Plant Archives Vol. 20, Supplement 1, 2020 pp. 2866-2868 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

ISOLATION AND CULTURE OF PROTOPLAST FROM *CHLOROCOCCUM HUMICOLA*

T.A. Al-Someidae*, Q.S. Al-Nema and Y.J. Al-Shahery
Department of Biology, College of Education for Pure Science, Mosul University, Iraq.
*Corresponding author Email: tahaalsomeidae@yahoo.com

Abstract

The present study was succeeded to isolate protoplast from microalgae *Chlorococcum humicola*. Four enzymatic solutions were tested to analyze digest cell walls. We have found that the solution, which consists of 2% Celullase R-10, 1% Pectinase, 1% Macerozyme R-10, releases protoplast with yield 1.2 x 10^6 cell ml^{-1} and viability 98.13 %. In this study, we also reculture protoplast on agar-solidified Knop-M medium giving regeneration rate of up to 100 %.

Keywords **:** Protoplast isolation, *Chlorococcum humicola*, viability, regeneration.

Introduction

Microalgae are the largest nutrients in the natural aquatic food chain and aquatic animal farms (Kusumaningrum & Zainuri, 2018). The algae has a simple structure, short life cycle, easy physiological and biochemical study. It also grows rapidly under normal dietary conditions. Therefore, the algae plays a vital role in production of many biologically active natural compounds, such as proteins, carbohydrates and fats to be as source of renewable energy (Abo-Shady, 2008). Its simple structure, short life cycle, easy physiological and biochemical study, and its rapid growth under normal dietary conditions make it an important biomass resource in the production of many biologically active natural compounds such as proteins, carbohydrates and fats, which are a good source of renewable energy such as biofuels (Abo-Shady, 2008). In addition, the algae are one of the most important green resources commercially and artificially. It provides the highest and cheapest biomass per unit of light and area, or analyzes other toxic pollutants in water, reduces the level of CO_2 in the atmosphere through the process of carbon stabilization. Also, several pharmaceutical products are extracted from the algae (Santhoshkumar, *et al*. 2016). Among these microalgae, *Chlorococcum humicola*, which belongs to the single-cell freshwater algae of the chlorophyta algae department (Bhagavathy *et al*., 2011).

Protoplast isolation technique has been applied in many microalgae and somatic hybridization between similar or different genus for improving the production of activated and important secondary metabolites compared to pathogenic strains algae *Haematococcus* (Tjahjono *et al*., 1994). The protoplasts has been isolated from *Chlorella* and *Dunalilla* (Kusumaningrum and Zainuri, 2014) and from *Penium margaritaceum* (Raimundo *et al*., 2018). Another study was succeeded to isolate protoplast from *Chlorella* and *Dunaliella* (Kusumaningrum & Zainuri, 2014). In other hand the protoplasts was isolated from *Penium margaritaceum* (Raimundo *et al*., 2018). Abo-Shady (2008) has referred to the possibility of isolating protoplast from *Chlorococcum* algae and he obtained 98% by Celullase R-1. However, isolation of protoplast from microalgae is still unclear. Therefore, the aim of this study is detect a protoplast for isolation of viable protoplast particularly from *Chlorococcum humicola*.

Materials and Methods

Microorganism and Cultivation Conditions

Chlorococcum humicola was obtained from the department of Life Sciences, College of Education, Ibn al-Haytham, University of Baghdad, Iraq. This algae was grown in the rich Chu-13 medium at 25 °C in glass flasks with a capacity of 250 ml and 500 ml of the development medium/flask (Yamaguchi *et al*., 1987).

Microalgae media : we dissolved the medium components, Chu-13 (KNO_3 400, K_2HPO_4 800, $CaCL_2$ 107, $MgSO_4.7H_2O$ 200, Citric acid 100, Ferric citrate 100, H_3BO_3 5.72, $CoCl_2$ 0.02, 4.4 $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ 0.16, $NaMoO_2$ 0.084, Mg/l) in an appropriate volume of distilled water. Then, we added a drop of H_2SO4 at a concentration of 0.072 N, and completed the final volume to 1 L pH 7.5. We distributed the agricultural medium on glass flasks in different sizes volume 20%, and then we sterilized the solution using autoclave at 121 °C, pressure 1 par. The culture media was inoculated with 5% for 6-7 days and then incubated in the shaking incubator at 25 °C. /100 shakes min, for 30 days. The cells were then harvested for more analysis (Yamaguchi *et al*., 1987).

