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Effects of 2,4-Dichlorophenoxyacetic acid and Thidiazuron on Callus Induction and Organogenesis in the Medicinal Plant *Calotropis procera*

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Abstract

Calotropis procera, a medicinally and industrially valuable plant of the Apocynaceae family, faces challenges in propagation due to declining seed germination potential and a lack of set mass production methods. This study evaluates the effects of plant growth regulators, specifically 2,4-Dichlorophenoxyacetic acidichlorophenoxyacetic acid and Thidiazuron, on callus induction, shoot formation, leaf development, and root induction in stem explants of *C. procera*. Tissue culture techniques were employed to address propagation challenges and conserve this species.

The highest callus induction (100%) was achieved using 7.5 mg/L 2,4-Dichlorophenoxyacetic acidichlorophenoxyacetic acid and 7.5 mg/L Thidiazuron, with auxins and cytokinins demonstrating a synergistic effect in promoting cell division. For shoot formation, the optimal combination was 1.25 mg/L 2,4-Dichlorophenoxyacetic acidichlorophenoxyacetic acid and 5 mg/L Thidiazuron, while leaf formation peaked with 1.25 mg/L Thidiazuron alone. Excessive Thidiazuron concentrations, however, inhibited leaf formation, underscoring the importance of hormonal balance. Root induction was most effective with 2.5 mg/L 2,4-Dichlorophenoxyacetic acid and 7.5 mg/L Thidiazuron, whereas treatments with high cytokinin concentrations were found to hinder root growth.

These findings align with prior research on other medicinal plants, such as *Catharanthus roseus* and *Calotropis gigantea*, demonstrating the complementary roles of auxins and cytokinins in plant tissue culture. This study highlights the potential of tissue culture as a scalable and sustainable method for the propagation and conservation of *C. procera*, ensuring the preservation of its medicinal and industrial applications.

This study confirmed that the proper balance between auxin and cytokinin is important for the successful induction of each growth phase in the plant. This study highlights the potential of tissue culture as a scalable and sustainable method for the propagation and conservation of *C. procera*, ensuring the preservation of its medicinal and industrial applications.

Keywords: Plant tissue culture, Plant growth regulators, Hormonal synergy, Callus induction, *Calotropis procera*

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Introduction

The medicinal plant *Calotropis procera* (commonly known as the giant milkweed or crown flower) is a valuable species in the Apocynaceae family, known for producing important secondary metabolites such as cardiac glycosides and natural latex (Van Quaquebeke et al., 2005). It holds significant pharmaceutical and industrial importance due to its bioactive compounds and applications. Native to various regions of Iran, including South Khorasan, Sistan and Baluchestan, Khuzestan, and Hormozgan, *C. procera* is commonly used for its medicinal and industrial properties (Mohebi, 2021).

The latex of *C. procera* has been reported to possess anthelmintic (Iqbal et al., 2005), anti-inflammatory (Alencar et al., 2006), antioxidant (Chavda et al., 2010), and anticancer properties (Magalhães et al., 2010). Additionally, its fibers are utilized in the textile industry, and its hydrocarbons serve as a potential source for biofuel production (Parić et al., 2011).

Tissue culture techniques, as a branch of advanced biotechnological methods, provide a promising tool for the propagation and conservation of valuable plant species (García González et al., 2010). These methods not only enable the mass propagation of healthy plants but also offer a controlled environment for producing valuable secondary metabolites. However, in a controlled laboratory condition culture of plants like *C. procera*, which secrete latex, faces challenges such as phenolic exudation, which can damage explants. Using explants like immature embryos or hypocotyls, which contain lower phenolic content, has been suggested as a solution to mitigate these issues (Abasi et al., 2017).

Phytohormones play a pivotal role in enhancing various tissue culture stages such as callus induction, shoot formation, and root formation. The auxin 2,4-Dichlorophenoxyacetic acid is a potent promoter of cell division and facilitates callus induction, while Thidiazuron, an effective cytokinin, is efficient in inducing shoot and leaf formation (George et al., 2008). The combination of these two hormones can regulate hormonal balance, optimizing the propagation process under laboratory conditions (Sidik et al., 2024; Rineksane et al., 2021).

Therefore, this study aims to evaluate the effects of 2,4-Dichlorophenoxyacetic acid and Thidiazuron on callus induction and organogenesis in *C. procera*. Achieving optimal results can contribute to the mass propagation and conservation of this valuable species, preventing its potential extinction.

Material and Methods

Plant material

Seeds of *C. procera* were collected from the Kahnuj region in Kerman, Iran (27.9514° N, 57.7002° E; elevation ~435 m above sea level).

Seed sterilization

The seeds were washed with distilled water and then immersed in 5% sodium hypochlorite solution for 15 minutes. They were later rinsed in autoclaved distilled water for 5 minutes, followed by 2 minutes in 70% ethanol. Afterward, they were rinsed three more times in autoclaved distilled water, each time for 5 minutes (Lindsey et al., 2017).

Seed cultivation and culture conditions

To induce germination and obtain sterile seedlings, seeds were cultivated on basal Murashige & Skoog medium (Murashige and Skoog, 1962) containing 1.25 mg/L 2,4-Dichlorophenoxyacetic acid and 1.25 mg/L Thidiazuron. Cultures were maintained in a growth room under a 16-hour light and 8-hour dark photoperiod at 23 ± 2°C. After three weeks, the aerial parts of sterile seedlings were used as explants for hormonal treatments.

