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Isolation and Morphological Identification of Two Freshwater Green Microalgae Isolated from Different Aquatic Habitats in Iraq

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ABSTRACT
Microalgae that live in freshwater form an important element to wetlands and there has been much interest developed surrounding their ecological significance and their potential use in biotechnological applications and remediation efforts for environmental conditions. We aim, in the current study, to examine the morphological characteristics of the samples taken from multiple aquatic ecosystems across Iraq's freshwater environments. Samples were obtained from a wastewater treatment basin at the Samarra Drug Industries Plant and along with two sample sites along the Tigris River, which receives wastewater effluent, within Baghdad (where discharge occurs). Isolation of the samples was performed using serial dilution and streak plate methods on CH-13 medium maintained under laboratory conditions. Purified cultures of axenic strains were produced that were free of bacterial and/or fungal contamination. Light microscopy and taxonomic keys were used in morphological assessments. Morphologically, the isolates were all different based on the size, shape, and structural characteristics of their cells, cell walls, chloroplasts, and reproduction. The *Chlorococcum humicola* isolate was characterized as large (spherical or subspherical) with thick walls, dark green pigment, and parietal chloroplasts. Alternatively, the *Chlorella vulgaris* isolate was described as small and spherical, with bright green pigmentation, cup-shaped chloroplasts, and reproducing via autospore formation. The fact that two species were isolated from contaminated aquatic habitats suggests there is some resilience to environmental stresses and points toward their potential application in wastewater treatment and bioremediation. The data generated here contributes to existing data regarding the microalgal biodiversity indigenous to Iraq, and provides information that can be utilised in future physiological and environmental studies.

KEYWORDS: *humicola, Chlorella vulgaris, Morphological Identification, Isolation, Freshwater Algae, Iraq*

1. INTRODUCTION

Microalgae constitute the largest group of diverse photosynthetic microorganisms inhabiting the aquatic ecosystems. They are the primary producers and cycling of nutrients, with the production of oxygen and balancing of eco-systems (1). The freshwater ecosystems comprise various species of microalgae that have managed to survive in widely ranging environmental conditions including pollution of habitats by industrial and pharmaceutical contaminants (2).

The onset of anthropogenic activities has ensured the outpouring of various pollutants into aquatic ecosystems. Wastewater discharged from the industrial, hospital, and municipal contributions forms a sizable portion of environmental pollution (3). Accordingly, this has led to the search for exotic microalgae, which are supposed to survive under such conditions due to their ecological importance and biotechnological applications (4).

Chlorella and *Chlorococcum*, the green microalgae present in many freshwater ecosystems throughout the world, are capable of changing their behaviours. Because of this ability to adapt physiologically to their environments, they are often found in various types of polluted aquatic systems (5). Likewise, their ability to withstand and use the available nutrients from the environment makes these groups of microalgae directly applicable for use in wastewater treatment and bioremediation programmes (6).

Morphology is one of the primary methods of identifying and classifying microalgae at an early stage based on their appearance. Some of the most important morphological characteristics used to determine algae species are their cell size; cell shape; chloroplast type; cell wall type; and reproductive method (7).

While Iraq has a large number of lakes and rivers, few studies have been done on the microalgae that grow in these water bodies that have been affected by pharmaceutical factories. Therefore, one of the primary objectives of this study is to collect and morphologically classify freshwater green microalgae from several different aquatic habitats in Iraq.

Despite the ecological importance of freshwater microalgae in Iraq, studies focusing on the isolation and characterization of indigenous microalgal species from wastewater-affected aquatic habitats remain limited. Therefore, the present study contributes to documenting native freshwater microalgal diversity and provides baseline information for future environmental and biotechnological applications involving locally adapted strains.

2-MATERIALS AND METHODS

Sampling Sites

Samples of water were gathered from three types of aquatic ecosystems in various parts of Iraq that differed in their amounts of contact with wastewater and man-made processes. Locations sampled were the Samarra Drug Industries Plant's wastewater treatment pond, a site next to Al-Karkh Maternity Hospital in the Tigris River, and the Medical City on the Tigris River in Baghdad. This sampling was completed to identify where possible inputs of pharmaceutical and municipal wastewater may influence the growth and diversity of freshwater microalgae. The geographic coordinates of all locations sampled are given in Table 1, and the locations of the sample sites are depicted in Figure 1.