Protoplast Isolation: Protoplast was isolated from algae C. *humicola* by centrifuging the cells cultures (10^6-10^8 cell/ml^{-1}) at a volume of 4 ml at speed (2600 rpm) for 5 minutes (Lu *et al*., 2011; Kusumaningrum & Zainuri, 2018). Pelleted cells were re-suspended in 4 ml of in 25 mM Tris buffer (pH 6.0) containing 0.6 M D-mannitol and centrifuged at 2600 rpm. 4 ml of the enzyme mixture solution were added to the supernatant in 9.0 cm dima. Plastic petri-dishes. Three enzyme mixture were prepared (Table 1) at pH 5.8, and filter sterilized using Millipore filter of 0.22 µm size pore (Lu *et al*., 2011).

Table 1: Enzymes solutions utilized in isolation of protoplast from *C. humicola* .

Enzymes / Mixture	Macerozyme R-10	Pectinase	Celullase R-10
1.0	1.0	0.0	0.0
2.0	1.0	1.0	0.0
3.0	1.0	1.0	1.0
4.0	2.0	1.0	1.0

Samples were stored at 30 °C for 12 hours and then 24 hours in the dark. The enzyme-protoplast mixture was centrifuged at 1,500 rpm for 2 min, and the supernatant was removed. Subsequently, protoplasts wear suspended in Tris-HCl buffer at 4 °C until use. (Lu *et al*., 2011).

The characterization of isolated protoplast: Structural protoplast volume was tested by adding 10 microliter of protoplast suspension to a glass slide. Cell dimensions and shapes were measured using X4, 10X and 40X magnification by using ocular stage.

Protoplast viability test: Phenosafranin staining was used to estimate the protoplast viability by staining the dead cells in red colors, the prepared stock solution at a concentration of 1 mg/ml was stored at 4 °C until the use. The dye was added to the protoplast suspension to obtain a final concentration of 0.01%. The samples were left for 5 minutes at room temperature, and then examined using a composite optical microscope to find the percentage of viability by finding the percentage between life cells and the dead (Lu *et al*., 2011).

Protoplast regeneration: we Prepared Knop-M-Agar solid medium by dissolving 1 gm

$Ca(NO_3)_2.4H_2O$, 0.25 gm KCl, 0.25 g KH_2PO_4, 0.25 gm $MgSO_4.7H_2O$,0.0125 gm $FeSO_4.7H_2O$, 91.35 gm D-mannitol in one liter of distilled water, pH 5.8. 15 gm agar were added to the solidified medium then sterilized at 1 bar 121 °C. (Hohe & Reski, 2002). The concentration of the obtained protoplast suspension was adjusted to 5×10^3 ml^{-1} cells, and then 100 microliters were Spreader disseminated on the surface of the regenerating medium in petri-dishes (9 cm). To investigate the effect of mannitol on protoplast regeneration, the D-mannitol-free Knop-Agar medium was used as a control medium. The protoplast was incubated at 30 °C and 2000-3000 LUX with 16: 8 hours of light and darkness respectively for 10-20 days. The number of renewed protoplasts was calculated as individual colonies on petri dishes.

Results

Protoplast Isolation: The results showed the successful isolation of the protoplast from *C. humicola* cells in terms of the results of the protoplast isolated by the enzymatic mixture used which amounted to 1.2×10^6 mL cells (Table 2). The light microscope also showed protoplast wear spherical as an oval shape (Fig. 1: a, b), and the diameter rang of isolated protoplast was 10.5-15.6 µm, compared to the diameters 6.5-10.4 µm. in the original cells.

Table 2: Protoplast yield using different enzymatic solutions.

Yield cells /ml^{-1}	Viability %	Releasing time %	Enzymes / Mixture
0.0	0	-----	1
0.0	0	-----	2
1.6×10^4	70	24 hour	3
1.2×10^6	98.13	24 hour	4

Protoplast viability: The results showed that the living protoplast was colorless compared to the dead colored protoplast (Fig. 1: d, c) and the viability ratio was 98.13%.

Protoplast regeneration: The culture of protoplast in agar solidified Knop-M medium showed initial cleavages to the formation of individual colonies of *C. humicola* (Fig. 1: e). After 20 days of incubation, the number of cells ranged was from 560 cells, in the rate of 100% of the cultured protoplast cells. The results showed that the protoplast did not grow on the Knop medium without osmosis factor (D-mannitol) compared to the original cells (Table 3).

