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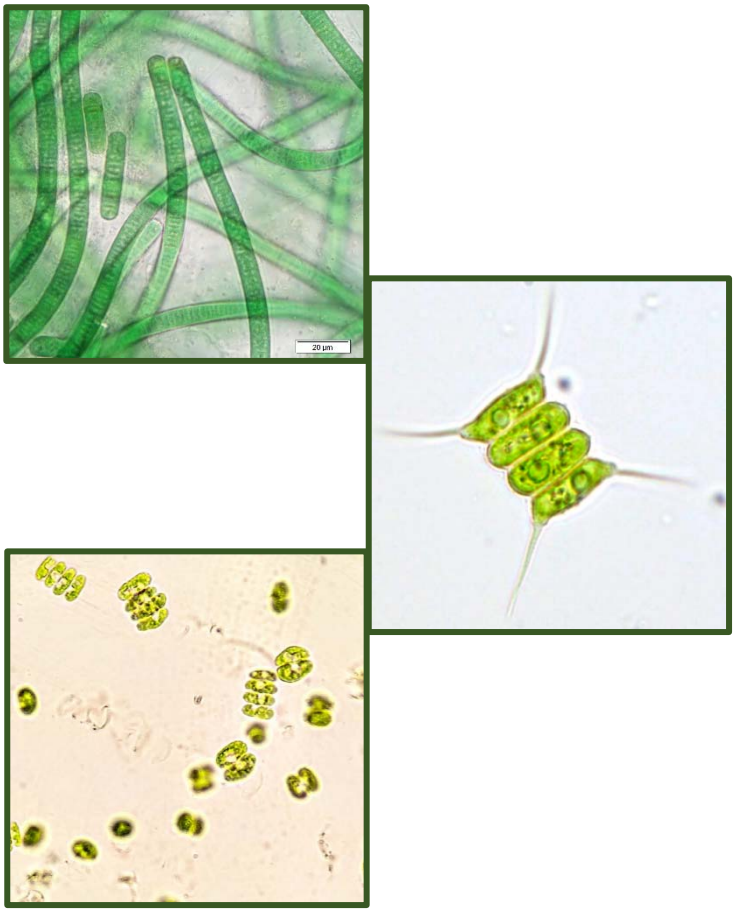
MICROALGAE PROTOCOLS II



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EDITORS

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INDEX

COLORIMETRIC DETERMINATION OF ANIONIC SURFACTANTS BY MBAS METHOD IN SURFACE WATERS AND WASTEWATER

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SUMMARY

This method is suitable for the determination of anionic surfactants in surface waters and wastewater. Anionic surfactants can be detected using a simple and precise method based on their activity with methylene blue active substance (MBAS). This method, derived and modified from IDEAM (2007), involves three consecutive extractions from an acidic aqueous solution, where an excess of methylene blue is added, into a chloroform organic phase. Subsequently, the blue color in the organic phase is measured by spectrophotometry at 652 nm (Standard Methods, 2005). However, it is important to consider the potential presence of other active substances that may react with methylene blue in the sample. Under controlled laboratory conditions, this methodology enables the precise quantification of MBAS concentrations commencing at 0.50mg/L.

INTRODUCTION

The discharge of untreated domestic and industrial wastewater into aquatic ecosystems presents a significant peril to water resources, biodiversity, and ecosystem services. Hence, it is imperative to investigate alternative techniques for wastewater treatment, including microalgae phytoremediation, which involves the removal or biotransformation of pollutants, such as nutrients and xenobiotics (Mishraa & Mohantya, 2019). The implementation of native microalgae strains in these systems may provide greater efficiency owing to their innate capacity to adapt to local conditions, resulting in better growth in untreated wastewater (Wen et al., 2017). However, given the susceptibility of microalgae to various pollutants, it is crucial to study and establish the limitations of these bioremediation approaches.

REAGENTS

- Distilled water for the preparation of all reagents and dilutions.