Table 1: Sampling locations.

Location	Latitude	Longitude
Samarra Drug Industries Treatment Basin	34.1865° N	43.8912° E
Tigris River near Al-Karkh Maternity Hospital	33.3402° N	44.3885° E
Tigris River near Medical City	33.3498° N	44.3845° E

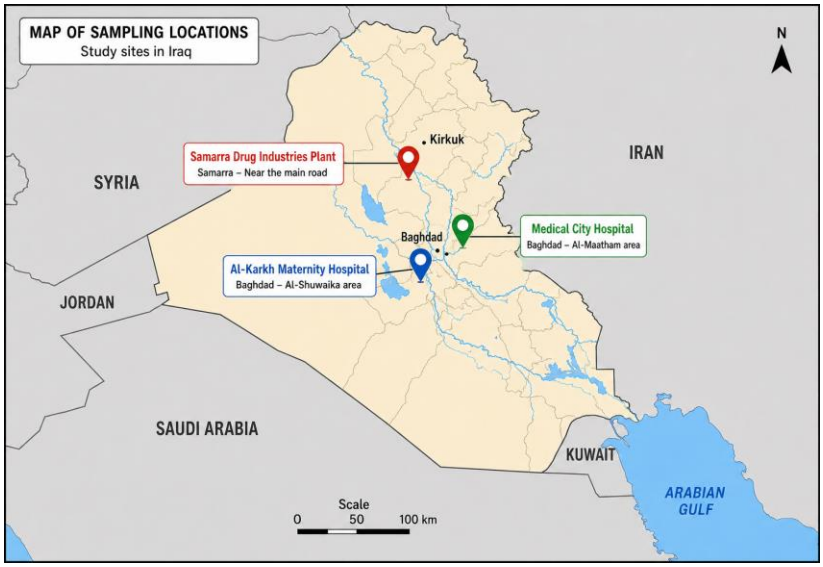

Figure 1: Geographic locations of sampling sites in Iraq.

Collection of Samples

Water samples were collected aseptically in sterile polyethylene containers and transported immediately to the laboratory for processing and cultivation (8).

Isolation of Microalgae

Microalgae were isolated using: Serial dilution method (9) and streak plate method (10). The isolates were cultivated on CH-13 medium supplemented with 1.5–2% agar (11).

Culture Conditions

Cultures were cultivated under controlled environmental conditions. The temperature was regulated at about 25 °C using an incubation period of 16 hours of light and 8 hours of darkness (16:8). Cultures were exposed to a constant light intensity of 3000 lux to maintain proper atmospheric conditions for the isolated microalgal species to grow and develop physiologically (12).

Purification of Cultures

Repeated subculturing was performed according to Pokorny *et al.* (13) until axenic cultures free from bacterial and fungal contamination were obtained..

Morphological Identification

Light microscopy was used to morphologically identify isolated microalgal strains. The procedure for identifying the species involved comparing the morphologic characteristics seen under the light microscope to those from authoritative algal descriptions, as well as standard taxonomic keys (14-16). The following diagnostic criteria were used in the identification: cell morphology, size and shape of cells (cell dimensions), type of cells (cell wall characteristics), structure and arrangement of chloroplasts, and reproductive morphology. The combination of these characteristics resulted in accurate taxonomic identification of the isolated microalgal species.

3-RESULTS

Efficiency of Isolation Techniques

The serial dilution and streak plate methods are highly effective for isolating freshwater microalgae from environmental samples (See Table 2 and Figure 2). Serial dilution produced lower densities of associated microorganisms, resulting in discrete algal colonies developing, especially at higher dilutions. Nevertheless, some plates were still contaminated by microbial growth, requiring further purification.

