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Rossana Sidari*

Department of Agraria, Mediterranea University of Reggio Calabria, Reggio Calabria, Italy

Study on mixed microalgal populations

*Correspondence: Rossana Sidari, Department of Agraria, Mediterranea University of Reggio Calabria, loc. Feo di Vito, 89122, Reggio Calabria, Italy. E-mail: rossana.sidari@unirc.it

ABSTRACT

To improve the study of mixed microalgal populations, three naturally evolved marine microalgal cultures were subjected to a light crushing mechanical treatment using a silicon spatula coupled with zymolyase treatment at four concentrations: 5, 10, 20 and 25 U/ml, for 15, 30, 45 and 60 min before being observed under a microscope. The enzyme concentration of 20 U/ml after 45 min reduces the size of macroscopic microalgal aggregates and improves the microscopic observation of the different microalgal species comprising the population. There was no improvement using the higher enzyme concentration. This paper proposes a new approach to the study of naturally evolved microalgal populations which is useful for distinguishing the morphology of the different species present in the population and allowing for the identification by classical keys, and also to obtain a pure culture from an inoculum of mixed species by using a micromanipulator for cell counting.

Key words: mixed microalgal populations, natural evolution, zymolyase, mechanical treatment, algal EPS

Introduction

Nowadays, microalgae are an interesting field to study and explore, as they allow for the production of an attractive form of alternative energy from lipids (Demirbas 2011). Due to high lipid production, marine microalgae have been considered potential sources of renewable energy (Jiang et al. 2011). The great majority of papers on this topic are about the optimization of uni-algal culture as regards growth rapidity and the high lipid accumulation in the cells (Duong et al. 2012; Lim et al. 2012; Xie et al. 2012). The use of mixed microalgae naturally evolved from water bodies resulted in more cost-effective processes. Few authors have reported about induced or natural mixed algal populations (Phatarpekar et al. 2000; Mohan et al. 2011; Wahlen et al. 2011).

This study points out some of the difficulties encountered in studying naturally evolved mixed microalgae populations, such as in the use of a spectrophotometer; in fact, the different species clump together, forming macroscopic aggregates that in some cases float and remain in suspension while in others they tend to sediment after mixing. The behaviour observed is probably attributable to the production of extracellular polysaccharides (EPS; Kristensen et al. 2008), which impairs the easy recognition of the different type of cells as well as the identification by classical keys. This makes it difficult to obtain pure cultures by micromanipulation and to achieve growth curves for the populations present in the mixed culture. Different enzymes such as chitinase, lysozyme and pectinase, coupled with other types of treatments, have been used to break down the algal cell walls to allow release of the lipid (Gerken et al. 2013). Some authors verified the effect of the treatments by microscopic observations (Allard et al. 2002; Versteegh & Blokker 2004), some others by electron microscopy (Jensen & Sicko 1971). No paper reports on the use of enzymatic treatment to attack the microalgal EPS to disaggregate the clumped cells naturally produced from water bodies.

The aim of this work is to propose an enzymatic based procedure that allows disaggregating the

78 macro-aggregates of mixed microalgal populations isolated from the marine environment in order to
79 facilitate the study of naturally evolved populations.

80

81 **Material and methods**

82 Three naturally evolved marine mixed microalgal blooms – indicated as A, B and C – were used.
83 The seawater was collected during the autumn season from different sites along the coast of Reggio
84 Calabria (Calabria, South Italy), passed through a 100 μm filter, enriched with Walne medium (1
85 ml/l) plus vitamins (0.1 ml/l) (Walne 1966) and kept at room temperature and natural photoperiod
86 conditions.

87 The samples were monitored daily by microscopic observation (Zeiss standard 20 optical
88 microscope) from the day of sampling to the time the macroscopic microalgal population
89 developed. Then, the development of the A, B and C blooms was monitored microscopically each
90 week.

