth rate of 0.11/h after 6 days of culture, decreasing to 0.01/h during the following 24 hours of culture; from this moment, µ was zero until the end of the assay. Under this condition the total accumulation of biomass in the culture medium was 40.99 mg/Lafter 15 days of incubation. However, when the microalgae was cultured in RL medium at 20ºC, a significant increase in the accumulated biomass (83.27±2.84 mg/L) in the growth medium was detected (Fig. 5a) showing a μ_{max} of 0.11/h after 9 days of culture, which was gradually reduced to 0/h within 24 hours of the culture.

S41 was the only strain that did not show good growth at 10°C (Fig. 4d) although it grew well at 20ºC (Fig. 5d). At 10°C, after 15 days of growth, a total biomass of 5.28±0.89 mg/L was obtained. However, when this strain was cultured at 20ºC, it grew very well, producing an accumulated biomass concentration of 125.54±12.98 mg/L; at this time, the specific growth rate was 0.10/h after 13 days of incubation time. Table 5 shows the maximum growth rate of the selected microalgae species isolated from the *La Caldera* Lake and also the maximum biomass accumulated after15 days of incubation in RL medium at 50% (p/v) nutrient concentration at temperatures of 10ºC and 20ºC. Biomass accumulation of the Microalga S21 (genetic related with *Chlorococcum minutum*, Table 5) was 393.73±3.16 mg/L at 20ºC; however, it can be observed that microalgae S120, S121, S41, S91 and S3 generated a biomass of 53.41, 59.69, 68.11, 78.85 and80.89% with respect to the strain S21 at 20ºC. On the other hand, in the assays performed at 10°C, the strain that showed higher biomass production(159.28 mg/L) in RL medium was S120 (*Scenedesmus* sp.). Other strains with high biomass production were S121 and S21, which showed values of 154.01 and 128.52, respectively. S3, S91,S41 and C41 strains showed lower growth rates at 10ºC and consequently lower biomass amounts in RL.

Our experiments clearly confirmed the importance of the incubation temperature on the growth and biomass production of selected microalgae from *La Caldera* Lake; it could be suggested that, in general terms, the incubation temperature of 20°Cproduces a higher growth rate and biomass accumulation in medium diluted RL medium. However, the results can be also influenced by the microalgae strain typology. Thus, *Scenedesmus* sp. showed the higher production of biomass at 10ºC. The importance of light and temperature interactions on microalgae growth has been reported since the early works of Kratz *and* Myers (1955) . Moreover, the importance of the kinetic data to evaluate the effect of temperature on algal growth was demonstrated already by Goldman *and* Carpenter (1974). In this context, many studies have reported that higher values of growth rates could represent higher values of biomass production if nutrients are available. Our study confirmed previous works and showed that, after 15 days of incubation, acclimation is achieved in most of the microalgae cultured in RL medium. Specifically, our data showed that, when growth rates was constant, a significant increase in biomass was detected in the microalgae cultures as suggested by Renauld *et al*. (2002).

According to our results, although values of specific rate of growth were similar at both 10°C and 20°C (values close to 0.10/h), biomass production was doubled in the assays at 20°C, suggesting that incubation temperature directly affects the specific rate of growth. A similar result was obtained by Renaud *et al.* (2002), who found that in the case of microalgae such as *Isochrysis sp.* and *Prymnesiophyte* sp., a temperature up to 30ºC can negatively affect microalgal growth and biomass production. Thus, the relationship between growth rate and biomass production will depend on the microalga used and cultivation conditions, as was seen in our assays.

CONCLUSION

This paper presents the microalgae culturable biodiversity of a high mountain lake (*La Caldera*) and concludes that each selected microalga has optimal nutritional conditions of cultivation as a function of the incubation temperature assayed. We found that RL medium at 50% (p/v) nutrient concentration could be considered a good culture medium for the production of biomass and particularly an excellent culture medium forS21and SX1 strains. Also, it was found that the growth rate is directly linked to the optimal conditions for growth and that the use of statistical tools such as MSR helps to optimise experimental design, saving time and reagents, as well as obtain information for postulating isolated natural environments with a possible biotechnological application.

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