Hormonal treatments for explants

A factorial experiment was conducted in a completely randomized design to evaluate the effects of 2,4-Dichlorophenoxyacetic acid and Thidiazuron on callus, root, and shoot induction. The treatments included five concentrations of 2,4-Dichlorophenoxyacetic acid (0, 1.25, 2.5, 5, and 7.5 mg/L) and five concentrations of Thidiazuron (0, 1.25, 2.5, 5, and 7.5 mg/L), with at least three replicates for each treatment. Explants measuring 2–3 cm were excised from the aerial parts of sterile seedlings under a laminar flow hood. Explants were placed on different media and maintained under the same growth room conditions. After four weeks, parameters such as callus induction percentage, root induction percentage, root number per explant, shoot induction percentage, shoot number per explant, leaf induction percentage, and leaf number per explant were evaluated.

Data Analysis

The data were analyzed using SPSS software (version 22, Allen et al., 2014). Duncan's multiple range test was employed to compare the means.

Results

Ten days after culturing the explants on media with different hormonal treatments, we observed callus formation, shoot induction, and root induction.

Callus Induction

The highest callus induction rate (100%) occurred with 7.5 mg/L Thidiazuron and 7.5 mg/L 2,4-Dichlorophenoxyacetic acid, highlighting the synergistic effect of these hormones (Figure 2). At lower concentrations of 2,4-Dichlorophenoxyacetic acid (0 and 1.25 mg/L), callus induction was significantly reduced.

Shoot Induction

Optimal shoot formation was achieved with a combination of 1.25 mg/L 2,4-Dichloro-

Fig. 1. Induction of callus, shoots, leaves, and roots in stem explants of *C. procera* cultured on Murashige & Skoog medium with varying concentrations of 2,4-Dichlorophenoxyacetic acid and Thidiazuron

phenoxyacetic acid and 5 mg/L Thidiazuron. Higher concentrations of 2,4-Dichlorophenoxyacetic acid negatively affected shoot formation, while low concentrations were more effective (Figure 3).

Leaf Induction

We observed the highest percentage of leaf formation and the maximum number of leaves at 1.25 mg/L Thidiazuron (Figure 4)—increasing Thidiazuron concentrations above 2.5 mg/L reduced leaf formation, indicating an inhibitory effect at higher concentrations.

Root Induction

We observed root formation only with the combined treatment of 2.5 mg/L 2,4-Dichlorophenoxyacetic acid and 7.5 mg/L Thidiazuron, achieving a 90% success rate. On average, this condition produced 2.5 roots per explant.

Discussion

The native Iranian plant *Calotropis procera*, a notable member of the family Apocynaceae, holds special importance. One of the challenges in propagating the medicinal species *C. procera* is the decline in seed germination potential over time as seeds are stored longer (Galal et al., 2015). Furthermore, no set method exists for the mass production of this plant, which is nearing extinction. In this context, research and modern tissue culture techniques have gained attention as effective solutions to address these challenges. Tissue culture can be employed for the propagation of these plant species, whose populations are dwindling, enabling production in an aseptic environment free from environmental contaminants. Plant growth and differentiation are notably influenced by the type and concentration of plant growth regulators and culture media composition. Auxins and cytokinins, the most commonly used plant growth regulators in plant tissue culture, play crucial roles in regulating developmental pathways. These substances can improve the plant's growth responses. For example, in the case of *C. procera*, data indicates that the use of 2,4-Dichlorophenoxyacetic acid is notably more effective for callus induction than Naphthaleneacetic acid or Indole-3-acetic acid. Moreover, the cytokinin-to-auxin ratio in this process is crucial and influences callus growth and plant regeneration (Tripathi et al., 2013).

This study demonstrated the synergistic effect of 2,4-Dichlorophenoxyacetic acid and Thidiazuron on callus induction and organogenesis in *C. procera*. The combination of 7.5 mg/L 2,4-Dichlorophenoxyacetic acid and 7.5 mg/L Thidiazuron achieved the highest callus induction (100%). The combined use of these hormones increased the expression of genes related to cell division, supporting previous findings (Kumar & Reddy, 2011). These findings align with previous studies that demonstrate the complementary roles of auxins and cytokinins in callus induction (George et al., 2008). The hormone 2,4-Dichlorophenoxyacetic acid, a strong auxin, promotes increased cell division, while Thidiazuron, a phenylurea-type cytokinin, acts as a regulator of cellular development. According to prior research, explants of *Calotropis gigantea* cultured on media containing a combination of BAP and 2,4-Dichlorophenoxyacetic acid present-

