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ORIGINAL PAPER

Quantification of phototrophic biomass on rocks: optimization of chlorophyll-*a* extraction by response surface methodology

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Abstract Biological colonization of rock surfaces constitutes an important problem for maintenance of buildings and monuments. In this work, we aim to establish an efficient extraction protocol for chlorophyll-*a* specific for rock materials, as this is one of the most commonly used biomarkers for quantifying phototrophic biomass. For this purpose, rock samples were cut into blocks, and three different mechanical treatments were tested, prior to extraction in dimethyl sulfoxide (DMSO). To evaluate the influence of the experimental factors (1) extractant-to-sample ratio, (2) temperature, and (3) time of incubation, on chlorophyll-*a* recovery (response variable), incomplete factorial designs of experiments were followed. Temperature of incubation was the most relevant variable for chlorophyll-*a* extraction. The experimental data obtained were analyzed following a response surface methodology, which allowed the development of empirical models describing the interrelationship between the considered response and experimental variables. The optimal extraction conditions for chlorophyll-*a* were estimated, and the expected yields were calculated. Based on these results, we propose a method involving application of ultrasound directly to intact sample, followed by incubation in 0.43 ml DMSO/cm^2 sample at 63°C for 40 min. Confirmation experiments were performed at the predicted optimal conditions, allowing chlorophyll-*a* recovery of 84.4 ± 11.6% (90% was expected), which implies a substantial improvement with respect to the expected recovery using previous methods (68%). This method will enable detection of small amounts of photosynthetic microorganisms and quantification of the extent of biocolonization of stone surfaces.

Keywords Chlorophyll-*a* · Incomplete factorial design · Response surface methodology · Stone biofilms · Ultrasonic methods

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Introduction

Stone surfaces exposed to the open air are inevitably colonized by a variety of organisms, some of which are responsible for biofilm formation. Among them, algae and cyanobacteria have great importance, since they feature an extracellular matrix composed primarily of exopolymers (EPS) which are involved in the formation of the biofilm and in the resistance of biofilms to adverse abiotic conditions [1–4]. Once established, algal-cyanobacterial biofilms are added to by heterotrophic bacteria and fungi, forming a microbial biocenosis [5]. Biofilms are responsible for apparent staining of rocks due to the biogenic pigments of phototrophic organisms [1, 6] and the formation of black patinas [7, 8], and may also affect the physicochemical properties of mineral materials [5]. This process is particularly important when the stone under consideration is the building material of monuments of historic and cultural interest [9–12].

Thus, determining the extent of algal and cyanobacterial colonization is crucial for the study of the deterioration of rocky works of art and for the development of methods to

control their deterioration, as well as for the development of bioreceptivity assays of building materials. Therefore, as a starting point, it is necessary to establish a reliable method for quantifying biocolonization on stone surfaces.

One of the most commonly used biomarkers for quantifying microalgal and cyanobacterial biomass is chlorophyll-*a*, which has been extensively used to estimate photosynthetic growth in water, liquid media, and soil, and to a minor extent for estimating algal biomass in rocky substrata [13–16]. Prieto et al. [17] compared several methods for biomass quantification on stone surfaces, and found that chlorophyll-*a* was a good estimator of biofilm biomass. However, they observed problems in the total extraction of chlorophyll-*a* from stone and obtained a relatively high value as a lower limit of detection, which would prevent early detection of stone biocolonization below this lower limit. These authors suggested that the stone itself impedes total chlorophyll-*a* extraction, and concluded that optimization experiments carried out on stone samples were necessary.

Traditional optimization methods examine a single factor at a time, while fixing all other variables at one level. Their major disadvantage is that these methods do not include the interactive effects among the variables studied. As a consequence, these techniques do not depict the complete effects of the parameters on the response. To avoid this problem, response surface methodology (RSM) was developed by Box and collaborators in the 1950s [18]. RSM is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data, which must describe the behavior of a data set with the objective of making statistical predictions. It can be applied when a response is influenced by several variables with the objective of simultaneously optimizing the levels of these variables to attain the best system performance [19]. Response surface designs are useful for modeling a curved quadratic surface to continuous factors. A response surface model can pinpoint a minimum or maximum response inside the factor region. Three distinct values for each factor are necessary to fit a quadratic function, so standard two-level designs cannot fit curved surfaces. Three-level full factorial designs are used, in which factors can take on three values: low, medium or center, and high. Generally, if midpoints or center points are added to a 2^k full factorial design then it will become a 3^k full factorial design, where k is the number of factors. However, the main disadvantage of this design (3^k) is the need for a large number of experimental runs, which produces unwanted high-order interactions, and in addition can be expensive and time consuming. Therefore, designs that present a smaller number of experimental points, such as the Box–Behnken method where the number of experiments required (N) is given by $N = 2k(k - 1) + C_0$, where k is the number of variables and C_0 is the number of center points, are more often used.