91 This procedure is a mix of light mechanical and enzymatic treatment. For this purpose, a flat
92 flexible silicon spatula and the enzyme zymolyase (Sigma-Aldrich, USA) were used. The
93 zymolyase was used at the final concentrations of 5, 10, 20 and 25 U/ml. About 3 ml of each sample
94 of macro-aggregates was transferred into a weighing vessel. The sample was gently and briefly
95 (from seconds to 1 min) treated by crushing the aggregates in the weighing vessel with a flat silicon
96 spatula to reduce their size. Then, for each sample four vials were prepared as follows: 200 μl of
97 culture added to 200 μl of zymolyase solution (5, 10, 20, 25 U/ml). The samples were then observed
98 under a microscope after the addition of 400 μl of Lugol's solution to fix the cells for 15, 30, 45 and
99 60 min. Trials performed either with mechanical or enzymatic treatment were also carried out. To
100 check the maintenance of cell vitality after the enzymatic treatment, before addition of the Lugol's

101 solution, a small quantity of each sample was inoculated on solidified (agar 1.5%) seawater and
102 incubated at 25°C with a photoperiod of 18 h light/6 h dark for 20 days.

103 Bloom A was micromanipulated (MSM System 400, Singer Instrument Co Ltd, UK) in order to
104 obtain purified species. Briefly, the culture was spread on one side of Petri plates containing
105 solidified seawater (plus Walne and vitamins). Cells with different morphology were placed at
106 defined positions on the plates (according to the MSM System instruction). The plates were then
107 incubated at 25°C with a photoperiod of 18 h light/6 h dark. The two genera of microalgae that were
108 isolated were grown in enriched (Walne plus vitamins) seawater for 14 days and cell counting was
109 carried out.

110

111 **Results**

112 After one week of culturing, the three samples were composed of different genera of microalgae
113 and associated populations of bacteria. During the development, the samples turned a green/brown
114 colour and showed persistent macroscopic aggregation that impaired the normal study of the
115 cultures.

116 The samples showed a natural evolution consisting of the appearance of some species and the
117 disappearance of others until the establishment of stable mixed populations composed of four/six
118 species for each bloom. According to the morphological features, bloom A was composed of
119 *Extubocellulus* cf. *spinifer*, *Nitzschia* sp., *Navicula* sp. and *Nannochloropsis* sp. Bloom B was
120 composed of *Pseudoanabaena* sp., *Cymbella* sp., *Nitzschia* sp. and *Navicula* sp. Bloom C was
121 composed of Pseudoanabaena sp., *Nodularia* sp., *Nitzschia* sp., *Navicula* sp., *Cylindrotheca* sp. and
122 *Nannochloropsis* sp. The population of bacteria normally associated with the natural population
123 sample was more evident after the enzymatic treatment because the bacterial cells were freed from
124 the microalgal matrix.

A reduction in size of the macro-aggregates was not observed with zymolyase treatment at concentrations of 5 and 10 U/ml; thus, neither concentration resulted in a disaggregation of different microalgal genera when observed under the microscope. At concentrations of 20 and 25 U/ml there was a visible reduction in size of the macro-aggregates. As an example, Figure 1 shows sample C treated with spatula and zymolyase at a concentration of 20 U/ml on glass slides. Compared with the microscopic features of the cultures observed before the mechanical plus enzymatic treatment, the best result was obtained using the enzyme concentration of 20 U/ml after 45 min, as shown in Figures 2–4 for the three blooms A, B and C observed under the microscope, respectively. The higher concentration of the enzyme did not produce any improvement compared to the concentration of 20 U/ml (Figure 5). Also, performing either mechanical or enzymatic treatment did not give satisfactory results, as shown for bloom B as an example (Figure 6). For all the experiments under the microscope the cells observed were intact; moreover, the three blooms succeeded in growing on solidified seawater plates, demonstrating that they were not negatively affected by the enzyme. Therefore, the treatment did not cause cell lysis. Figure 7 shows the growth curves of *Extubocellulus* cf. *spinifer* and *Nannochloropsis* sp. isolated from bloom A by micromanipulation.

