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ALGINATE BEADS UTILIZATION FOR LONG-TERM STORAGE OF MICROALGAL ISOLATES

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SUMMARY

Microalgae reckon one of the most promising organisms due to their numerous applications in biotechnology, such as, their biomass utilization to extract various primary and secondary metabolites. These compounds benefit the food and pharmaceutical industries and the energy field, such as, biofuel and hydrogen gas production. Therefore, it is necessary to find various sustainable ways to actively preserve the isolates and productive strains with purity for an extended period without affecting their genetic characteristics and productive ability to grow and regenerate. The latest study aimed to compress several microalgae to form alginate beads using sodium alginate with five types of pure algal cultures, i.e., *Scenedesmus quadricauda*, *Scenedesmus dimorphus*, *Chlorella vulgaris*, *Chlorococcum humicola,* and *Chlamydomonas* sp. The vitality and activity of all the strains studied through the storage period showed the success of preparing alginate beads and staying viable for 18 months at 4 °C in the refrigerator under dark conditions. Therefore, encapsulating microalgae with sodium alginate is a possible and helpful method for preserving algae isolates for a prolonged period in a pure form. The survival of algae in alginate beads is an essential step to apply in the future as one of the viable methods to preserve pure algae isolates for a long time.

Keywords: *Scenedesmus*, *Chlorella, Chlorococcum, Chlamydomonas*, microalgae, alginate bead, sodium alginate, long-term storage

Key findings: Encapsulating microalgae with sodium alginate is a possible and valuable method for preserving algae isolates for a long time in a pure form. The process successfully prepared alginate beads and stayed viable for 18 months at 4 °C in the refrigerator under dark conditions.

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INTRODUCTION

Maintaining a microalgae culture is monotonous and a time-consuming laboratory work. Therefore, researchers pursued developing new technologies for preserving these microorganisms (Abreu *et al*., 2012). In recent years, microalgae usage in biotechnology has improved because small single cells and updated cell-freezing techniques have evolved to solve these obstacles in the biotechnological processes for these algae (Moreno-Garrido, 2008; Kareem *et al*., 2022).

The long-term stability and vitality of microalgae populations' maintenance cannot prosper in serial sub-cultivation with liquid and solid media because of the labor-intensive contamination risks and genetic alteration. Therefore, cryopreservation is the only way to preserve microalgae cell viability at low temperatures for an extended term. The technology also protects them from genetic changes and requires minimal maintenance, storing them under appropriate conditions that minimize the risk of contamination by other microorganisms (Demirel *et al*., 2018). Controlling microalgae includes preventing the free movement of biological matter, with possible application to bacteria, yeasts, molds**,** and algae.

The first time freezing of algae cells occurred in the mid-1980s. The application of agar and alginate used granules with a bead-like structure, which is the most common form for application (Goss *et al.,* 2020). In stock culture and long-term storage of algae seeds for producing biomass and secondary metabolites, the recommended extended preservation of algae cultures is in sodium alginate beads form. Such a method of algae preservation can also aid wastewater treatment and heavy metal removal (Santhoshkumar *et al*., 2016).

Alginate dependence was superior to agar concerning long-term storage (Kaur *et al*., 2019). The fixation and encapsulation of microalgae comprised the cell retention techniques that helped fix the algae. Polymers, such as calcium alginate, and hardening agents, such as $CaCl_2$, form a hydrogel bead where algae can quickly grow. Thus, separating algae beads can be fast during laboratory experiments (Sewiwat *et al*., 2016). Sodium alginate is one of the most widely used polymers in formal algae immobilization, and inactivated microalgae have been beneficial in removing nutrients in wastewater (Soo *et al*., 2017). Romo and Pherez-Martinez's (1997) study proved successful with their assessment of a new method for long-term storage of algae cultures, wherein the laboratory relies on cell encapsulation technology. Encapsulating the filamentous cyanobacteria *Pseudanabaena galeata* cells in sodium alginate gained storing in the refrigerator for 14–18 months**.** Santhoshkumar *et al*. (2016) indicated the possibility of lengthened storage of the alga *Chlorococcum humicola* by regulating algae cells in sodium alginate granules, which showed viability for up to 14 months.