Conversely, the streak plate method produced more distinct, uniform colonies and, thus, allowed for simpler recognition and selection of colonies for subsequent purification and identification. Additionally, adequate spacing between colonies facilitated the establishment of axenic cultures and the minimization of cross-contamination.

Using both methods together provided us with the most reliable means of isolating the microalgae. The initial method involved serial dilutions to obtain an initial growth phase while controlling the density of the other microorganisms.

The second method used was streak-plating to isolate a pure sample of a single colony of microorganisms and thus fully purify the colony of microorganisms. By using this combined approach we achieved the greatest success in isolating and obtaining pure cultures of the microalgae that allow for complete morphological characterization and taxonomic identification of the microalgae from these pure cultures.

Table 2: Efficiency of isolation techniques used for obtaining pure microalgal colonies.

Isolation Technique	Colony Formation	Pure Colony Percentage (%)	Remarks
Serial dilution	Moderate	65–75	Presence of accompanying microorganisms in some plates
Streak plate	High	80–90	Clear and morphologically uniform colonies
Serial dilution + streak plate	Very high	>90	Highest efficiency for isolation and purification

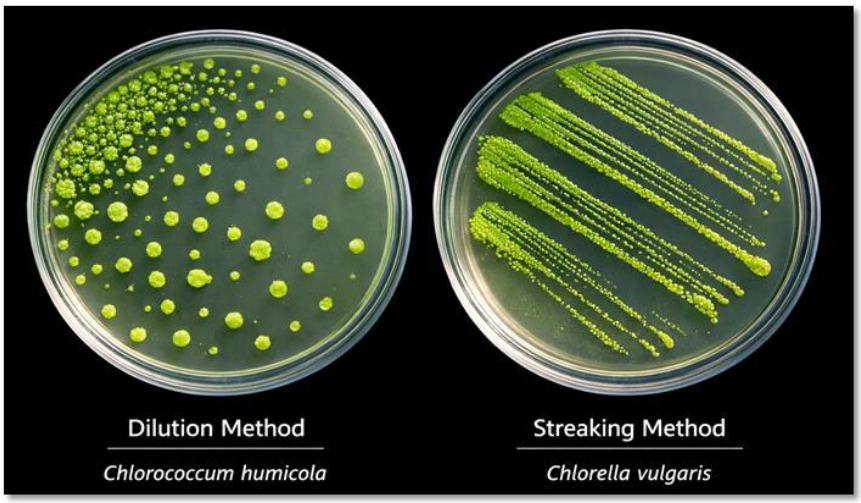

Figure 2. Growth of microalgal colonies on solid culture media using serial dilution and streak plate techniques.

Isolation and Purification of Microalgal Cultures

The comparison of the bacterial counts during wet The isolation methods in this research-used serial dilution and streak plating successfully yielded pure cultures of microalgae (Figure 1). The repeated subculturing and purifying techniques were effective at removing all associated microorganisms, thereby producing axenic cultures (cultures that are sterile) for further morphological characterization and experimental studies before morphological study and experimental testing of each isolate could be performed. In the purification process, purity was continually assessed using microscopic examination and testing for microbial contamination, and no bacterial colonies, fungal growth, or other contaminants were observed from any of the cultures during the entire period of observation (Table 2). Additional confirmation of cellular morphology showed that all isolates were homogenous with regard to cellular morphology, above and beyond contamination from foreign microbial cells. Additionally, the purified isolates continued on and maintained stable growth and displayed the known morphological characteristics of the microalgal species from which they came. Finally, the results of this study indicate that the methods used to isolate and purify *Chlorococcum humicola* and *Chlorella vulgaris* cultures were efficient in yielding uncontaminated cultures of these microalgal species, thus providing reliable cultures for subsequent taxonomic identification and physiological investigations.

Table 3. Purity assessment of isolated microalgal cultures.

Species	Bacterial Growth	Microscopic Contamination	Culture Status
Chlorococcum humicola	Absent	Absent	Axenic
Chlorella vulgaris	Absent	Absent	Axenic

The findings shown in Table 3 indicate that both of the isolates were entirely devoid of bacterial or fungal contamination. Therefore, the cultures that were obtained were concluded to be axenic cultures and thus, were suitable for more detailed work involving morphological identification and future laboratory studies. Establishing axenic cultures is one of the most important components of all areas of microalgal research because it minimizes the impacts of experimental Variables presented by microorganisms that are associated with an isolate and provides for a more accurate assessment of isolated species characteristic features.