In this work, we tried various combinations of mechanical and ultrasonic methods as pretreatments to improve the extraction efficiency of chlorophyll-*a* from stone samples, as this is the primary photosynthetic pigment present in organisms responsible for biofilm formation on building materials. To find the optimal conditions of the three independent variables (sample-to-extractant ratio, temperature, and extraction time) potentially influencing the efficiency of phytopigment extraction with dimethyl sulfoxide (DMSO), we performed a response surface analysis following a Box–Behnken design. Finally, we compare the results achieved by the different pretreatments and discuss the adequacy of these methods for quantification of the extent of biocolonization on rock surfaces.

Materials and methods

Preparation of samples

Experiments were carried out with a mixed culture of the filamentous N_2-fixing heterocyst-forming cyanobacteria strains *Nostoc* PCC 9025, *Nostoc* PCC 9104, and *Scytonema* CCC 9801, grown in $BG11_0$ medium. The mixed inoculum consisted of 0.42 g (dry weight) of each strain per liter of medium. Ten milliliters of mixed culture (equivalent to 12.7 g total dry weight and 90.5 μg chlorophyll-*a*) was sprayed onto the surface of 6 × 6 × 1 cm^3 blocks cut from a granite rock. The blocks were placed in a climatic chamber for 2 days under stationary conditions at 25°C, 95% humidity, and 12 h of light (1,600 lx) to induce biofilm formation before pigment extraction was carried out.

To determine the chlorophyll-*a* content from the culture mixture, five replicate aliquots of 5 ml culture were filtered, and the filters were added to 5 ml DMSO and heated to 65°C for 1 h as described in [17]. After filtration of the samples to remove filter fragments, absorbance of the extracts was measured at 649.1 and 665.1 nm wavelength ($A_{665.1}$ and $A_{649.1}$). The concentration of chlorophyll-*a* (C_a) was calculated using the equation proposed by Wellburn [20]:

$$C_a = 12.47A_{665.1} - 3.62A_{649.1}.$$

Mechanical pretreatment of samples

Three different block pretreatments were assayed to determine the best procedure for complete chlorophyll-*a* extraction (Table 1). For the first method (pretreatment A), 15 inoculated blocks were crushed to obtain fragments no larger than 0.25 cm^3, which were added to DMSO following the protocol described by Prieto et al. [17]. For pretreatment B, 15 inoculated blocks were

Table 1 Independent and dependent variables employed in this study

Sample pretreatment		Nomenclature	
Experiment			
Block crushing		A	
Block crushing and sonication in DMSO (ultrasonic bathing 30 min)		B	
Whole blocks sonication in DMSO (tip ultrasonic generator 2 min 30 s)		C	
Variable	Nomenclature	Units	Variation range
Independent variables			
Extractant/sample (v/v) ratio[a,b] or extractant volume/sample surface[c]	*ES*	ml/cm^{3}[a,b] or ml/cm^{2}[c]	1.39–1.94[a]; 1.67–2.22[b]; 0.28–0.56[c]
Temperature	*T*	°C	30–80
Time	*t*	min	30–90
Variable	Nomenclature	Definition	Variation range
Dimensionless, coded independent variables			
Dimensionless extractant/sample ratio	x_1	(*ES*-1.67)/1.39[a]; (*ES*-1.94)/1.67[b]; (*ES*-0.42)/0.28[c]	(−1, 1)
Dimensionless temperature	x_2	(*T*-55)/40	(−1, 1)
Dimensionless time	x_3	(*t*-60)/30	(−1, 1)
Variable	Nomenclature	Units	
Dependent variables			
Chlorophyll-*a*	y_1	μg	

[a] Pretreatment A

[b] Pretreatment B

[c] Pretreatment C

crushed and introduced into 250-ml glass flaks, DMSO was added, and the flasks were introduced into an ultrasonic bath (Transonic T780, Elma™) filled with enough water to apply ultrasound to the whole of each sample, during 30 min (the water temperature was measured during this process to ensure that no heating was taking place). For pretreatment C, 15 intact inoculated blocks (without crushing) were placed onto Petri plates containing DMSO and sonicated by inserting the narrow tip of an ultrasonic generator (UP200S; Dr Hielscher GmbH) into the extractant. Sonication was for 5 × 30 s (0.5 duty cycle, 60% amplitude), with 30 s breaks to avoid overheating.