- Standard solution of sodium dodecyl sulfate (1000 mg/L), expressed as 1000 mg MBAS/L. The sodium salt of dodecyl sulfate ($C_{12}H_{25}NaO_4S$, MW = 288.38 g) with a purity of 96% was employed. Dissolve 1.042 g of sodium dodecyl sulfate salt in 500 mL of distilled water and dilute to 1000 mL. Store the solution refrigerated.
- Working solution of 10 mg/L of sodium dodecyl sulfate. Take 10 mL from the 1000 mg MBAS/L stock solution and transfer it to a class A 1000 mL volumetric flask. Bring to volume. Prepare this solution daily. When preparing this solution from the 1000 mg MBAS/L stock solution, it is crucial to homogenize the stock solution by bringing it to 25 °C, as slight salt precipitation may occur at temperatures below the laboratory's working temperature.
- Alcoholic phenolphthalein indicator solution. Dissolve 80 mg of phenolphthalein in 100 mL of absolute methanol.
- 1N Sodium hydroxide solution. Dissolve 40 g of 100% sodium hydroxide in 1 liter of distilled water.
- Sulfuric acid, H_2SO_4, 1N and 6N.
- Chloroform, $CHCl_3$. CAUTION: Chloroform is toxic and a potential carcinogen. Take appropriate precautions to prevent inhalation and skin exposure (Always work in an organic vapor hood and use safety equipment).
- 0.1% Methylene blue solution: Dissolve 100 mg of methylene blue (Eastman No. P573 or equivalent) in 100 mL of distilled water.
- Methylene blue reagent: Transfer 30 mL of the 0.1% methylene blue solution to a 1000 mL flask. Add 500 mL of distilled water, 41 mL of 6N H_2SO_4, and 50 g of $NaH_2PO_4 \cdot H_2O$ previously dissolved in 200 mL of distilled water. Stir until dissolved, then bring to volume.
- Washing solution: Add 41 mL of 6N H_2SO_4 to 500 mL of distilled water in a 1000 mL flask. Add 50 g of $NaH_2PO_4 \cdot H_2O$. Stir until dissolved and dilute to 1000 mL
- Hydrogen peroxide, H_2O_2, 30%.

MATERIALS

- Spectrophotometer with a 1 cm light path, to be used at 652 nm.
- Rotary evaporator.
- Organic vapor hood.
- Separatory funnels, preferably 250 and 500 mL in size, equipped with TFE stoppers and keys.
- Small glass filtration funnels with long stems.
- Class A volumetric flasks of 100, 200, 250, 500 and 1000 mL.
- Funnel supports.

- Beakers of 50, 100, 250, and 500 mL.
- Pipettes of 1, 2, 3, 4, 5 and 10 mL.
- Class A graduated cylinders of 20, 25, 50, and 100 mL.
- Glass wool or glass fiber.

Ensure all equipment is washed using soap or detergent that does not contain active substances, rinse with a diluted solution of sulfuric acid, and thoroughly rinse with abundant distilled water.

PREPARATION OF THE CALIBRATION CURVE

Prepare a series of separatory funnels containing 5, 10, 20, and 30 mL of a working solution of sodium dodecyl sulfate (SDS) at a concentration of 10 mg MBAS/L. The final concentrations of these standards after the extraction process will be 0.5, 1.0, 2.0, and 3.0 mg MBAS/L, respectively. Instead of using the suggested linear alkylbenzene sulfonate in the Standard Methods (5540 C), utilize sodium dodecyl sulfate salt for the assays. Ensure that each funnel is supplemented with sufficient distilled water to reach an approximate volume of 100 mL, as specified in Table 1.

Table 1: Calibration curve for MBAS determination.

	Concentration	Distilled water	Standard solution MBAS	Final Volume
	mg MBAS/L	**(mL)**	**(mL)**	**(mL)**
Blank	0	100	0	
STD 1	0.5	95	5	
STD 2	1	90	10	100
STD 3	2	80	20	
STD 4	3	70	30	
STD 5	5	50	50	
STD CTRL 1*	0.5	95	5	
STD CTRL 2*	2.5	75	25	

*Control standard; MBAS standard solution 10 mg/L.

Process each standard and the blank following the described procedure (treat the samples uniformly) and construct a calibration curve by plotting the absorbance against mg MBAS/L of sodium salt.

ANALYSIS PROCEDURE

Sample Extraction

Pour the indicated volume of the standard or an appropriate volume of the sample as follows: 100 mL if the expected MBAS concentration is below 5.0 mg MBAS/L, or an adequate volume to achieve a concentration between 0.5 and 5.0 mg MBAS/L. Add the necessary volume of distilled water to reach a total volume of 100 mL in a separatory funnel of either 250 mL or 500 mL capacity, as appropriate.

To each sample or standard, add 3 drops of phenolphthalein indicator, followed by a dropwise addition of 1N NaOH until a persistent pink color is obtained. Then, add 1N H_2SO_4 dropwise until the pink color disappears.

Figure 1: Neutralization (Standards with phenolphthalein indicator).