Morphological Characteristics of the Isolated Microalgae

Morphological investigation of the isolated algae cultures resulted in a number of features that assisted in the identification of the taxa. The two examined isolates represented green algae, and displayed nonmotile vegetative cells that were spherical to subspherical in shape; however, there were differences regarding cell size, cell wall structure, morphology of the chloroplast and in the method of reproduction.

The first isolate had large (8-30µm) cells, occurring as solitary cells or in irregular clusters, and with a thick, multilayered cell wall. The chloroplasts were located near the cell's periphery (parietal) and comprised a significant portion of the cellular volume, giving rise to a very dark green color. Reproductive structures were observed as autospores and aplanospores, which are standard reproductive structures associated with *Chlorococcum humicola*.

The second isolate had much smaller cells (2-10µm), which were mostly solitary with a thin cell wall. Each cell had a single cup-shaped chloroplast, creating a bright green color. Most reproduction was asexual through autosporogenesis, which is the reproductive characteristic associated with *Chlorella vulgaris*.

The summit provided by morphological characteristics showed good agreement with standard taxonomic descriptions for both types, as well as with many references to standard taxonomic reference. A combination of cell size, shape, chloroplast structure, cell wall characteristics and reproductive characteristics will provide good diagnostic criteria for identifying the two isolates and confirming their taxonomic standing.

Table 4: Morphological characteristics of the isolated microalgal species

Character	*Chlorococcum humicola*	*Chlorella vulgaris*
Cell shape	Spherical to subspherical	Spherical to subspherical
Cell size (µm)	8–30	2–10
Cell arrangement	Solitary or irregular colonies	Mostly solitary
Cell wall	Thick and multilayered	Thin
Chloroplast	Single parietal	Single cup-shaped
Color	Dark green	Bright green
Reproduction	Autospores/Aplanospores	Autospores
Motility	Non-motile	Non-motile

Cell size, chloroplast configuration and cell wall characteristics were the most important morphologic differences between these two strains; they represent important taxonomic characteristics for identification at the species level. As such, these data support the conclusion that the two strains are *Chlorococcum humicola* and *Chlorella vulgaris* respectively (Figures 3 and 4).

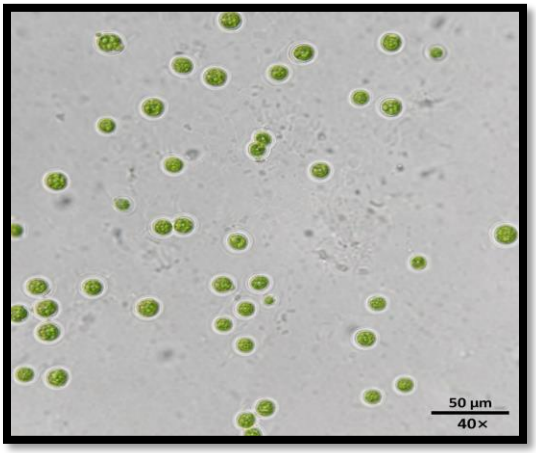

Figure 3: Light micrograph of *Chlorococcum humicola*.

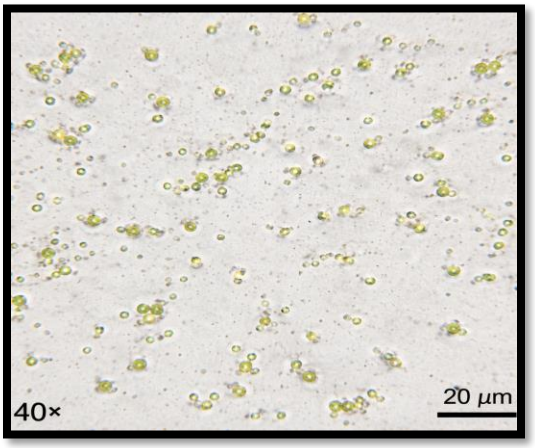

Figure 4: Light micrograph of *Chlorella vulgaris*.