Design of experiments

For each pretreatment we performed a Box–Behnken design for three-variable optimization with 13 experimental points plus 2 additional experiments at the central point (three central replicates), to study the influence of the experimental conditions on the extraction yield. (Note that this makes a total of 15 experiments, which in comparison with a 3^3 design with 27 experiments, is more economical and efficient.) The independent variables used in this study and their variation limits are listed in Table 1. *ES* corresponds to the extractant/sample ratio, expressed as volume of DMSO/volume of the sample blocks (v/v) for the crushed samples (pretreatments A and B) and as volume of DMSO/sample surface (cm^2) for the intact blocks (pretreatment C); *T* corresponds to the temperature of extraction (°C); and *t*, to the extraction time (min). The levels of the variable were coded, namely each studied real value was transformed into coordinates inside a scale with dimensionless values proportional to its location in the experimental space. Codification enables the investigation of variables of different orders of magnitude without the greater influencing the evaluation of the lesser [19]. The standardized (coded) dimensionless independent variables employed, having variations limits (−1, 1), were defined as x_1 (coded extractant/sample ratio), x_2 (coded temperature), and x_3 (coded extraction time). The correspondence between coded and uncoded variables was established by linear equations deduced from their respective variation limits (Table 1). The dependent variable considered was chlorophyll-*a*, measured by the variable y_1. After incubation in DMSO at their corresponding conditions, samples were filtered, and the concentration of chlorophyll-*a* was determined, as described before.

Model fitting

To evaluate the importance of the independent factors studied in the extraction of chlorophyll-*a*, for each pretreatment, the experimental data were subjected to analysis of variance (ANOVA). Tests on equality of the different factor levels were performed, and main (linear and quadratic) and interaction (linear by linear) effects were estimated. The main effect of a factor is the change in the average response produced by a change in the level of that factor. The interpretation of the quadratic main effects is analogous to that of the linear main effects; this is, the estimated quadratic main effect is the difference between the medium setting and the average of the low and high settings for the respective factors. Interaction effects exist if the difference in response between the levels of one factor is not the same at all levels of another factor. Replicates at the center point allow estimation of the pure error associated with repetitions. Thus, the sum of the square of residuals (SS_{res}) can be separated into two contributions: the sum of squares due to pure error (SS_{pe}) and the sum of squares due to lack of fit (SS_{lof}). To evaluate the adequacy of the model, a lack-of-fit test was performed. The key idea of this test is that, if the mathematical model is well fitted to the experimental data, the mean of the squares of the lack of fit (MS_{lof}) should reflect only the random errors inherent to the system. Additionally, the mean of the square of the pure error (MS_{pe}) is also an estimate of these random errors, and it is assumed that these two values are not statistically different. Thus, it is possible to use the Fisher distribution (*F*-test) to evaluate whether there is some statistical difference between these two means:

$$MS_{lof} / MS_{pe} \approx F\, v_{lof}, v_{pe},$$

where v_{lof} and v_{pe} are, respectively, the degrees of freedom associated with the lack of fit and the pure error variances. If this ratio is higher than the tabulated value of *F*, it is concluded that there is evidence of lack of fit and that the model needs to be improved [19].

Optimization of experimental variables

Finally, the experimental data were analyzed by the response surface methodology [18] using Statistica software (version 8.0; Stat Soft Inc., Tulsa, OK [http://www.statsoft.com]), which allowed the development of empirical models describing the interrelationship between operational and experimental variables by equations including linear, interaction, and quadratic terms:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2, \quad (1)$$

where *y* is the dependent variable, *b* denotes the regression coefficients (calculated from experimental data by multiple regression using the least-squares method), and *x* denotes the independent variables. This model enabled the prediction of optimal conditions (x_1, x_2, and x_3 values) corresponding to the best extraction yield (maximum y_1).

Influence of concentration on the accuracy of spectrophotometric estimation of chlorophyll-*a*

Chlorophyll-*a* was extracted with DMSO from five aliquots of a mixed culture of the three cyanobacteria (0.24 g of each strain per liter), as described above. Serial dilutions (1, 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64) were made, and the concentration of chlorophyll-*a* of the 35 samples (7 dilutions × 5 replicates) was estimated using the equations proposed by Wellburn [20]. The mathematical relationship between chlorophyll-*a* concentration and dilution factor was determined by regression analysis using the JMP statistical package (version 8.0; SAS Institute, Cary, NC [http://www.jmp.com]). The same analysis was performed with a subset of samples corresponding to the four most diluted cases (1/8, 1/16, 1/32, and 1/64). In each case, the coefficient of determination (R^2) and the 99% confidence interval were calculated.