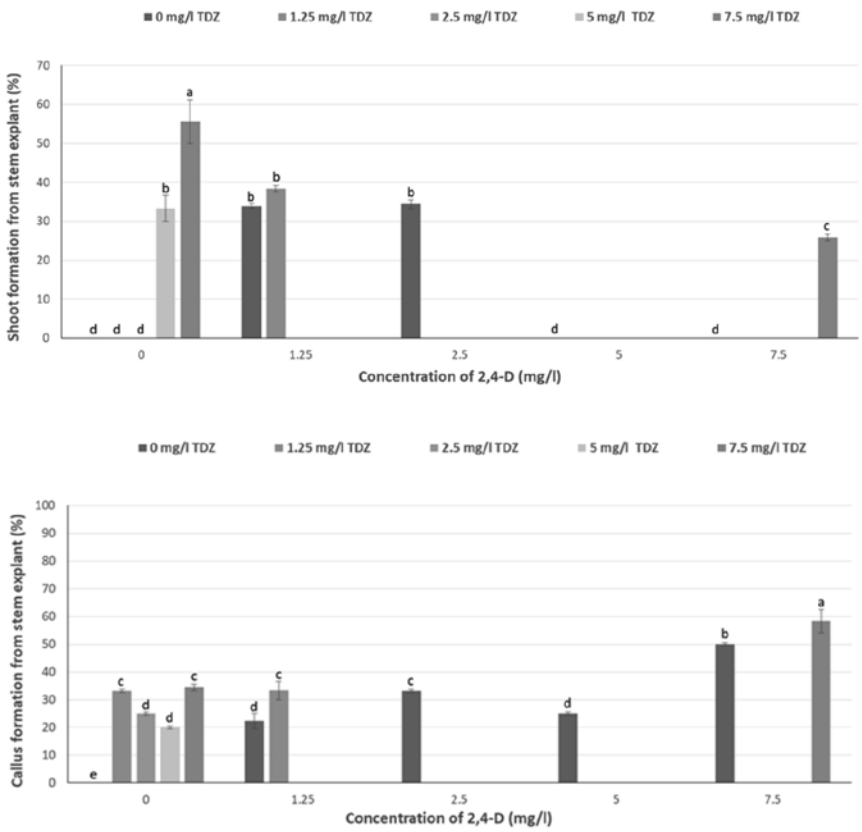

Fig. 3. The effect of different concentrations of the hormones 2,4-Dichlorophenoxyacetic acid and Thidiazuron on the percentage of shoot formation (A) and the number of shoots (B) in stem explants of *C. procera* after 4 weeks. Data represent the mean values of at least three replicates ± standard error. Different letters indicate significant differences between means at a probability level of $P \leq 0.05$

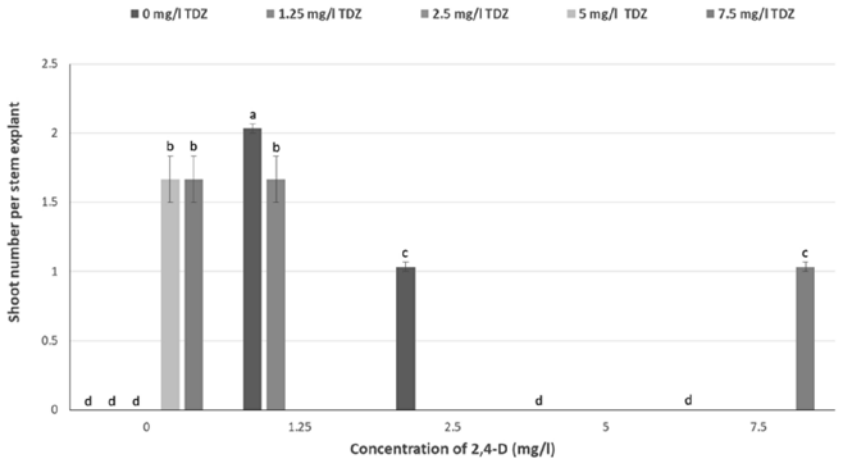

Fig. 4. The effect of different concentrations of the hormones 2,4-Dichlorophenoxyacetic acid and Thidiazuron on the percentage of leaf formation (A) and the number of leaves (B) in stem explants of *C. procera* after 4 weeks. Data represent the mean values of at least three replicates ± standard error. Different letters indicate significant differences between means at a probability level of $P \leq 0.05$

ed callus formation, whereas explants on media lacking 2,4-Dichlorophenoxyacetic acid failed to form calluses (Muthi'ah et al., 2023). These findings are consistent with the results of our experiments and underscore the complementary and sometimes antagonistic roles of auxins and cytokinins in regulating growth processes. Furthermore, the demonstrated ability to manipulate hormone concentrations for optimal outcomes emphasizes the potential of tissue culture as a scalable method for the conservation and propagation of *C. procera*. Our data show that Thidiazuron alone cannot induce callus formation but enhances its performance in the presence of auxin.

For shoot formation, the optimal combination was 1.25 mg/L 2,4-Dichlorophenoxyacetic acid with 5 mg/L Thidiazuron, while leaf formation peaked at 1.25 mg/L Thidiazuron. However, higher Thidiazuron concentrations inhibited leaf formation, emphasizing the importance of hormonal balance. These findings align with studies on *Catharanthus roseus* and *Calotropis gigantea* (Talitha et al., 2023; Dhandapani et al., 2008). Cytokinins such as Thidiazuron play a key role in this process by stimulating signaling pathways associated with stem meristem proliferation (Malik et al., 2023).

The findings reported by Rout et al. (2000) underscore the critical role of cytokinins in enhancing leaf formation. Leaf formation occurred at a concentration of 5 mg/L 2,4-Dichlorophenoxyacetic acid when we used only 2,4-Dichlorophenoxyacetic acid as the hormonal treatment in the culture medium. According to studies (Talitha et al., 2023) on *Calotropis gigantea*, leaf formation is influenced by specific concentrations of auxins and cytokinins. Among hormonal treatments, the one containing only Indole-3-butyric acid in the culture medium was found to be optimal for increasing leaf numbers compared to other treatments. This is because auxins stimulate the action of gibberellin hormones in increasing internode length, which later enhances the number of nodes and leaves. Agustina et al. (2020) have noted that both auxins and cytokinins have long been recognized to work synergistically and antagonistically in regulating various key growth processes.

Root induction required the combined treatment of 2.5 mg/L 2,4-Dichlorophenoxyacetic acid and 7.5 mg/L Thidiazuron. Similar results were reported for *Zingiber officinale*, where Thidiazuron combined with low auxin concentrations was most effective (Lincy & Sasikumar, 2010). Research findings (Talitha et al., 2023) have shown that in *Calotropis gigantea*, low concentrations of BAP increased the number of roots formed. However, there was an inverse relationship between increased BAP concentration and the number of roots formed. Optimal root formation occurred in the absence of BAP treatment in the culture medium. This is supported by prior research by Chen et al., which states that high concentrations of cytokinins can hinder root growth (Chen et al., 2020).

In conclusion, modern tissue culture techniques, guided by a deeper understanding of plant growth regulator interactions, hold promise not only for the sustainable production of *C. procera* but also for broader applications in conserving other endangered

medicinal plants.