Taxonomic Confirmation

The taxonomic identification of the isolated microalgal strains was confirmed via a close morphological examination, along with comparisons to standard taxonomic descriptions and identification keys. A number of diagnostic characteristics (including size, shape, morphology of the cell wall, structure of the chloroplast, and methods of reproduction) were used to identify the isolates at species level. The characteristics observed matched very closely with published descriptions of *Chlorococcum humicola* and *Chlorella vulgaris* (Table 5), providing a reliable identification of both species.

Table 5: Diagnostic characteristics used for species identification.

Species	Diagnostic Characteristics
Chlorococcum humicola	Large spherical cells, thick cell wall, parietal chloroplast
Chlorella vulgaris	Small spherical cells, cup-shaped chloroplast, autospore formation

Table 5 clearly shows how to tell two different isolates apart; namely, by their size, thickness of their cell walls, and shape of their chloroplasts. The larger cells with thick cell walls and parietal chloroplasts would represent *Chlorococcum humicola*; while the smaller cells with cup-shaped chloroplasts and producing autospores would indicate *Chlorella vulgaris* morphology. This confirms the taxonomic status of the isolated strain.

4-DISCUSSION

Two freshwater green microalgae, *Chlorococcum humicola* and *Chlorella vulgaris*, have been isolated and morphologically characterized in the current study from water samples that receive wastewater effluent. These species' successful recovery from contaminated environments is a demonstration of their significant ecological versatility and ability

to persist in environments characterised by changing nutrient concentration factors and human induced pollutants (17). The recovery of these green microalgae in wastestream impacted habitats also supports earlier studies suggesting that among photosynthetic microorganisms found in freshwater ecosystems, green microalgae represent some of the most resilient groups of species (18).

Isolated cultures obtained through the employed methods of isolation in the study were shown to be adequately pure for use in subsequent taxonomic characterization of the cultures. The combination of serial dilution and streak plating resulted in improved isolation efficiency because these two methods reduced the effects of microbial interference, thereby allowing better separation of individual colonies (19). Similar findings were presented by Benítez *et al*. (20) when they showed that implementing dilution and plate-based methods improved the ability to recover axenic microalgal cultures. Contamination-free establishment of cultures was especially critical since bacterial or fungal contamination could negatively impact algal growth and morphology; therefore resulting in erroneous taxonomic and experimental conclusions (21).

Morphological analysis showed distinct morphological differences between each isolate, particularly with respect to cell morphology, chloroplast morphology, and cell wall morphology. The characteristics of *Chlorococcum humicola*, which included large, spherical shaped cells; thick multilayer cell walls; and parietal chloroplasts, closely match the original descriptions provided in standard taxonomic references. *C. humicola* exhibits thick cell walls that may confer an adaptation for increased resistance against numerous environmental stressors associated with pollutants such as toxic chemicals and fluctuations of osmotic pressure; both of these factors are common within polluted aquatic systems (22). Therefore, the structural adaptations associated with *C. humicola* can be attributed to the presence of this organism in aquatic environments that have been impacted by industrial and pharmaceutical wastes.

Also, the morphological characteristics seen in *Chlorella vulgaris* were consistent with earlier observations found in literature. The morphology comprised of small, round cells, thin cell wall, cup-shaped chloroplast, and autospore development seen in the current study are indicative of the species. The relatively small cell size of *C. vulgaris* provides a higher surface area to volume ratio which may increase the efficiency of nutrient uptake and allow it to grow rapidly in nutrient rich environments (23). This feature is often considered to be a primary reason for the wide distribution of *Chlorella* species throughout a variety of aquatic habitats (24). The ability of both species to survive in habitats associated with treated wastewater may also point to their ability to metabolize high amounts of organic and inorganic nutrients. Wastewater discharges typically contain high concentrations of nitrogen, phosphorus and dissolved organic matter that promote algal proliferation (25). Many studies have shown that *C. vulgaris* has a large capacity for nutrient uptake and biomass production when grown in wastewater systems, making it one of the most studied microalgal species for environmental applications (26, 27).