Results and discussion

Design of experiments

Tables 2, 3, 4 show the set of experimental conditions assayed for the 3^3 Box–Behnken design (expressed in terms of both coded variables and real values), as well as the experimental data obtained for chlorophyll-*a* (y_1). It should be noted that experiments 1–12 allowed the calculation of the regression coefficients, whereas experiments 13–15 were replications at the central point of the design to estimate the influence of experimental error (pure error) and to determine whether quadratic effects should be included.

The most efficient extraction was achieved by crushing the sample blocks followed by ultrasonic bathing (pretreatment B), with subsequent incubation under the following conditions: in 1.94 (v/v) DMSO at 80°C for 90 min.

Optimization of chlorophyll-*a* extraction

Experimental results were employed to construct a second-order model with linear, quadratic, and interaction effects, which can predict the total weight of chlorophyll-*a* extracted (dependent variable) as a function of the extractant/sample ratio, temperature, and extraction time

Table 2 Operational conditions for the factorial design considered in this study, and experimental results for phytopigment extraction achieved after sample crushing (pretreatment A)

Experiment	Independent variables			Dependent variable
	ES (x_1) (v/v)	*T* (x_2) (°C)	*t* (x_3) (min)	y_1 (μg)
1	1.67 (0)	80 (1)	30 (−1)	77.6
2	1.67 (0)	30 (−1)	30 (−1)	56.7
3	1.67 (0)	80 (1)	90 (1)	70.2
4	1.67 (0)	30 (−1)	90 (1)	47.0
5	1.39 (−1)	80 (1)	60 (0)	96.1
6	1.39 (−1)	30 (−1)	60 (0)	49.7
7	1.94 (1)	80 (1)	60 (0)	109.1
8	1.94 (1)	30 (−1)	60 (0)	41.2
9	1.39 (−1)	55 (0)	30 (−1)	45.3
10	1.39 (−1)	55 (0)	90 (1)	70.2
11	1.94 (1)	55 (0)	30 (−1)	57.4
12	1.94 (1)	55 (0)	90 (1)	71.1
13	1.67 (0)	55 (0)	60 (0)	49.4
14	1.67 (0)	55 (0)	60 (0)	67.9
15	1.67 (0)	55 (0)	60 (0)	60.7

Levels for coded dimensionless variables are indicated in brackets

Table 3 Operational conditions for the factorial design considered in this study, and experimental results for phytopigment extraction achieved after sample crushing and ultrasonic bathing (pretreatment B)

Experiment	Independent variables			Dependent variable
	ES (x_1) (v/v)	*T* (x_2) (°C)	*t* (x_3) (min)	y_1 (μg)
1	1.94 (0)	80 (1)	30 (−1)	102.5
2	1.94 (0)	30 (−1)	30 (−1)	61.6
3	1.94 (0)	80 (1)	90 (1)	115.4
4	1.94 (0)	30 (−1)	90 (1)	62.3
5	1.67 (−1)	80 (1)	60 (0)	97.1
6	1.67 (−1)	30 (−1)	60 (0)	53.4
7	2.22 (1)	80 (1)	60 (0)	104.4
8	2.22 (1)	30 (−1)	60 (0)	63.1
9	1.67 (−1)	55 (0)	30 (−1)	59.5
10	1.67 (−1)	55 (0)	90 (1)	79.9
11	2.22 (1)	55 (0)	30 (−1)	72.8
12	2.22 (1)	55 (0)	90 (1)	82.0
13	1.94 (0)	55 (0)	60 (0)	75.0
14	1.94 (0)	55 (0)	60 (0)	90.1
15	1.94 (0)	55 (0)	60 (0)	89.4

Levels for coded dimensionless variables are indicated in brackets

(independent variables). The sums of squares (SS) and *F*-tests associated with the combined linear and quadratic effects and their interactions are presented in Table 5. The mean of squares due to pure error (MS_{pe}) and the mean of squares due to lack of fit (MS_{lof}) were also estimated. In all cases (pretreatments A, B, and C), the ratios MS_{lof}/MS_{pe} were higher than the tabulated value of $F(v_{lof},v_{pe})$, indicating that the model fitness can be considered satisfactory.