Fixing microalgal strain cells in alginate beads gave success in preserving microalgae isolates, such as, *Chroococcus* sp., *Anabaena variabilis, Oscillatoria tenuis, Chlorella vulgaris, Chlorococcum humicola, Scenedesmus bijuga,* and *Selenastrum bibrainum* (Goss *et al.,* 2020). The concerned study sought to prepare industrial seeds from sodium alginate of five types of microalgae in beads-like structure and then store them in the refrigerator for a long time. The second part of this study focused on following up on the numbers of growing cells from those algae fixed in alginate beads by cultivating them in an algae medium every three months to ascertain their ability to grow again after long-term storage.

MATERIALS AND METHODS

Microalgae used

The five algal isolates (*Scenedesmus quadicoda*, *Scenedesmus dimorphus*, *Chlorella vulgaris*, *Chlorococcum humicola,* and *Chlamydomonas* sp.) procured from several sources served as samples in the present research (Table 1).

Table 1. Microalgae isolates used in the research.

No.	Isolation name	Environment	Source
1	*Chlorella vulgaris*	Egyptian	Algae Biotechnology Unit/National Research Center, Cairo, Egypt
2	*Chlorococcum humicola*	Iraqi	Baghdad University, Iraq
3	*Scenedesmus dimorphus*	Egyptian	Mansoura University, Egypt
4	*Scenedesmus quadicauda*		
5	*Chlamydomonas* sp.		

Preparing alginate beads

The use of sodium alginate aided in preparing the artificial seeds of the microalgae. The preparation of 3% sodium alginate ran in the Chu 13 medium (free of $CaCl_2.2H_2O$) with continuous stirring at 60 °C in a water bath, then, adjusting the pH to 5.8, sterilizing the mixture continued in an autoclave for 20 min, based on the methodology by Santhoshkumar *et al*. (2016) and Al-Mula (2022). Taking 5 ml from each pure liquid culture of microalgae at the age of two weeks underwent centrifuging with added speed and condition of centrifugation. The sediment washing followed with distilled water twice, with 1 ml of sterile distilled water added to the cell pellet, then mixed with 20 ml of sanitized sodium alginate solution earlier prepared. Then, dropping the mixture into a disinfected solution containing 0.2 M calcium chloride, used a sterile pipette of beads-shaped loaded with algae cells. After leaving the beads for 30 min to harden, washing them with sterile distilled water 3–4 times took place. Then, drying proceeded under aseptic conditions on sterile filter paper.

Testing alginate bead's vitality after a long period

The alginate beads' viability and activity testing transpired every three months. Five beads underwent cultivation on 50 ml of Chu 13 medium for 20 days. The number of cells counted growing in the medium per 1 ml received evaluation for their vitality after each incubation period (Santhoshkumar *et al*., 2016).

RESULTS AND DISCUSSION

Formation of alginate beads

Encapsulation of microalgae using sodium alginate as industrial seeds can be a profitable method, as it requires less energy and is easier to handle. Pure cultures and re-seeding stocks and microalgae contribute to various applications for extended storing while maintaining structural integrity of cells. Their vitality and natural physiological activities over a long period removed long-term storage culture methods for pure isolates, which can be an alternative to frequent re-cultivation to maintain the energy of microalgae and reduce pollution, costs, and efforts.

Engaging various technologies included phytoremediations, algae treatment, the production of important secondary metabolites from algae, and biofuel and hydrogen production. Despite the existence of limited studies in this aspect, the recent research successfully prepared alginate beads in a sterile manner for five isolates of microalgae, i.e., *Scenedesmus quadicoda*, *Scenedesmus dimorphus*, *Chlorella vulgaris*, *Chlorococcum humicola,* and *Chlamydomonas* sp**.** The results showed that the alginate grains formed were spherical to oval when dropped into a calcium chloride solution (Figure 1). The diameter of those beads ranged between 6–7 mm and appeared homogeneous regarding the distribution of algal cells inside said beads (Figure 2). Immobilizing the microalga *Scenedesmus quadricauda* for cultivation in wastewater indicated that the beads' diameters were 3.4, 4.0, and 4.7 mm (Porkka, 2021).