Discussion

The current study was focused on the isolation of protoplasts from microalgae *C. humicola* cells. This study is a unique one in the field of isolation of protoplast from unicellular algae in our country Iraq. The adoption of several enzymatic solutions, some of which are of two or more types, all of which are available enzymes Cellulase and Pectinase is necessary for the selection of the appropriate enzymatic solution (AL-Nema, 2013). The enzyme potential of 2% Celullase R-10, 1% Macerozyme R-10 and 1% Pectinase in the presence of 10.9% D-mannitol in attaining

The highest protoplast yield compared with the rest of the solutions used in the study is likely to attribute different enzyme types and concentrations to the resulting interference. It also explains the survival of this protoplast retaining its vitality and its spherical shape after its plasma membrane exposed to ocean conditions and not bursting to the appropriate concentration of added mannitol to maintain the osmotic pressure in the isolation conditions of this protoplast.

Table 3 : Effect of Mannitol on Regeneration of Isolated Protoplast and Progenitor Cells.

Type of transplanted cells	Knop-M-Agar Medium	Knop-Agar-Medium
	Number of developing cells/petri dish	Number of developing cells/ petri dish
Protoplast cells	≈560	No growth
Original cells	510≈	530≈

*The number of transplanted cells ranges between 450-550 cells.

The protoplast is not grown on the mannitol-free regeneration medium due to cell burst, once of the medium is in contact with the water, the gradient of the plasma membrane between the protoplast cells and the components of the medium and the absence of a cell wall protects the protoplast cells from the effect of high osmosis of the plasma membrane in the protoplast cells. Unlike the growth of progenitor cells on the mannitol-free medium, they are not affected by that cause and contain the cell wall that protects them from explosion (Davey *et al*., 2010). It is generally noted that, the technique of isolating protoplast requires learning of many skills and many requirements (Davey *et al*., 2005). The success of transplantation of *C. humicola* protoplasts was attributed to the fact that its cells were able to directly divide and fit into the approved conditions and that the approved density of transplantation was very appropriate (Al-Mallah and Al-Nema, 2017).