Table 4 Operational conditions for the factorial design considered in this study, and experimental results for phytopigment extraction achieved after sonication of the intact block samples without crushing (pretreatment C)

Experiment	Independent variables			Dependent variable
	ES (x_1) (v/cm^2)	*T* (x_2) (°C)	*t* (x_3) (min)	y_1 (μg)
1	0.42 (0)	80 (1)	30 (−1)	88.8
2	0.42 (0)	30 (−1)	30 (−1)	52.7
3	0.42 (0)	80 (1)	90 (1)	67.0
4	0.42 (0)	30 (−1)	90 (1)	68.4
5	0.28 (−1)	80 (1)	60 (0)	71.3
6	0.28 (−1)	30 (−1)	60 (0)	56.4
7	0.56 (1)	80 (1)	60 (0)	69.9
8	0.56 (1)	30 (−1)	60 (0)	58.7
9	0.28 (−1)	55 (0)	30 (−1)	65.6
10	0.28 (−1)	55 (0)	90 (1)	51.2
11	0.56 (1)	55 (0)	30 (−1)	76.1
12	0.56 (1)	55 (0)	90 (1)	78.0
13	0.42 (0)	55 (0)	60 (0)	64.8
14	0.42 (0)	55 (0)	60 (0)	79.2
15	0.42 (0)	55 (0)	60 (0)	90.6

Levels for coded dimensionless variables are indicated in brackets

Table 6 presents estimates of the main (linear and quadratic) and interaction effects. These represent the difference in process performance caused by a change from low (−1) to high (+1) levels of the corresponding factor [21]. Both Student tests (*t*-tests) and their associated probabilities (*P*-values) were used to confirm the significance of the factors studied. Temperature of incubation was the most relevant variable with respect to pretreatment for recovery of chlorophyll-*a* (*T*, $P < 0.05$). Raising the temperature from 30°C to 80°C led to an increase in chlorophyll-*a* extraction, on average, of 39.61, 44.76, and 15.20 μg (after pretreatments A, B, and C, respectively). The *P*-values for the main effects of *ES* and *t* were higher than 0.05, and their corresponding coefficients had very low values in the equations proposed by the coded models. Consequently, these parameters will not have a great influence on the values of y_1. This result contrasts with the observations made in similar studies [22], where it was found that the extraction efficiency of DMSO for epilithic biofilms was greatly influenced by both the ratio of extractant volume to sample weight and extraction time. However, in our case, the volume of DMSO (*ES*) varied within a range that was probably too narrow to have a significant effect, the limits being defined by experimental

Table 5 Results of ANOVA and lack-of-fit tests for pretreatments A, B, and C

Factor	SS	*df*	MS	*F*	*P*
Pretreatment A (crushing)					
ES	38.13	1	38.13	0.44	0.58
ES × *ES*	153.09	1	153.09	1.76	0.32
T	3,137.27	1	3,137.27	36.02	0.03*
T × *T*	252.56	1	252.56	2.90	0.23
t	57.29	1	57.29	0.66	0.50
t × *t*	82.89	1	82.89	0.95	0.43
ES × *T*	116.64	1	116.64	1.34	0.37
ES × *t*	31.63	1	31.63	0.36	0.61
T × *t*	1.17	1	1.17	0.01	0.92
Lack of fit	1,011.52	3	337.17	3.87	0.21
Pure error	174.21	2	87.10		
Total SS	5,070.59	14			$R^2 = 0.77$
Pretreatment B (crushing and ultrasonic bathing)					
ES	131.12	1	131.12	1.80	0.31
ES × *ES*	273.77	1	273.77	3.75	0.19
T	4,007.05	1	4,007.05	54.96	0.02*
T × *T*	39.73	1	39.73	0.54	0.54
t	234.68	1	234.68	3.22	0.21
t × *t*	26.85	1	26.85	0.37	0.61
ES × *T*	1.46	1	1.46	0.02	0.90
ES × *t*	31.49	1	31.49	0.43	0.58
T × *t*	37.13	1	37.13	0.51	0.55
Lack of fit	42.55	3	14.18	0.19	0.89
Pure error	145.82	2	72.91		
Total SS	4,982.84	14			$R^2 = 0.96$
Pretreatment C (sonication without crushing)					
ES	182.60	1	182.60	1.09	0.41
ES × *ES*	224.05	1	224.05	1.34	0.37
T	462.22	1	462.22	2.77	0.24
T × *T*	146.23	1	146.23	0.88	0.45
t	42.91	1	42.91	0.26	0.66
t × *t*	26.07	1	26.07	0.16	0.73
ES × *T*	3.51	1	3.51	0.02	0.90
ES × *t*	65.98	1	65.98	0.40	0.59
T × *t*	350.69	1	350.69	2.10	0.28
Lack of fit	179.05	3	59.68	0.36	0.79
Pure error	333.62	2	166.81		
Total SS	1,975.24	14			$R^2 = 0.74$