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Investigating the effect of nutrients on cocultivation of *Haematococcus pluvialis* and *Xanthophyllomyces dendrorhous*

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Abstract

Microalgae *Haematococcus pluvialis* and yeast *Xanthophyllomyces dendrorhous* are two microorganisms known for their ability to produce astaxanthin, a valuable carotenoid with huge applications in various industries. This study aimed to investigate the optimal culture medium for the coculture of these two species, focusing on providing appropriate nutrients for their growth, particularly before *H. pluvialis* enters the red phase. Growth curves of *H. pluvialis* and *X. dendrorhous* were obtained in their standard media, Bold's Basal Medium (BBM) and Yeast Malt (YM) medium, respectively. Four candidate media were prepared based on BBM and YM constituents: BBM with glucose (BG), BBM with glucose and malt (BGM), BBM with glucose and peptone (BGP), and BBM with glucose and yeast extract (BGY). Cell numbers of both species were compared after 6 days of coculture incubation. Results showed that *H. pluvialis* exhibited the highest cell densities in BGM and BGY media, reaching 1.22×10^5 and 1.488×10^5 cells/mL, respectively. In contrast, the highest growth of *X. dendrorhous* was observed in BG medium, with a maximum cell density of 3.8×10^5 cells/mL. BGM demonstrated the balanced growth for both species, while BGY resulted in the highest cell concentration for *H. pluvialis* and controlled the growth of *X. dendrorhous*. The study highlights the importance of selecting a culture medium that balances the growth of both species and ensures controlled nutrient competition for a productive co-culture system. These findings contribute to the development of efficient co-cultivation strategies to enhance the cell growth rate and productivity.

Keywords: *Haematococcus pluvialis*, *Xanthophyllomyces dendrorhous*, Co-culture, Microalgae, Yeast, Culture medium

Introduction

Cultivating multiple microbial species under controlled conditions, known as microbial co-culture, can boost productivity and efficiency in various biotechnology applications. This approach promotes symbiotic relationships among microorganisms, improving performance compared to monoculture. Microbial co-cultures offer numerous advantages in industrial biotechnology, including increased yields, improved product quality, and the ability to utilize cheaper

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substrates (Bader et al., 2010).

Microalgae and yeast co-cultures have gained significant attention because of their potential to enhance biomass production and valuable compound synthesis. These interactions are characterized by a symbiotic relationship in which microalgae act as oxygen generators for yeast, while yeast provides CO_2 and organic acids to microalgae (Arora et al., 2019). This mutually beneficial arrangement can increase productivity and reduce cultivation costs. Studies have shown that co-culturing microalgae and yeast can substantially improve their biomass and lipid production. For instance, a co-culture of *Rhodotorula glutinis* and *Scenedesmus obliquus* in a photobioreactor demonstrated a 40-50 % increase in biomass and a 60-70 % increase in total lipids compared to single culture batches (Yen et al., 2015). Similarly, a coculture of *Chlorella pyrenoidosa* and *Rhodotorula glutinis* at a 3:1 ratio achieved maximum biomass concentration and lipid productivity, with total fatty acid productivity reaching twice that of monoculture (Liu et al., 2018). Microalgae-yeast coculture interactions offer promising opportunities for improving biomass and enhancing the synthesis of valuable compounds. These synergistic effects can be attributed to gas exchange, nutrient sharing, and gene expression alterations (Arora et al., 2019; Karitani et al., 2024; Xu et al., 2024). The co-culture system also demonstrated synergistic effects on dissolved oxygen and pH levels, which were mutually adjusted by the two organisms (Liu et al., 2018). Despite the numerous advantages of co-culturing, it also presents disadvantages that need to be addressed. A key issue is maintaining an optimal inoculum ratio, as deviations can reduce efficiency or harm the culture (Karitani et al., 2024). Furthermore, in some cases organic acids produced by microalgae may inhibit yeast growth (Naseema Rasheed et al., 2023).

Medium optimization plays a crucial role in enhancing the performance of microalgae and yeast co-cultures. The composition of the cultivation medium significantly affects growth, metabolite production, and the symbiotic relationship between microalgae and yeast (Xu et al., 2024; Qin et al., 2019). Studies have shown that optimizing the macronutrient composition, particularly carbon and nitrogen sources, can substantially improve co-culture performance. For instance, the addition of glucose with peptone in the co-culture of *Chlorella vulgaris* or *Chlorella sorokiniana* with a strain of *Saccharomyces cerevisiae* significantly enhanced biomass production of the co-culture by approximately 2-fold compared to that in monocultures (Xu et al., 2024). Furthermore, the addition of *Rhodotorula glutinis* yeast residual cell-free medium to *Chlorella vulgaris* cultures enhanced the production of specific fatty acids, such as nervonic acid and behenic acid, by 9 and 6 times, respectively (Ashtiani et al., 2021). By optimizing the nutrient composition, researchers can enhance biomass production and synthesis of valuable metabolites. This approach improves the economic feasibility of microbial metabolite production and opens up new possibilities for sustainable biofuel and biochemical production (Arora et al., 2019; Kitcha and Cheirsilp, 2014).

Haematococcus pluvialis is a freshwater unicellular green microalga that is widely recognized as one of the best natural sources of astaxanthin (Lee et al., 2016). It has the most significant capacity to accumulate astaxanthin among non-genetically modified organisms, with the ability to compose up to 4-7 % of its total dry weight (Gherabli et al., 2023; Mota et al., 2022). *H. pluvialis* undergoes a unique lifecycle, transitioning from green vegetative motile cells (green phase) to red hematocysts under stress conditions (red phase), during which it develops a thick, rigid three-layered cell wall (Kim et al., 2022).