Likewise, members of the *Chlorococcum* genus have also been reported to have high tolerances for contaminated sites, and can help remove nutrients through biological assimilation mechanisms (28, 29).

Both species are considered promising candidates for phycoremediation processes due to their ability to tolerate nutrient-rich and wastewater-impacted environments. *Chlorella vulgaris* has been extensively investigated for nitrogen and phosphorus removal, whereas *Chlorococcum* species have demonstrated significant capacities for pollutant uptake and biomass production. These characteristics support their potential utilization in wastewater treatment systems and sustainable biomass generation.

Another key takeout from the work described here is that the isolated strains had successfully adapted to laboratory cultivation conditions. The cultures were able to achieve stable rates of growth under controlled temperature, light intensity and photoperiod regimes, showing that the selected environmental conditions were suitable for supporting their physiological activities. The ability of these isolates to grow in the laboratory allows for future research into their growth kinetics, biochemical composition, pollutant removal efficiency and biomass production (30).

The ecological significance of the findings go beyond providing information on the taxonomy of the isolated strains. Indigenous species of microalgae may be better adapted than non-indigenous strains to local environmental conditions and therefore may perform better than non-indigenous strains in terms of use in environmental remediation programs (31). The isolation of native strains of *C. humicola* and *C. vulgaris* from Iraq has provided a potential source of biological material for future research into wastewater treatment, nutrient recovery, carbon dioxide sequestration and the production of bioactive compounds. The successful identification of these species provides valuable information on the freshwater microalgal biodiversity of Iraq, which is still a relatively under researched area in light of the importance of the aquatic ecosystems of Iraq (32).

The findings of this study show that *Chlorococcum humicola* and *Chlorella vulgaris* can both thrive in aquatic environments affected by wastewater, as both species display morphological and ecological features that make them candidates for application in biotechnology and the environment. Morphological characteristics should be examined in light of additional studies using molecular methods and physiological assessments so as to further understand the ecological roles and biotechnological properties of these indigenous algal strains (33).

Study Limitations and Future Perspectives

Although the identification of the isolated microalgae was conducted using detailed morphological observations and standard taxonomic keys, morphology-based identification alone may have limitations due to phenotypic plasticity exhibited by many microalgal taxa. Therefore, molecular approaches such as 18S rRNA gene sequencing are recommended in future studies to provide more robust taxonomic confirmation and phylogenetic resolution of the isolated strains.

In addition, a limitation of the present study is the absence of physicochemical measurements of the sampling sites, including pH, dissolved oxygen, electrical conductivity, and nutrient concentrations. Consequently, correlations between environmental conditions and the occurrence of the isolated microalgal species were beyond the scope of the present study. Future studies integrating environmental monitoring with taxonomic analyses are recommended to better understand the ecological preferences and distribution patterns of these indigenous microalgae.

CONCLUSION

The current research successfully isolated and morphologically identified two freshwater green microalgal species, *Chlorococcum humicola* and *Chlorella vulgaris*. Using an isolation method that incorporated serial dilution and streak plate techniques was an effective way to provide an axenic culture so that their shapes could be measured and classified taxonomically. The morphological examinations of each species provided distinct and diagnostic characteristics that clearly indicated the species and confirmed the taxonomic identity of the isolated micro algal species. Furthermore, the presence of both species of microalgae in environments containing pollution demonstrated that these two species could withstand environments with plenty of pollution and that they have the ability to tolerate environmental stressors and adapt to anthropogenically contaminated environments. In addition, the plant growth of both species of microalgae under laboratory conditions provides them with a basis for the development of future environmentally and biotechnologically related applications, particularly with respect to the treatment of wastewater, performing bioremediation, removing nutrients, and producing biomass. This study, by determining additional native species within Iraq, helps document the microorganism biodiversity in Iraq, and provides a valuable foundation for future physiological, molecular, and ecological studies of native microalgal species.

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