To prevent decolorization of methylene blue by sulfides, add three drops of 30% H_2O_2. Agitate the funnel and add 25 mL of methylene blue reagent along with 25 mL of chloroform. Agitate the funnel vigorously for 30 seconds, venting after each extraction, and let the phases separate. Excessive agitation can lead to emulsion formation. In case of persistent emulsions, add a small volume of isopropanol (<10 mL); the same volume of isopropanol should be added to all standards and the blank. Typically, calibration curves do not exhibit emulsion formation. Note that some samples may require a longer phase separation time than others. Before draining the chloroform layer, gently swirl and allow it to settle.

Figure 2: Standards with Methylene Blue and Chloroform (Phase separation for extraction).

Transfer the chloroform layer by pouring it into a second separatory funnel or a 100 mL beaker. Rinse the stem of the first funnel with a small amount of chloroform. Repeat the extraction two more times using 25 mL of chloroform each time. If the blue color of the aqueous phase weakens or disappears, discard it and repeat with a smaller volume of the sample. Combine all the chloroform extracts in the second separatory funnel. Add 50 mL of washing solution and agitate vigorously for 30 seconds; no emulsions should form at this stage. Allow it to settle. Extract the chloroform layer through a small glass funnel with a narrow stem containing a cotton plug into a 100 mL Class A volumetric flask; the filtrate should be clear. Extract the washing solution once more with 25 mL of chloroform. Collect the chloroform extracts in the 100 mL flask, fill it up to the calibration mark with chloroform, and mix thoroughly.

A) B)

Figure 3: A) Chloroform extracts with washing solution. **B)** Calibration curve for measurement at 652 nm.

Spectrophotometric Measurement

Turn on the UV-Vis Spectrophotometer. Ensure that the 1 cm glass cell is perfectly clean. If you observe any blue stains, leave it in soapy water, wash it with 5% HCl, and rinse it thoroughly with distilled water.

To begin the photometric readings, place the reagent blank and label it as BLANK. Proceed with the control standards in ascending order, starting from the lowest concentration, and read the samples at 652 nm.

Data Processing and Calculation of Results

The spectrophotometer provides results in Absorbance. The concentration in mg MBAS/L is determined by interpolating the measured absorbance values of the sample using the calibration curve equation while considering the dilution factor of each sample. This calculation is carried out using the following formula:

$$y = m * x + b$$

$$x = \frac{(y - b)}{m} * (Fd)$$

x = Calculated Surfactant Concentration (mg MBAS /L).
y = (Absorbance at 652 nm) Reading taken by the spectrophotometer.
b = Y-intercept.
m = Slope of the calibration curve.
Fd = Dilution factor.

RESULTS

This research aimed to evaluate the detergent tolerance of a native strain of *Chlorella vulgaris*, both in suspension and immobilized in alginate beads, to assess its effectiveness and applicability in domestic wastewater treatment.

A unialgal culture of *Chlorella vulgaris* strain LMPA-40 was obtained from the culture collection belonging to the Natural Science Faculty of the National University of Patagonia San Juan Bosco (Biological Data National System, SNDB-173). This culture was maintained on Murashige Skoog (MS) synthetic culture medium supplemented with sucrose (3% w/v) and indoleacetic acid (1 mg/L) as a growth regulator (MS, Murashige and Skoog 1962) in Erlenmeyer of 250 mL containing 50 mL of culture media. Cultures were kept at 24 ± 2°C in a shaker at 100 rpm, with

mixotrophic conditions and a photoperiod of 16 h PAR (14,000k, 400 µmol photon/ m2 /s). The experimental design involved *in vitro* culture of the microalgae and two treatments with commercially available detergents: (Det-A) for dishwashing and (Det-B) for laundry. Twenty-five and seventy-five µL of each detergent were tested by a supplement in 50 mL of synthetic wastewater (WS) (Hyungseok et al., 1999). *C. vulgaris* culture was used as inoculum to suspension cultures (20%) or immobilized in alginate beads (20%) in Erlenmeyer flasks following the methodology described in Marconi et al. (2022). The cultures were maintained as described above. Six replicates were employed for each treatment.

The concentration of anionic surfactants in each volume was determined using the protocol for MBAS determination in wastewater, resulting in 55.6 and 143.1 mg/L for (Det-A), and 13.7 and 44.2 mg/L for (Det-B). MS and WS media were used as controls.

After three days of treatment, biomass production was estimated through Neubauer chamber counting, dry weight, chlorophyll content, also, growth rate (µ), and doubling time (dt) were estimated as described in Sanchez Novoa (2020).