Xanthophyllomyces dendrorhous, formerly known as *Phaffia rhodozyma*, is also capable of synthesizing astaxanthin (Domínguez-Bocanegra et al., 2007; Rodríguez-Sáiz et al., 2010). Although both organisms are considered major sources of natural astaxanthin production, their cultivation methods and astaxanthin yields vary. *H. pluvialis* can produce up to 9.2 mg/g of astaxanthin under optimal conditions, wherease *X. dendrorhous* has achieved yields of up to 9 mg/g through genetic engineering and optimized fermenter conditions (Domínguez-Bocanegra et al., 2007; Gassel et al., 2014). Both *H. pluvialis* and *X. dendrorhous* are of significant interest to the biotechnology industry for the production of natural astaxanthin, a valuable carotenoid used in aquaculture, nutraceuticals, cosmetics, and pharmaceuticals (Rodríguez-Sáiz et al., 2010; Mota et al., 2022). Ongoing research focuses on improving cultivation techniques, enhancing astaxanthin yields, and developing cost-effective production methods to compete with synthetic astaxanthin.

Co-cultivation of *X. dendrorhous* and *H. pluvialis* has been explored as an innovative approach to enhance astaxanthin production while simultaneously addressing environmental concerns by in situ carbon dioxide fixation. This method exploits the complementary metabolic abilities of these two astaxanthin-producing microorganisms (Domínguez-Bocanegra et al., 2007). In a mixed culture system, CO_2 generated by *X. dendrorhous* during fermentation was fixed by *H. pluvialis* through photosynthesis. Concurrently, the oxygen produced by *H. pluvialis* stimulates growth and astaxanthin production in *X. dendrorhous*. This symbiotic relationship resulted in significantly increased biomass and astaxanthin concentrations compared to pure cultures of either species (Dong and Zhao, 2004). This co-culture approach presents a novel method for improving the yield of high-value bio-products while simultaneously achieving in situ CO_2 fixation. By combining the strengths of both microorganisms, this strategy addresses the limitations of individual cultures and offers a more sustainable and efficient means of improving production (Dong and Zhao, 2004).

This study aims to investigate the optimal culture medium for cocultivation of the two species, *H. pluvialis* and *X. dendrorhous*. The focus was on understanding how nutrients influence the cell growth of each species during coculture, particularly before the microalga transitioning into the red phase.

Material and methods

Inoculum preparation

The yeast *X. dendrorhous* (IBRC-M30167) was purchased as a lyophilized ampoule from the Iranian Biological Resource Center. The yeast was activated by adding Yeast Malt (YM) medium (10 g/L glucose (Merck, Germany), 5 g/L peptone, 3 g/L yeast extract, and 3 g/L malt extract (Quelab, Canada) to the ampoule and grown on YM Petri dishes (Villegas-Méndez et al., 2021). A sample of grown yeast was transferred into a 250 mL Erlenmeyer flask filled with 50 mL of YM medium. The mixture was then incubated for 48 h at 23 °C with continuous stirring at 110 rpm in an orbital shaker incubator (KTG, Iran). This culture served as the inoculum for subsequent experiments.

Liquid *H. pluvialis* was purchased from the algae bank of the Research Institute for Industrial Biotechnology, Academic Center for Education, Culture, and Research (ACECR), Mashhad, Khorasan Razavi Province, Iran. It was grown in a Bold's Basal Medium (BBM) (Samhat et al., 2024) and incubated for 14 days at 23 °C under 12 h daily white illumination (30 µml m^{-2} s^{-1}).

Growth curve of X. dendrorhous

To determine the growth curve of the yeast over 5 days, experiments were conducted in duplicate using 250 mL Erlenmeyer flasks containing 50 mL YM medium. The flasks were inoculated with 10 % (v/v) of the prepared inoculum suspension. The cultures were incubated at 23 ± 1 °C with continuous shaking at 110 rpm. Growth was monitored by measuring the optical density at 600 nm using a spectrophotometer and counting cell numbers with a hemocytometer at 24-hour intervals for five consecutive days. The samples were appropriately diluted when necessary to ensure that the readings fell within the linear range of the spectrophotometer. The obtained OD values and cell numbers were plotted against time to generate the yeast growth curve, allowing for the identification of different growth phases.

Growth curve of H. pluvialis

Growth curve experiments were conducted to determine the growth characteristics of microalgae. Microalgal cultures were inoculated with 10 % (v/v) inoculum into sterile 250 mL Erlenmeyer flasks containing 50 mL BBM medium. The flasks were incubated in a shaker incubator at 23 ± 1 °C under 12 h of daily illumination (30 µmol m^{-2} s^{-1}) and agitated manually three times per day. Cell density was measured daily using a hemocytometer, and the optical density (OD) was recorded at 680 nm using a spectrophotometer (UNICO S-2150, USA). Samples were collected every 24 h for 15 days. The experiment was performed in duplicate, and the average values were used to plot growth curves.

Preparation of media

Four candidate media were prepared based on BBM (standard medium of *H. pluvialis*) and the constituents of YM (standard medium of *X. dendrorhous*). Since yeast growth requires a carbon source, all media contain glucose. The media used were as follows.

1-BBM with the addition of 10 g/L of Glucose (BG)

2-BBM with 10 g/L glucose and 3 g/L Malt (BGM)

3- BBM with 10 g/L glucose and 5 g/L Peptone (BGP)

4-BBM with 10 g/L glucose and 3 g/L Yeast

extract (BGY).

The cell numbers of *H. pluvialis* and *X. dendrorhous* after 6 days of coculture incubation were compared with their standard medium, BBM, and YM, respectively.