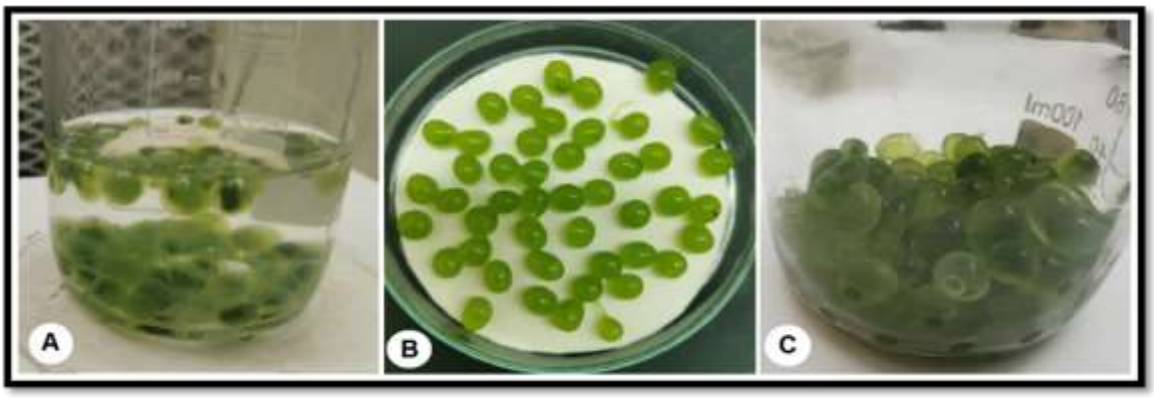

Figure 1. Stages of preparing artificial seeds loaded with microalgae.
A: Dropping artificial seeds into a solution of calcium chloride at a concentration of 0.2 M.
B: Dried industrial seeds on sterile filter paper after washing them with distilled water 3–4 times.
C: Industrial seeds in sterilized glass container at 4 °C.

Figure 2. Shape of the beads and their diameters ranging between 6–7 mm.

The difference in the size of the beads was due to the diameters of the holes prepared in the pipette before dropping the beads into the sodium chloride solution (Al-Mula, 2022).

Testing the vitality of microalgae inside alginate beads

When studying the vitality of algae encapsulation with sodium alginate, the results showed the stages of releasing the algae from the alginate beads with time during growing the algae on Chu 13 medium for testing its vitality after every three months (Figure 3). Developing algae, after 20 days of incubation (Figure 4), revealed the algae used were able to grow after being cultured on the medium for regular periods until the last 18th month. The results also showed the number of microalgae cells grown on the culture medium to detect their strength (Table 2). A continuous decrease in the number of cells to each ml^{-1} after each storage period, even reaching its lowest level in the last month (18th), emerged (Figure 5).

The number of developing cells also decreased to their lowest numbers resulting from the continuous decline during the storage period as earlier discussed, indicating that the physiological state of the algae cells had the storage passing of time affecting it, or perhaps due to the accumulation of carbon dioxide gas in the storage vessels during the continuous respiration process. Moreover, the components of a culture medium may have influenced the