Discussion

The proposed method facilitates the study of mixed microalgal populations, especially for laboratories that do not have expensive technologies available. The observation of intact algal cells is consistent with the results of other authors (Allard et al. 2002; Versteegh & Blokker 2004) due to the high resistance to degradation of most microalgal cell walls. Sander & Murthy (2009) reported that, depending on the species, the inner cell wall layer – often composed of cellulose – may be enclosed in layers of other substances such as mucilage, pectin, proteins, lipids and sulfonated polysaccharides that together can make the cell wall less susceptible to enzymes. This study

provided evidence of the maintenance of cell vitality after the enzymatic treatment and of the ability of the three bloom communities to grow on solidified seawater after treatment. The reduction in size of the aggregates could be determined by the zymolyase attacking the microalgal EPS. This study is an initial contribution that proposes an approach to study mixed algal populations. Different authors have reported on the diversity of cell walls in algae (Domozych et al. 2012; Popper et al. 2014) and of the EPS in various groups of microalgae (de Jesus Raposo et al. 2013), in cyanobacteria (Parikh & Madamwar 2006) and in diatoms (Chiovitti et al. 2003; Higgins et al. 2003). This approach takes into account the possible use of different enzymes according to the differences of cell walls and EPS of the cells in the mixed population. Moreover, for the optimal results of this proposed method, it is important to highlight the role of the light mechanical treatment coupled with the use of the enzymes. In fact, the brief mechanical treatment by crushing is needed to reduce the macro-aggregates (that still remain macroscopically visible) to enable the withdrawal of the sample as homogeneously as possible to be added to the enzyme. The treatment of the sample with the spatula was only effective for up to a minute as the size of the aggregates no longer varied. Also, performing the enzymatic treatment directly was not feasible because the aggregates were too large to be homogeneously taken up using normal pipette tips.

The proposed approach is useful for distinguishing the morphology of the different species present in a natural population, allowing for their identification by classical keys; furthermore, it allows a pure culture to be obtained from an inoculum of mixed species using a micromanipulator, and allows cell counting, particularly if the bloom does not consist of filamentous species.

Acknowledgements

The author would like to thank Prof. Athena Economou-Amilli for her kind help in microalgae identification.

Disclosure statement

No potential conflict of interest was reported by the author.

Funding

This work was supported by the project funded by the European Union under grant MED-ALGAE – Production of biodiesel from algae in selected Mediterranean countries, Project reference number I-B/2.2/099.

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242 **Figure legend**

243 **Figure 1.** Marine microalgal mixed bloom (sample C) on glass slides (a) before and after (b) 15
244 min, (c) 30 min, (d) 45 min and (e) 60 min of mechanical and zymolyase (20 U/ml) treatment.

245 **Figure 2.** Microscopic observation (300×) of the marine microalgal mixed bloom A (a) before and
246 (b) after 45 min of mechanical and zymolyase (20 U/ml) treatment.

247 **Figure 3.** Microscopic observation (300×) of the marine microalgal mixed bloom B (a) before and
248 (b) after 45 min of mechanical and zymolyase (20 U/ml) treatment.

249 **Figure 4.** Microscopic observation (300×) of the marine microalgal mixed bloom C (a) before and
250 (b) after 45 min of mechanical and zymolyase (20 U/ml) treatment.

251 **Figure 5.** Marine microalgal mixed bloom (sample B): (a) on glass slide after 45 min of treatment
252 with zymolyase (20 U/ml) and (b) observed under the microscope (300×); (c) on glass slide after 45
253 min of treatment with zymolyase (25 U/ml) and (d) observed under the microscope (300×).