* $P < 0.05$; *SS* sum of squares, *df* degrees of freedom, *MS* mean square, R^2 coefficient of determination

constraints. In experiments A and B, the receptacles had to be filled up to a level that covered the crushed blocks with DMSO completely without diluting the phytopigments too much. In experiment C, smaller volumes could be employed, as only the bottom face of the blocks had to be immersed in DMSO, but the volume had to be sufficient to allow introduction of the sonicator tip, and the upper limit was defined by the plate capacity. Anyway, the fact that *ES* did not have a significant effect enables the use of small volumes of extractant and consequently reduces experimental costs. Similarly, a 30 min extraction time was probably long enough to extract the maximum amount of chlorophyll-*a* under the test conditions; as a consequence, no improvement was observed at longer extraction times. Our results show that the problem of chlorophyll-*a* extraction from rock materials cannot be simply overcome by longer extraction periods, enabling the use of shorter incubation times than used by other authors [13, 17, 23].

The regression coefficients for equation Eq. 1 are presented in Table 7. These were used to construct the response surfaces for pretreatments A, B, and C, depicted in Fig. 1. The surfaces illustrate how higher temperatures, mainly, and longer extraction times, to a lesser extent, improved extraction of chlorophyll-*a* in these experiments. Visual inspection of the surfaces reveals the quadratic effects: variables with positive values for the regression coefficients of linear effects and negative coefficients for quadratic effects exhibit a maximum inside the experimental region.

Our model predicts the total weight of chlorophyll-*a* that will be extracted as a function of *ES*, *T*, and *t*. It thus allows determination of the optimum conditions at which maximum extraction will be achieved. Table 8 shows the optimal conditions for extraction of chlorophyll-*a* for pretreatments A, B, and C and also the predicted results, both in terms of total weight of chlorophyll-*a* and extraction yield. The model predicts that, under the optimal conditions, pretreatments A and B will yield the maximum weight of chlorophyll-*a* compared with pretreatment C. However, these values correspond to extraction yields superior to 100%, indicating a systematic error in the estimation of the chlorophyll-*a* concentration derived from the fact that the utilization of crushed blocks necessitates the use of large volumes of extractant, and therefore the total weight of chlorophyll-*a* is estimated from absorbances measured from very dilute solutions. This was corroborated by a complementary experiment in which the relationship between microorganism and chlorophyll-*a* concentration was studied with serial dilutions of a mixed culture with characteristics similar to those used in the optimization experiment. A strong linear relationship between concentrations of microorganism and chlorophyll-*a* was observed when all samples were considered ($R^2 = 0.99$, $P < 0.0001$), but this relationship was weakened when only the most diluted samples were taken into account ($R^2 = 0.85$, $P < 0.0001$) (Fig. 2). Thus, we conclude that the precision of the spectrophotometric method for calculation of chlorophyll-

Table 6 Estimates of main and interaction effects analysis for significant models of chlorophyll-*a* extraction after pretreatments A, B, and C

Factor	Effect (μg)	Pure error	*t*-value	*P*-value	Significance	Regression coeff.[#]
Pretreatment A (crushing)						
Mean/intercept	65.98	2.69	24.49	0.002	**	65.98
ES	4.37	6.60	0.66	0.576		2.18
ES × *ES*	−6.44	4.86	−1.33	0.316		−3.21
T	39.61	6.60	6.00	0.027	*	19.80
T × *T*	−8.27	4.86	−1.70	0.230		−4.14
t	5.35	6.60	0.81	0.503		2.68
t × *t*	4.73	4.86	0.98	0.432		2.37
ES × *T*	10.80	9.33	1.16	0.367		5.40
ES × *t*	−5.62	9.33	−0.60	0.608		−2.81
T × *t*	1.08	9.33	0.12	0.918		0.54
Pretreatment B (crushing and ultrasonic bathing)						
Mean/intercept	79.51	2.46	32.25	<0.001	***	79.51
ES	8.10	6.04	1.34	0.312		4.05
ES × *ES*	8.61	4.44	1.94	0.192		4.30
T	44.76	6.04	7.41	0.018	*	22.38
T × *T*	−3.28	4.44	1.94	0.192		−1.64
t	10.83	6.04	1.79	0.215		5.42
t × *t*	2.70	4.44	0.61	0.606		1.35
ES × *T*	−1.21	8.54	−0.14	0.900		−0.61
ES × *t*	−5.61	8.54	−0.66	0.579		−2.81
T × *t*	6.09	8.54	0.71	0.549		3.05
Pretreatment C (sonication without crushing)						
Mean/intercept	67.00	3.73	17.97	0.003	**	67.00
ES	9.56	9.13	1.05	0.405		4.78
ES × *ES*	7.79	6.72	1.16	0.366		3.89
T	15.20	9.13	1.66	0.238		7.60
T × *T*	6.29	6.72	0.94	0.448		3.15
t	−4.63	9.13	−0.51	0.662		−2.32
t × *t*	2.65	6.72	0.40	0.731		1.33
ES × *T*	−1.87	12.92	−0.15	0.898		−0.94
ES × *t*	8.12	12.92	0.63	0.594		4.06
T × *t*	−18.73	12.92	−1.45	0.284		−9.36