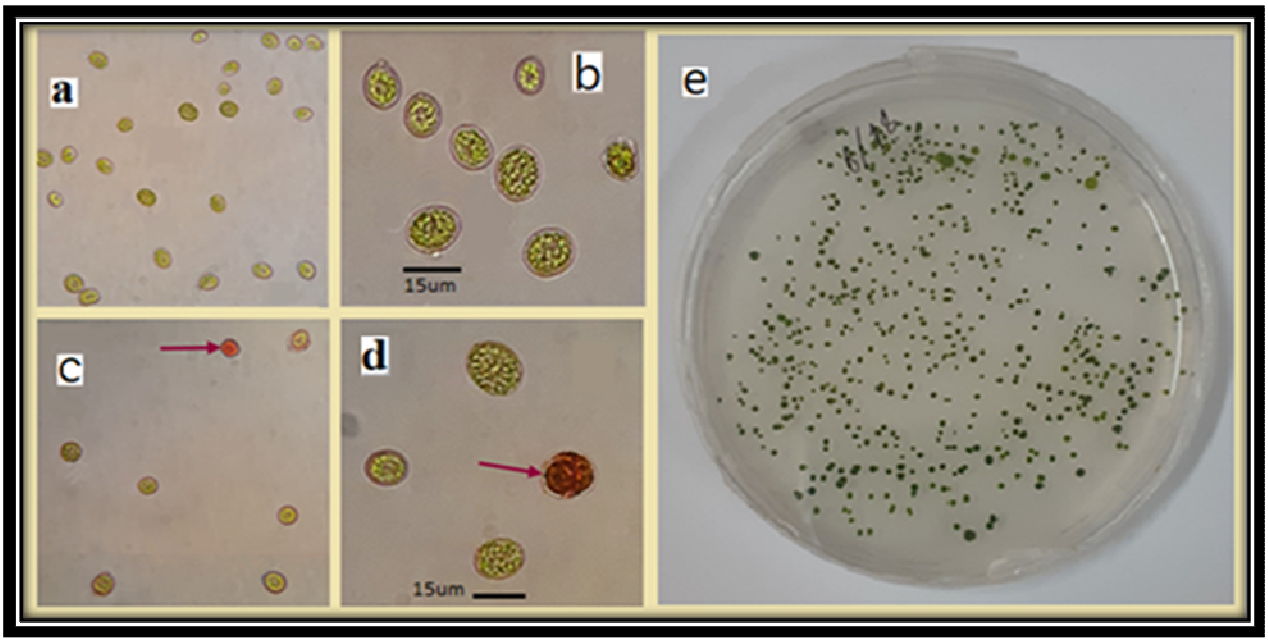

Fig. 1 : Isolation and culture of protoplast from *C. humicola.* a: free protoplast yield (10x), b: protoplast cells at a 40X magnification, c, d: Protoplast cells dyed with Phenosafranin stain showing dead cells dyed in red with an arrow point in 10x & 40x magnification respectively (Size 15 µm), e: Colonies resulting from the growth and regeneration of isolated and cultured protoplast cells on a Knop-M-Agar medium.

References

Abo-Shady, A.M. (2008). Protoplast Isolation and fusion from *Chlorella sp*. and *Chlorococcum sp*. Egypt. J. Biotechnol. 28: 223-233.

Al-Mallah, M.K. and Al-Nema, Q.S. (2016). Plant protoplast isolation, cultivation and applications. Dar Al-Wadah Publishing House, Amman, Jordan, 131.

Al-Nema, Q.S. (2013). Electrofusion between protoplast of two types verities of Sugarbeet and Seedlings Interaction with GUS-labelled *Gluconacetobacter diazotrophicus* assessed GUS gene. Ph.D. thesis / College of Education for Pure Sciences / University of Mosul / Iraq. P129.

Bhagavathy, S.; Sumathi, P. and Jancy S.B.I. (2011). Green algae *Chlorococcum humicola* a new source of bioactive compounds with antimicrobial activity. Asian Pacific Journal of Tropical Biomedicine, 1: S1–S7.

Davey, M.R.; Anthony, P.; Patel, D. and Power, J.B. (2010). Plant protoplasts: isolation ,culture and plant regeneration . In: Davery, M. R. and Anthony, Plant Cell Culture Essential Methods. Wiley-Blachwell, UK, 153-155.

Davey, M.R.; Anthony, P.; Power, J.B. and Lowe, K.C. (2005). Protoplasts: status and biotechnological perspectives. Biotech. Adv., 23: 131-171.

Hohe, A. and Reski, R. (2002). Optimisation of a bioreactor culture of the moss *Physcomitrella patens* for mass production of protoplasts. Plant Science, 00. 1/6.

Kusumaningrum, H.P. and Zainuri, M. (2014). Optimization and stability of total pigments production of fusan from protoplast fusion of Microalgae Dunalilla and Chlorella in vivo: Attempts on production of sustainable Aquaculture natural food. International J. of Marine and Aquatic Resource Conservation and Co-existence Research Article. 1(1):1-5.

Kusumaningrum, H.P. and Zainuri, M. (2018). Improvement of nutrition production by protoplast fusion techniques in *Clorella vulgaris*. J. of Food Process. Technol., 9: 1-5.

Lu, Y.; Kong, R. and Hu, L. (2011). Preparation of protoplasts from *Chlorella protothecoides*. World J. Microbiol Biotechnol. Published online: 03.

Raimundo, S.C.; Sorensen, I.; Tinaz, B.; Ritter, I. and Rose, J.K.C. (2018). Isolation and manipulation of protoplast from the unicellular green alga *Penium margaritaceum*. Plant Methods 14: 18.

Santhoshkumar, K.; Prasanthkumar, S. and Ray, J.G. (2016). *Chlorococcum humicola* (Nageli) Rabenhorst as a Renewable Source of Bioproducts and Biofuel. Journal of Plant Studies, 5(1): 48-57.

Tjahjono, A.E.; Kakizono, T.; Hayama, Y. and Nishio, N. (1994). Isolation of resistant carotenoid biosynthesis inhibitor for a green Alga *Haematococcus pluvialis* and their Hybrid formation by protoplast fusion for breeding of higher Astaxanthin producers. J. of Fermentation and Bioengineering. 77(4): 352-357.

Yamaguchi, K.; Nakano, H.; Murakami, M.; Konosu, S.; Nakayama, O.; Kanda, M.; Nakamura, A. and H. Iwamoto (1987). "Lipid composition of a green alga, *Botryococcus braunii*". Agric. Biol. Chem. 51(2): 493-498.