254 **Figure 6.** Marine microalgal mixed bloom (sample B) treated either by (a) mechanical treatment or
255 (b) by enzymatic treatment (20 U/ml) after 45 min.

256 **Figure 7.** Growth curves and microscopic images (300×) of (a) *Extubocellulus* cf. *spinifer* and (b)
257 *Nannochloropsis* sp. isolated from bloom A by micromanipulation.

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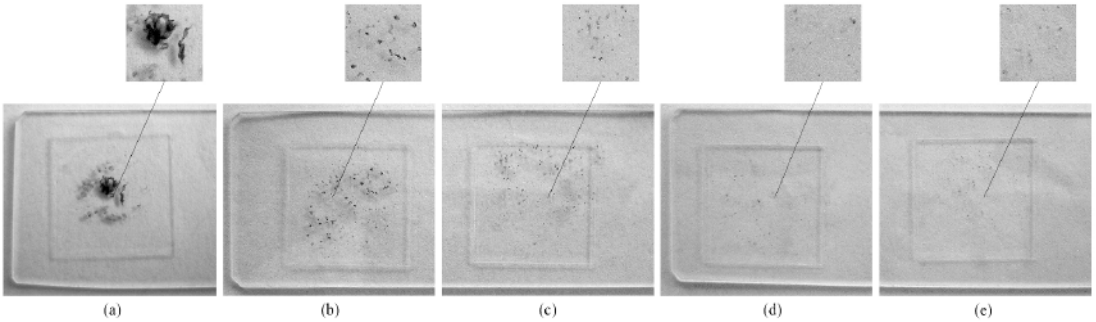


Fig. 1

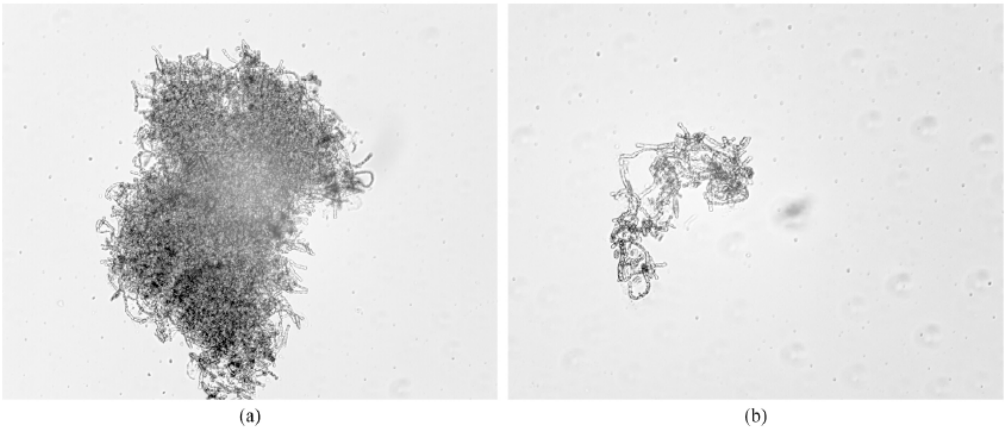
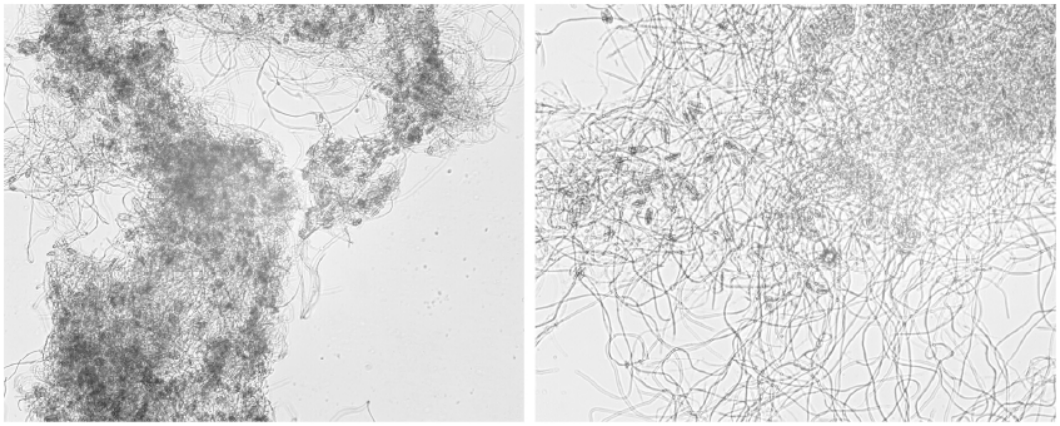