Incubation

The experiments were performed in 250 mL Erlenmeyer flasks containing 50 mL of the prepared media. All flasks were started at an initial cell concentration of 2×10^4 cells/mL for each species. The flasks were kept in an illuminated shaker incubator for 6 days at 23 °C under 12 h of daily illumination (30 µmol m^{-2} s^{-1}) and shaken at 110 rpm. The growth of both microalgae and yeast was measured at the end of six days of incubation by counting cell numbers under a microscope using a hemocytometer, and all experiments were performed in duplicate.

Statistical analysis

All data are expressed as mean ± standard error of the mean. Statistical analysis was performed using the Minitab software. A one–way ANOVA analysis was performed to detect whether the data were significantly different by using a p-value of $p < 0.05$.

Results and Discussion

Growth curve of X. dendrorhous

The *X. dendrorhous* culture in YM medium demonstrated a typical growth curve, as indicated by both cell concentration and OD measurements (Figure 1). However, the initial lag phase was too short to be observed. The exponential growth phase occurred within the first 48 hours, during which the cell concentration increased from 1.94×10^6 cells/mL at inoculation to 1.5×10^8 cells/mL at the end of day 2. Following this period, the culture entered a stationary phase, with cell numbers stabilizing around 1.6×10^8 cells/mL after 24 h, and a slight decrease was observed to 1.4×10^8 cells/mL by the end of day 5. Parallel optical density measurements at 600 nm revealed similar growth patterns. The OD values increased from 0.185 at inoculation to 2.213 after 2 days, corresponding to the exponential growth phase, and remained constant until the end of the measurements, indicating the stationary phase.

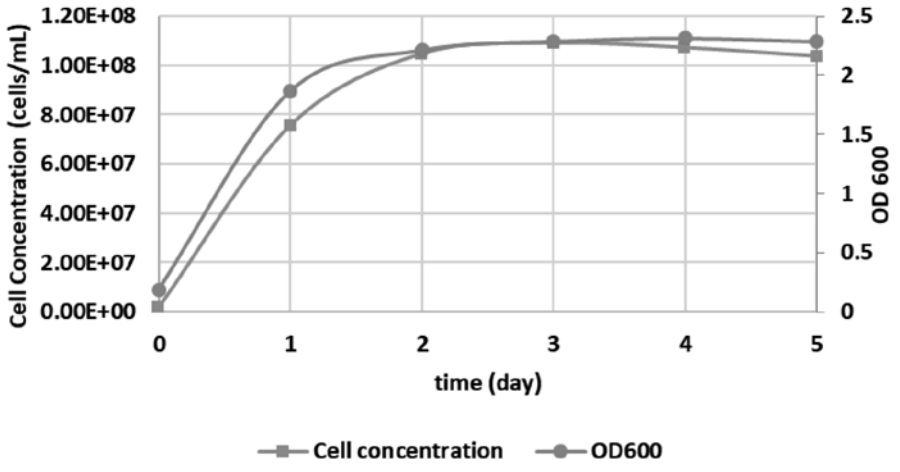

Fig. 1. Growth curve of *X. dendrorhous* in 5 days measured by cell concentration and optical density

X. dendrorhous typically exhibits a growth curve with distinct phases, including exponential and stationary phases (Castelblanco-Matiz et al., 2015). Recent studies have mainly focused on carotenoid biosynthesis, particularly astaxanthin, which is often induced during the late exponential growth phase (Lodato et al., 2007). Notably, growth curve and carotenoid production can be significantly influenced by various factors such as pH and nutrient sources, as well as availability. For example, the carbon source plays a crucial role in determining the timing of carotenoid biosynthesis. When grown on a non-fermentable carbon source, such as succinate, carotenoid production begins at the start of the growth cycle. It is approximately three times higher than when grown on glucose, a fermentable carbon source. In the presence of glucose, carotenoid production typically occurs at the end of the exponential phase (Wozniak et al., 2011).

The impact of nitrogen sources on carotenoid biosynthesis in *X. dendrorhous* is not thoroughly documented. However, the carbon to nitrogen (C/N) ratio in the growth medium significantly influences carotenoid production. As the C/N ratio increases, both cell growth and total astaxanthin accumulation increase, though the astaxanthin content per cell decreases. This indicates that the balance of carbon and nitrogen plays a crucial role in the process (Pan et al., 2017). Nitrogen sources such as peptone and yeast extract are known to enhance yeast metabolism and growth. For instance, in *Saccharomyces cerevisiae,* peptone increases biomass production (Da Cruz et al., 2002), while in *Penicillium canescens*, yeast extract is optimal for enzyme production, with the best results achieved by combining yeast extract and peptone (Bakri et al., 2003).

A comparison of the growth curves of the two species (Figures 1 and 2) shows that *X.*