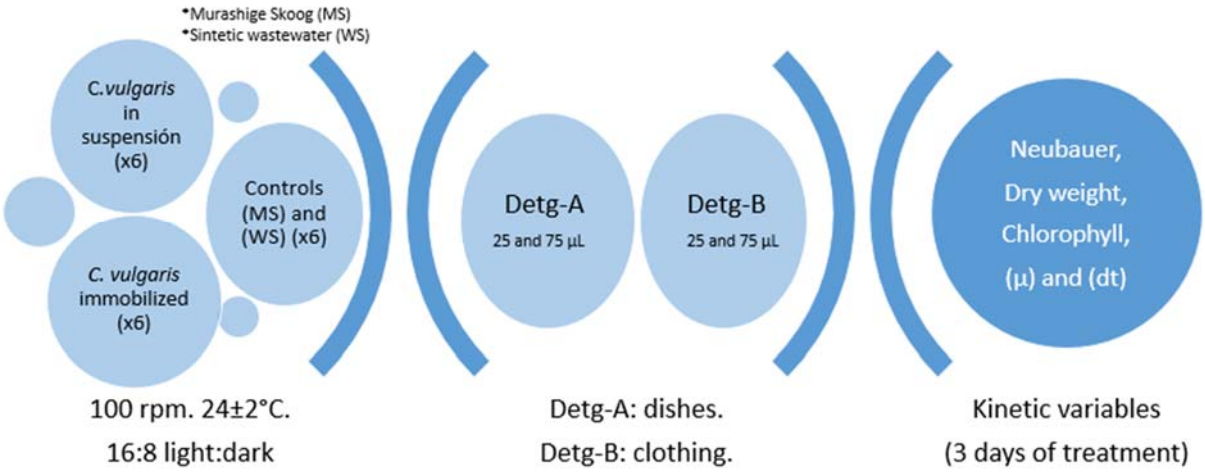

Figure 4: Methodological process diagram cultivation of *Chlorella vulgaris* with detergents.

The results indicated that algal growth was supported in (WS), except when treated with Det-A, which exhibited a toxic effect resulting in cell death (Sánchez-Novoa et al., 2023).

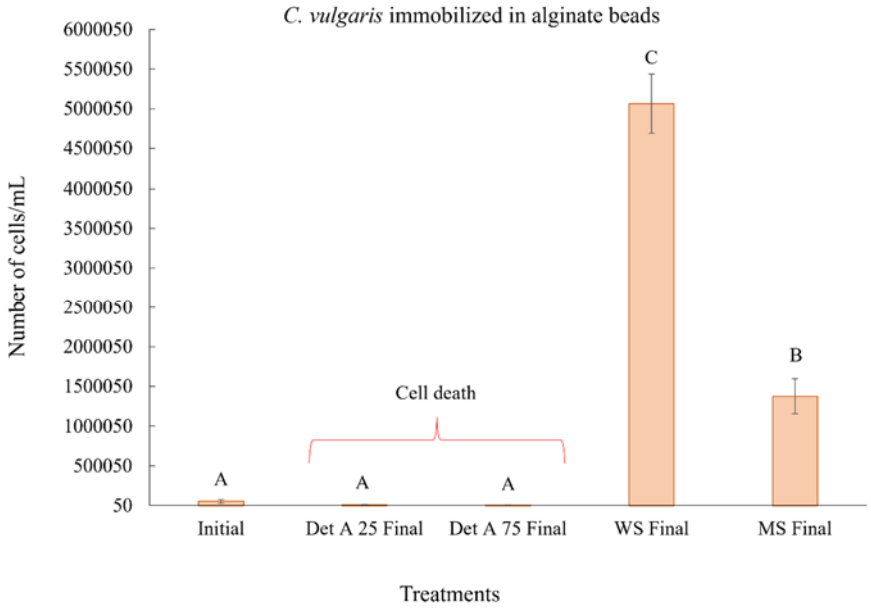

Figure 5: Biomass production of immobilized *Chlorella vulgaris* in alginate beads with (Det-A).

Comparing immobilized systems to suspension, it was observed that *C. vulgaris* demonstrated higher tolerance to Det-B in the first one. Immobilization in beads proved to be the most effective system when microalgae were exposed to detergents, achieving a dt of 0.65±0.06 and 3.02±1.7 days, and μ of 1.08±0.10 and 0.28±0.12 for low and high concentrations of Det-B, respectively. The immobilized system showed an increase in biomass production at low concentrations (2.96×10^6) as depicted in Figure 6. Additionally, there was an increase in chlorophyll content, although it was lower compared to the MS and WS controls.