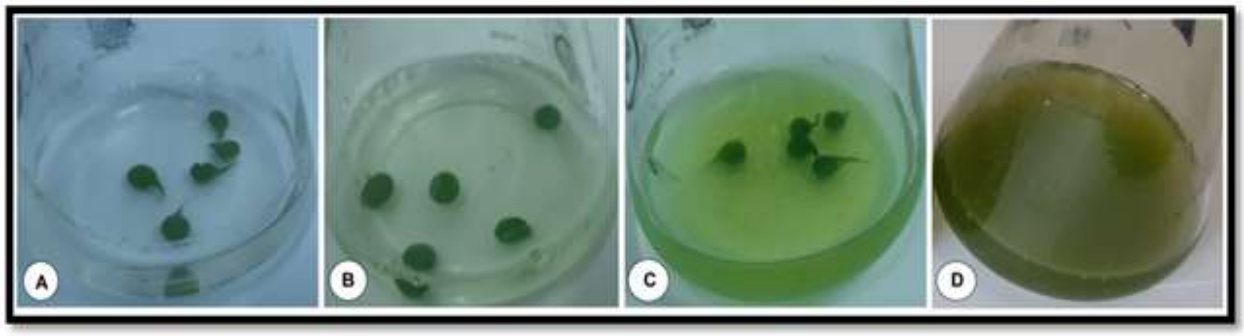

Figure 3. Growth stages of industrial seeds loaded with microalgae after cultivating them on Chu 13 medium**.**
A: Five beads cultured on Chu 13 medium.
B: Algae growth and its liberation from artificial seeds after 5 days on Chu 13 medium.
C: Growth and development of algae and their liberation from artificial seeds after 10 days on Chu 13 medium.
D: Growth and development of algae and their liberation from artificial seeds at 20 days on Chu 13 medium.

Figure 4. Growth of the algae under study at 20 days after their liberation from alginate beads.

Table 2. The number of algal cells under study calculated over the course of 18 months ($2.5–3.5\times10^6$ cell ml^{-1}).

Number of months	Microalgae isolate				
	S. quadricauda	*S. dimorphus*	*C. vulgaris*	*Chlo. humicola*	*Chlamydomonas* sp.
3	15.52×10^6	15.84×10^6	17.60×10^6	12.48×10^6	12.32×10^6
6	14.24×10^6	11.87×10^6	13.92×10^6	09.92×10^6	09.60×10^6
9	13.44×10^6	09.42×10^6	08.48×10^6	08.44×10^6	07.04×10^6
12	07.04×10^6	05.12×10^6	03.68×10^6	06.56×10^6	05.12×10^6
15	05.60×10^6	04.21×10^6	01.54×10^6	03.90×10^6	03.09×10^6
18	02.69×10^6	02.81×10^6	00.34×10^6	01.77×10^6	00.66×10^6