* $P < 0.05$; ** $P < 0.01$; [#] For coded variables

a concentration decreases when samples are much diluted, probably as a consequence of the spectrometer precision per se and the inadequacy of the equations of Wellburn et al. [19] in this range. An additional drawback derived from the use of large volumes of extractant is that there is a lower detection limit (sensitivity) bellow which fewer colonizing organisms cannot be detected [17]. Moreover, the experimental error (intrinsic and derived from device precision) is magnified when chlorophyll-*a* total weight values are inferred from large volumes. All these problems are overcome when chlorophyll-*a* is extracted and quantified from whole blocks, making the quantification of chlorophyll-*a* and the estimation of microbial biomass much more accurate. As a final consideration, this method is more convenient from a practical perspective, since it avoids labor-intensive and time-consuming crushing of rock materials.

Our results predict that direct extraction from whole blocks after ultrasonic treatment (treatment C) will enable extraction yields over 90% for chlorophyll-*a*. To experimentally confirm the prediction of the model, granite blocks were inoculated with 10 ml mixed culture of cyanobacteria of known chlorophyll-*a* content. After 2 days, chlorophyll-*a* was extracted by direct sonication of the intact blocks in 0.43 ml DMSO/cm^2 sample at 63°C for 40 min, which enabled 84.4 ± 11.6% chlorophyll-*a* recovery. Optimization of the sonication conditions (time, intensity, and/or frequency) might eventually improve the efficiency of extraction. To compare the efficacy of this protocol with other methods, we compare our results with

Table 7 Regression coefficients (b_n) and lack-of-fit analysis for the proposed regression models for extraction of chlorophyll-*a* (y_1) from rock biofilms following sample pretreatments A, B, and C

Coefficient	A y_1	B y_1	C y_1
Linear			
b_0	290.11	−455.59	−82.47
b_1	−292.83	473.55	327.27
b_2	−2.00	0.24	2.27
b_3	1.24	0.97	0.56
Quadratic			
b_{11}	83.45	−111.60	−403.82
b_{22}	0.01	0.01	−0.01
b_{33}	−0.01	0.00	0.00
Interaction			
b_{12}	0.78	−0.09	−0.27
b_{13}	−0.34	−0.34	0.97
b_{23}	0.00	0.00	−0.01

those reported by Prieto et al. [17], who applied a procedure similar to pretreatment A, in which they crushed the granite sample blocks and incubated in 0.83 ml DMSO/cm^3 sample (v/v) at 65°C for 1 h, obtaining a 68% extraction yield for the same amount of inoculated chlorophyll-*a*. The method we propose therefore implies a substantial improvement of extraction efficacy compared with previous studies.

Conclusions

From the obtained results it can be concluded that:

(a) The application of ultrasonic methods improved extraction of chlorophyll-*a* from biofilms developed on rocky substrata.
(b) The precision of the spectrophotometric method for calculation of chlorophyll-*a* concentration decreases when samples are highly diluted, thus methods requiring a lower volume of extractant are more convenient.
(c) The temperature of extraction (*T*) is the most important factor (compared with *t* and *ES*) in chlorophyll-*a* extraction from biofilms on rocky substrata.