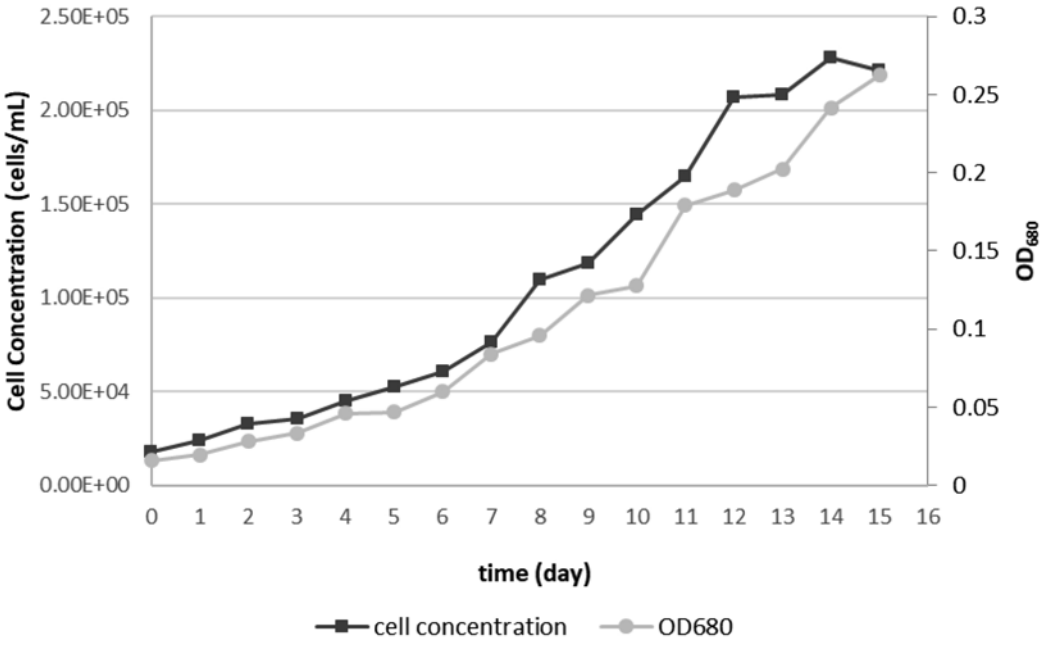

Fig. 2. Growth curve of *H. pluvialis* in 15 days measured by cell concentration and optical density

dendrorhous has a much faster growth rate than *H. pluvialis*, which necessitates the need to control the growth of yeast in the co-culture. Therefore, in this study, components of YM medium were added to BBM to propose different culture media for the controlled growth of *X. dendrorhous* in co-culture with *H. pluvialis*. This approach can be valuable for optimizing cultivation conditions to enhance biomass production.

Growth curve of H. pluvialis

The growth curve of *H. pluvialis* was monitored over 15 days using cell number and OD measurements (Figure 2).

The experiment began with an initial cell density of 1.75×10^4 cells/mL. A lag phase was observed during the first 3 days, with a minimal increase in cell number. Exponential growth started on day 4, with cell numbers increasing rapidly to reach 2.07×10^5 cells/mL by day 12. The growth rate decreased between days 12 and 15, indicating the start of the stationary growth phase. By day 15, the culture reached a final cell density of 2.21×10^5 cells/mL. The OD graph also showed the same growth pattern as the cell number, except for the stationary phase. The growth of *H. pluvialis* in BBM has been extensively studied, with several studies reporting favorable results. BBM has been found to provide optimal conditions for the vegetative growth of *H. pluvialis* compared to other common media, such as BG11 and 3NBBM (Nahidian et al., 2018). In autotrophic cultivation, BBM yielded the highest cell density of 1.5×10^5 cells/mL among the tested media (Tripathi et al., 1999). Interestingly, the growth curve of *H. pluvialis* in BBM can be further optimized by adjusting the nutrient concentrations. For instance, increasing phosphate levels in modified BBM led to up to an 86 % increase in growth rate and the highest cell density and the optimal concentrations of micronutrients such as boron (0.185 mM) and iron (0.046 mM) were found to enhance growth rates, although these optima depend on inoculum size (Nahidian et al., 2018). BBM consistently performed well for *H. pluvialis* growth, with a maximal biomass productivity of 86.54 mg/L/day (Zhao et al., 2019). However, the growth curve can be significantly improved by optimizing the nutrient concentrations, particularly phosphate, nitrogen and carbon. It's worth noting that while BBM supports excellent vegetative growth, other media may be more suitable for subsequent astaxanthin production in a two-stage cultivation strategy (Zhao et al., 2019; Fábregas et al., 2000). Wang et al. (2013) investigated the impact of varying concentrations of nitrate on biomass and astaxanthin production from *H. pluvialis*. The initial nitrate concentration significantly influenced the final biomass density; specifically, higher initial nitrogen concentrations in the culture led to greater final biomass density at the end of the 10-day culture period. The observed increases in final biomass density were attributed to both an increase in cell number and size (Wang et al., 2013).

Pang and Chen (2017) studied the effects of C5 organic carbon on the growth and cellular activity of *H. pluvialis*. They compared phototrophic, heterotrophic, and mixotrophic cultures using sodium acetate as the carbon source. The results showed

that the highest cell density was achieved under mixotrophic conditions, which was more than double that of the heterotrophic culture and higher than the phototrophic culture. This indicates that light is crucial for the growth and cell division of *H. pluvialis*. The researchers also examined nine different organic carbon sources, finding that ribose ,mannose ,fructose ,and sodium acetate resulted in the highest cellular densities (Pang and Chen, 2017).

BBM medium was selected as the base for potential co-culture media because of the necessity of excellent growth in the green phase of *H. pluvialis* for its co-culture with yeast.

Medium optimization for the co-culture

The results of the comparative study on cell concentration of *X. dendrorhous* and *H. pluvialis* in six different culture media (YM, BBM, BG, BGM, BGP, and BGY) are presented in Figure 3.

Growth of X. dendrorhous in different media in co-culture

Investigation of the growth of *X. dendrorhous* in the six different culture media in co-culture with *H. pluvialis* revealed that BG medium exhibited the highest cell numbers at the end of the co-cultivation, reaching a maximum of 3.8×10^5 cells/mL after 6 days (Figure 3). This was followed by BBM with glucose and peptone (BGP), which showed a peak cell density of 2.925×10^5 cells/mL. The BGM medium supported moderate growth at 1.725×10^5 cells/mL. In contrast, BGY and YM media showed controlled growth of yeast cells in the co-culture. The standard BBM medium without supplements showed no growth, with a final cell density of 5×10^3 cells/mL. This indicates that the additional nutrients, particularly the presence of glucose in the other media, significantly enhanced *X. dendrorhous* growth.