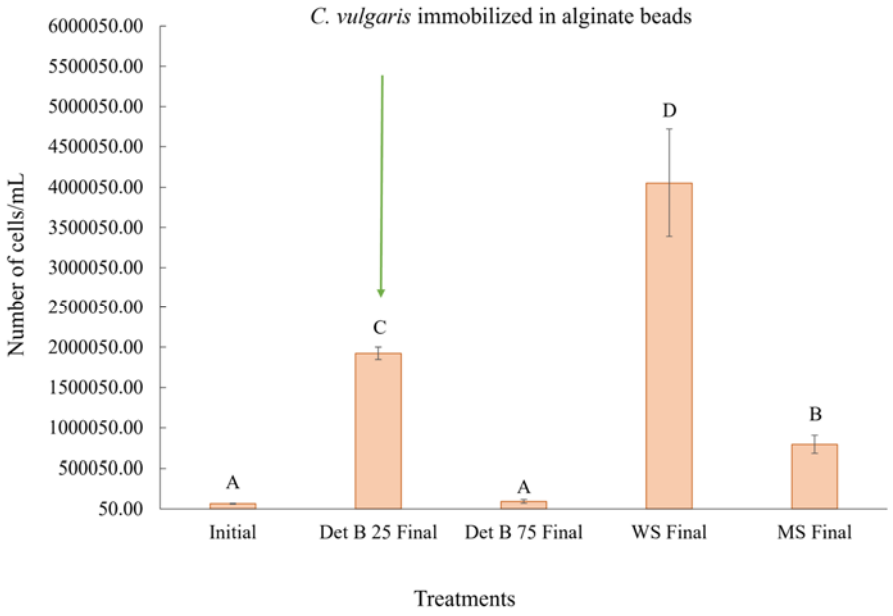

Figure 6: Biomass production of immobilized *C. vulgaris* in alginate beads with (Det-B).

In the suspension treatment, Det-B exhibited a dt of 5.20±0.95 and 10.38±1.27 days, and μ of 0.11±0.06 and 0.04±0.11 for low and high concentrations, respectively. The biomass production was (2.92 x 10^5) as illustrated in Figure 7.

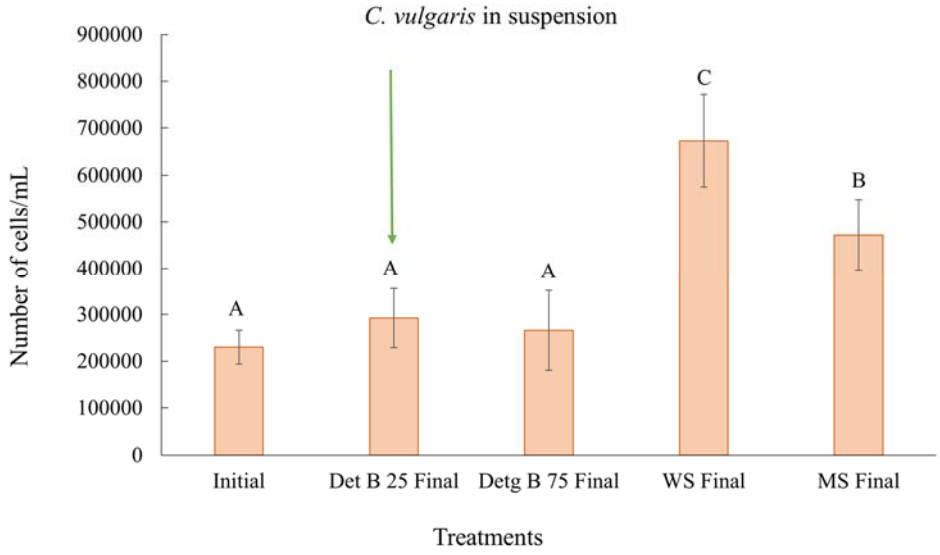

Figure 7: Biomass production of *Chlorella vulgaris* in suspension with (Det-B).

The tolerance of *C. vulgaris* to anionic surfactant substances decreases with increasing concentrations, with Detg-A reaching toxic levels beyond 50 mg/L. Nevertheless, the microalgae-based wastewater treatment system remains viable for wastewater containing MBAS levels higher than the guideline values established by the different national (Res. 79.179/90 and Law 26.221; < 5 mg/L) and provincial (ACUMAR: Res. 46/17 and ADA Res. 336/03; < 10 mg/L) that regulate the industrial and commercial discharges of liquid effluents in Argentina.

In conclusion, the results demonstrate a negative impact on microalgal biomass production when exposed to detergents in comparison to treatments without detergents.

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