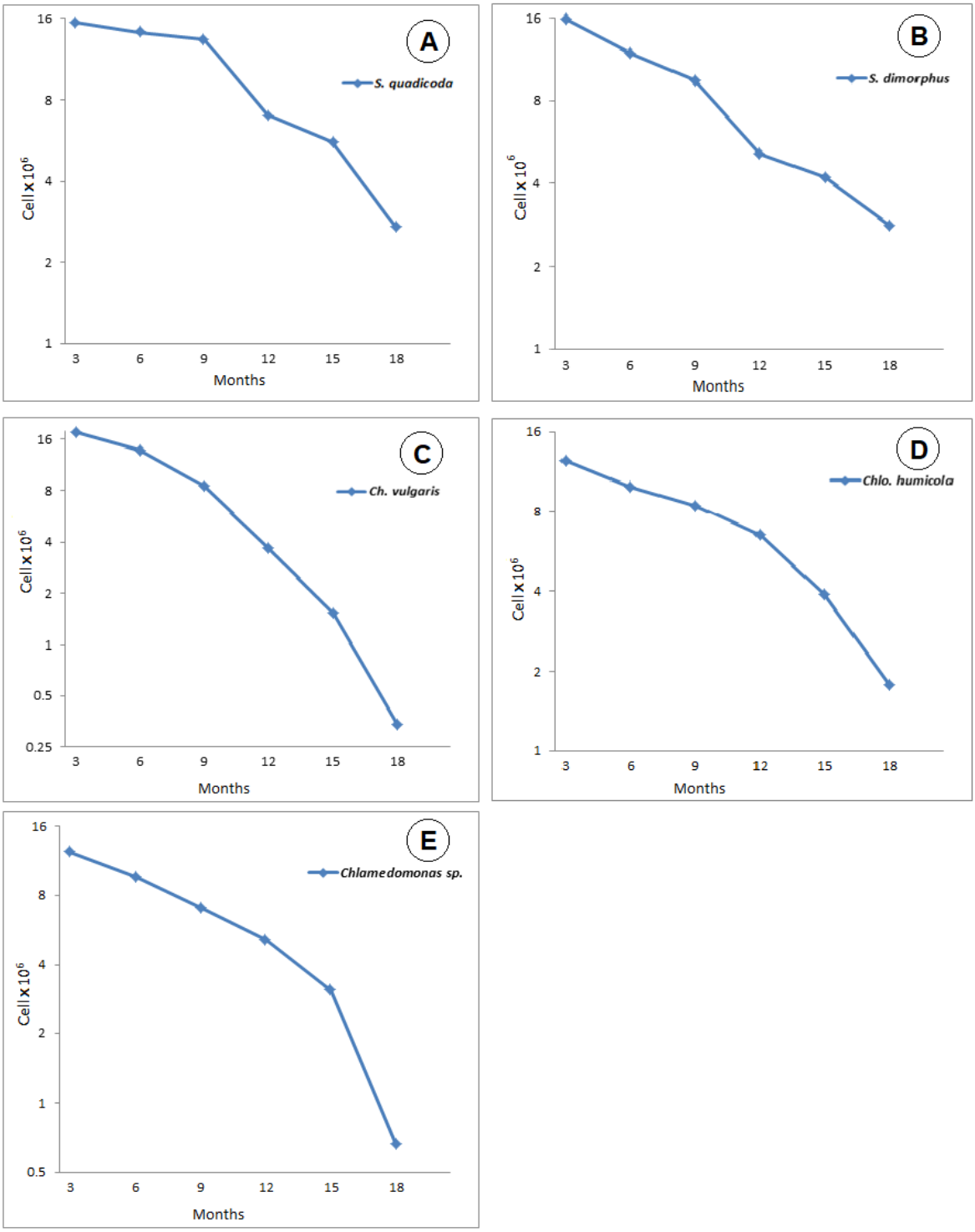

Figure 5. The number of growing microalgal cells after each storage period for 18 months.
A: The number of *S. quadicoda* cells decreased progressively throughout the storage period.
B: The number of *S. dimorphus* cells decreased progressively throughout the storage period.
C: The number of *Chlo. humicola* cells decreased progressively throughout the storage period.
D: The number of *C. vulgaris* cells decreased progressively throughout the storage period.
E: The number of *Chlamydomonas* sp. cells decreased progressively throughout the storage period.

preparation of alginate beads (Chu 13), as compared with the study of Santhoshkumar *et al*. (2016), who observed the stability in the number of growing cells of the alga *Chlo. humicola* during a bioassay of alginate beads for 14 months using a BBM medium.

For the number of cells, differences occurred between the types of microalgae in terms of cell number and their ability to persist with the immense number of cells from the first month until the last month (Figure 5, Table 2). In the 18th month of storage, notably, the best of these algae were *S. dimorphus* and *S. quadicoda*, which came close in the number of developing cells in the last month at 02.81×10^6 and 02.69×10^6, respectively, perhaps due to their belonging to the same genus, followed by the alga *Chlo. humicola.* Based on the lowest numbers of developing cells, it came from the alga *Chlamydomonas* sp. and *C. vulgaris* at 0.66×10^6 and 0.34×10^6, respectively, which appears more sensitive to survival for a lengthy period in alginate beads compared with the rest of the algae under study.

The studied algae proved their ability to remain vigorous for 18 months, a relatively extended period. Senko *et al*. (2022) found that using PVA cryogel (polyvinyl alcohol) to immobilize the cells of various phototrophic microorganisms led to successful storage for two years at -20 °C without reducing their metabolic activity. Other studies also indicated that the algae *Euglena gracilis* fixed in alginate beads remained active for over two years (Abdel-Hameed and Hammouda, 2007). The findings of Romo and Perez-Martinez (1997) signified that the algae *Pseudanabaena galeata* (Cyanobacteria) loaded on alginate beads, and stored for a long time, maintained its viability. Yean-Chang (2001) also kept the green alga *S. quadricauda* as alginate beads for a long time, where the cells retained their physiological activity and survival for three years in complete darkness. It means that cells can maintain their physiological activities for the long term by consuming their reserves of pyrenoids (Moheimani *et al*., 2015). Yean-Chang (2001) also confirmed the survival of alga *Isochrysis galbana* cells actively loaded with sodium alginate after a year of storage, which is the same period as mentioned by Abdel-Hameed (2005) on algae *C. vulgaris* cells encapsulated by alginate.

CONCLUSIONS

The results revealed that encapsulating the microalgae with alginate sodium is possible with great benefits to preserve the algal isolates for a long time in a pure form. Despite the decrease in the number of cells during the storage period, the survival of algae active in the alginate beads is an essential step for its application in the future.

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