Fig. 2



(a) (b)

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275 **Fig. 3**

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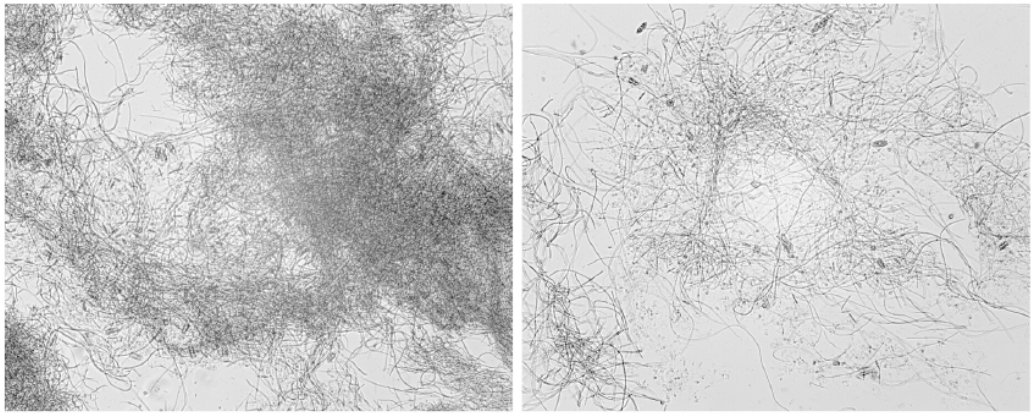

(a) (b)

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278 **Fig. 4**

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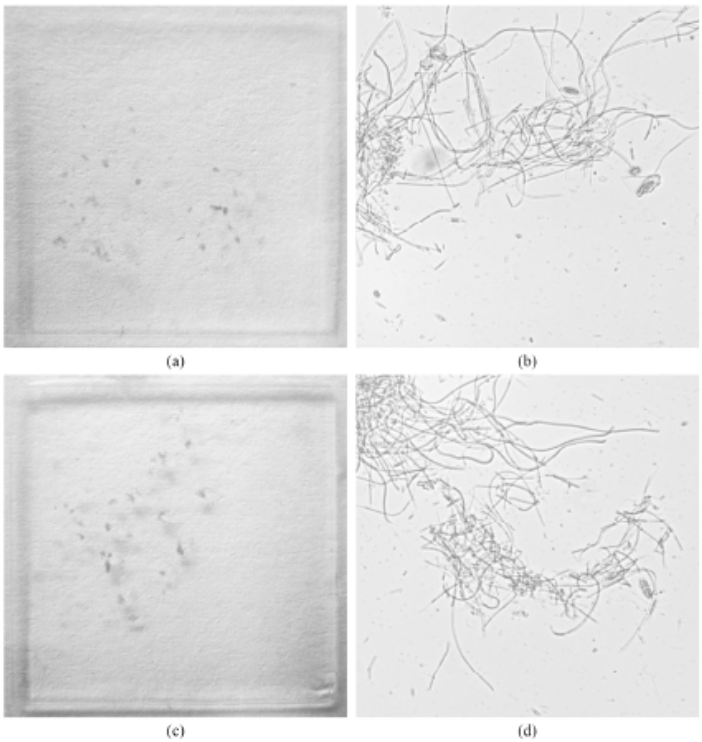