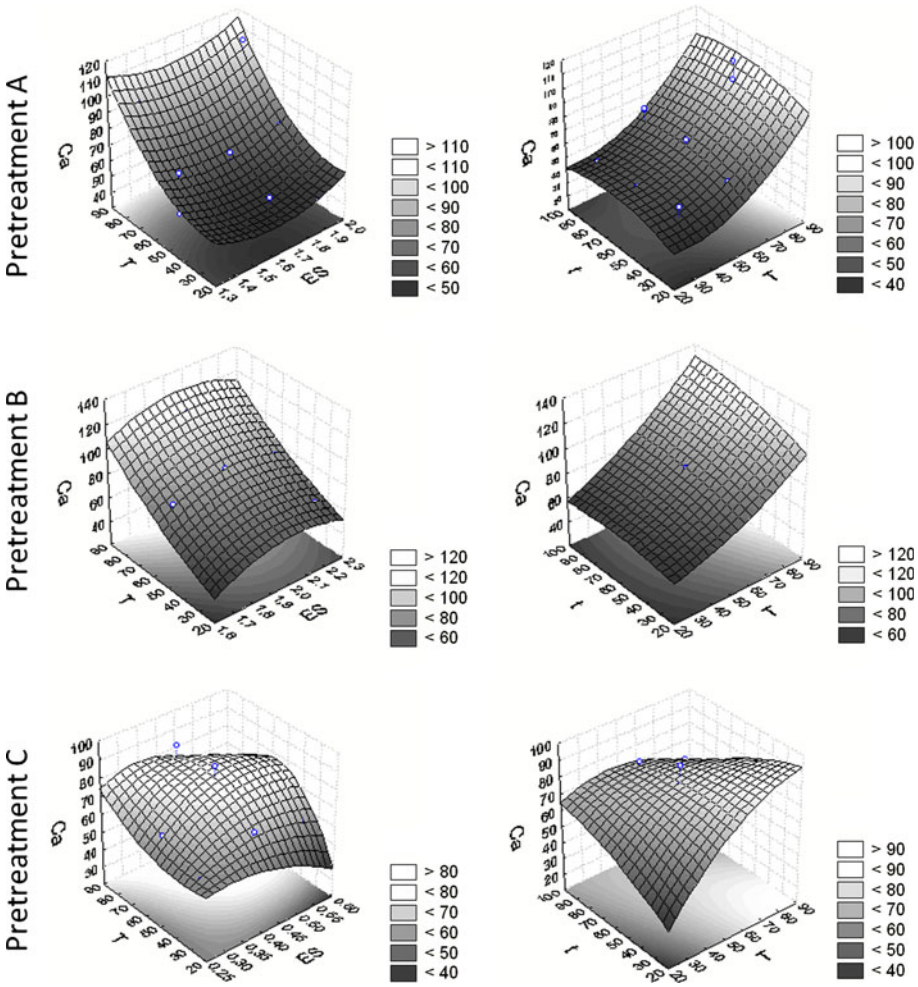

Fig. 1 Response surfaces for pretreatments A, B, and C showing dependence of chlorophyll-*a* (expressed in μg) on extractant-to-sample ratio (*ES*) and temperature (*T*) predicted for samples extracted during 60 min (*left*) and on extraction time (*t*) and temperature (*T*) for samples extracted in 1.67 DMSO:sample (v/v), 1.94 DMSO:sample (v/v) or 0.42 ml/cm^2 (pretreatments A, B, and C, respectively) (*right*)

Table 8 Chlorophyll-*a* recovery predicted by the model after extraction with DMSO under the optimal experimental conditions after pretreatments A, B, and C

	Independent variables			Dependent variables	
	ES ratio[a]	*T* (°C)	*t* (min)	Chlorophyll-*a* total dry weight (μg)	Chlorophyll-*a* extraction yield (%)
Pretreatment A	2.00	80	61	105.77	117
Pretreatment B	1.95	78	90	115.40	128
Pretreatment C	0.43	63	40	82.35	91

[a] Units are ml/cm^3 for pretreatments A and B, and ml/cm^2 for pretreatment C

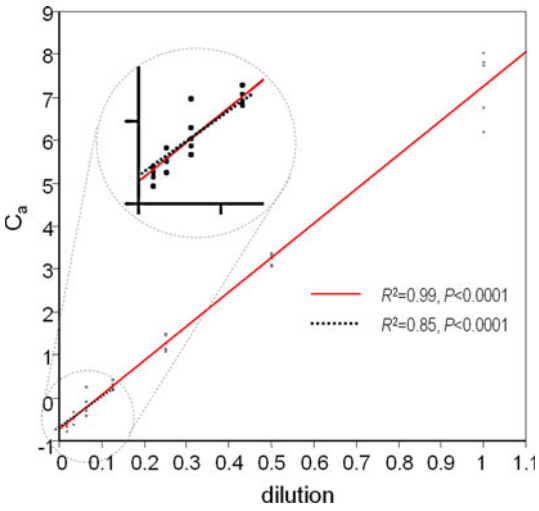

Fig. 2 Regression analysis between microorganism and phytopigment concentration. Chlorophyll-*a* concentration was estimated with the help of Wellburn's equations. Measurements correspond to serial dilutions of a mixed culture of cyanobacteria at 0.72 g dry weight/l (1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 dilutions corresponding to 0.72, 0.36, 0.18, 0.09, 0.045, 0.0225, 0.01125 g/l)

Thus, we propose the application of ultrasound directly to uncrushed rock samples followed by incubation in 0.43 ml DMSO/cm^2 sample (vol/surface) at 63°C for 40 min, as an efficient method for chlorophyll-*a* extraction from rock materials, which will allow detection of small amounts of photosynthetic microorganisms and quantification of the extent of biocolonization.

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