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285 **Fig. 5**

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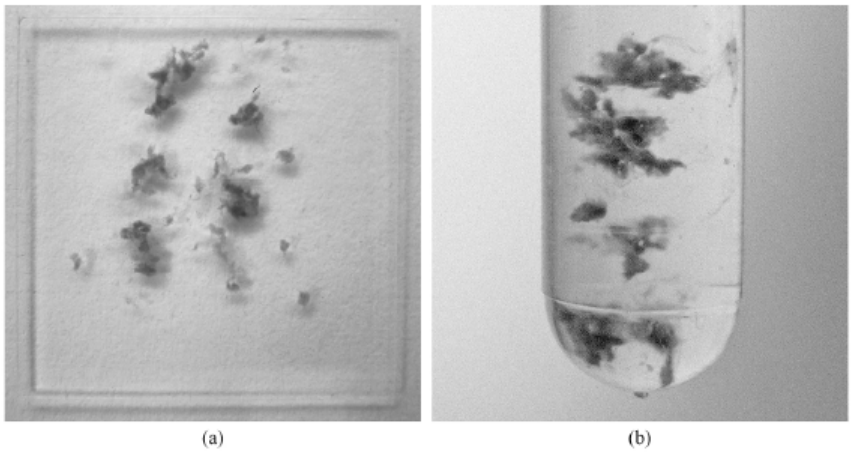

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288 **Fig. 6**

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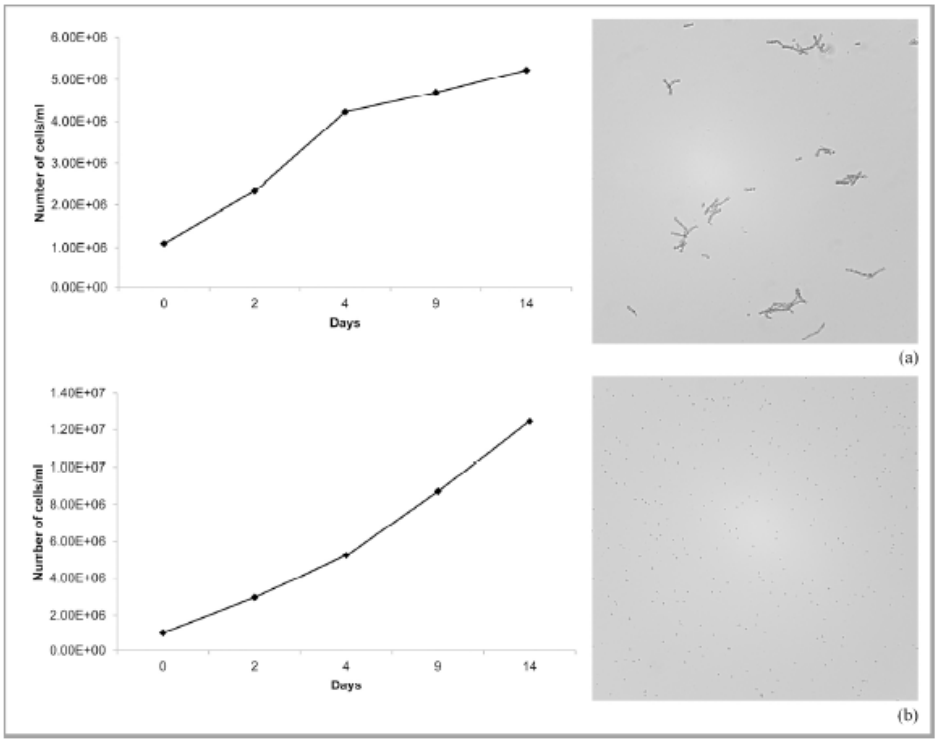

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292 **Fig. 7**

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