Growth of H. pluvialis in different media in co-culture

After six days of co-cultivation, *H. pluvialis* exhibited varying levels of cell growth across the six tested culture media (Figure

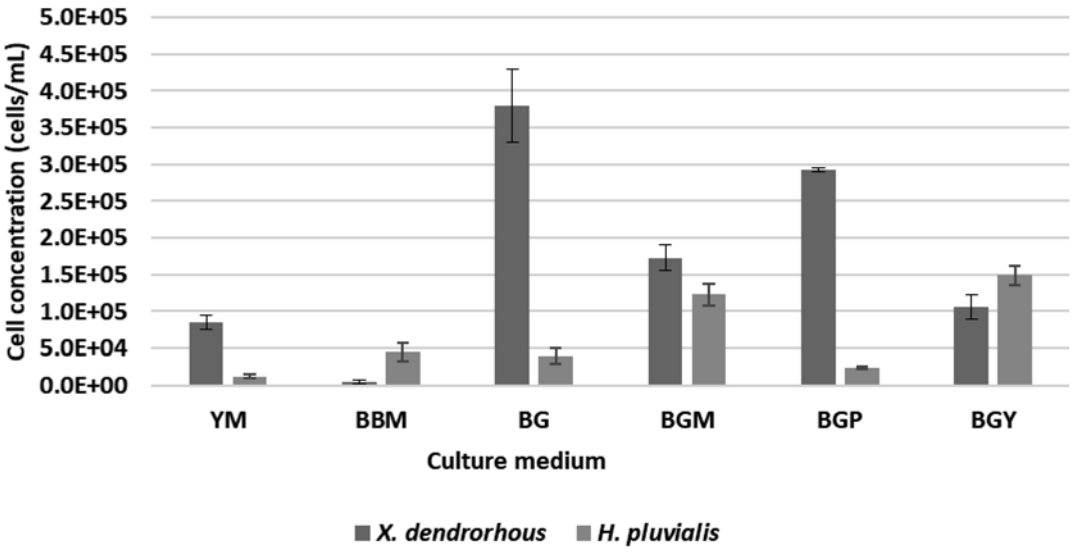

Fig. 3. Growth of *X. dendrorhous* and *H. pluvialis* in different culture media after 6 days of co-culture

3). The highest cell densities were observed in the BGM and BGY, with 1.22×10^5 and 1.488×10^5 cells/mL values, respectively. This was followed by BBM and BG, which had cell counts of 4.5×10^4 cells/mL and 4×10^4 cells/mL, respectively. The standard BBM medium demonstrated non-significant growth compared to the supplemented media. Among the variations in BBM, the addition of malt and yeast extract resulted significant increase in cell numbers, whereas peptone had the least impact on the growth enhancement of the microalga. As expected, the YM medium showed no growth compared to the BBM-based culture media, resulting in a final cell count of 1.25×10^4 cells/mL. These results indicate that supplementing BBM with certain compounds, specifically malt and yeast extract, significantly improves the growth of *H. pluvialis* in co-culture.

Comparison of the growth of H. pluvialis and X. dendrorhous in the co-culture

The growth of *X. dendrorhous* and *H. pluvialis* was compared across various coculture media (Figure 3), revealing distinct patterns and interactions between the two species. In the standard medium for *X. dendrorhous*, YM, the yeast's cell concentration was seven times higher than that of *H. pluvialis*. This indicates that *H. pluvialis* cannot utilize the complex organic nutrients present in YM. On the other hand, in BBM, the standard medium for *H. pluvialis*, *X. dendrorhous* showed no growth due to a lack of carbon sources, but the cell density of *H. pluvialis* increased to 2.25 times the initial cell concentration.

The BG medium allows both species to thrive effectively; however, the cell density of *X. dendrorhous* was 9.5 times higher than that of *H. pluvialis*. The yeast rapidly consumed all available glucose, which affected the growth of the microalga. *X. dendrorhous* is known to efficiently utilize glucose as a carbon source for growth. Marcoleta et al. (2011) noted that high glucose concentrations in the medium result in high cell growth but low carotenoid production, indicating that the yeast rapidly consumes glucose (Marcoleta et al., 2011). This suggests that while glucose is essential for yeast growth, the rapid consumption of glucose by *X. dendrorhous* may allow it to outcompete *H. pluvialis* in a co-culture situation, preventing the microalgae from utilizing the glucose present in the medium.

The rapid growth of yeast, driven by glucose consumption, leads to the expectation that the carbon dioxide produced during this process will promote the growth of microalgae. However, the results do not support this assumption. This indicates that the high levels of carbon dioxide generated in a short time from yeast growth are insufficient to meet the carbon needs of microalgae.

In the BGP medium, the high growth of *X. dendrorhous* also led to nutrient depletion, which hindered the growth of *H. pluvialis,* and similar to the BG medium, the CO_2 produced by the yeast in a short time was insufficient for the optimal growth of the microalga.

The BGM medium was superior for both species as it contained essential nutrients and promoted balanced growth. The similar cell concentrations observed in BGM for both species indicate its potential for creat-

ing a controlled co-culture environment. Interestingly, the BGY medium resulted in the highest cell concentration for *H. pluvialis* while effectively preventing the growth of *X. dendrorhous*. This suggests that the BGY medium facilitates efficient nutrient utilization and gas exchange between the two species.

It should be noted that, according to Figure 3, the presence of glucose in the medium is necessary for yeast growth, while malt and yeast extract are the most effective additives for enhancing microalgae growth.

A consistent pattern emerged across all media: when the growth of *X. dendrorhous* reached its maximum (as observed in BG and BGP media), the growth of *H. pluvialis* was inhibited due to competition for nutrients and inadequate CO_2 production. This highlights the importance of selecting a culture medium that balances the growth of both species, ensuring controlled nutrient competition and optimal gas exchange. In conclusion, a successful co-culture of *X. dendrorhous* and *H. pluvialis* necessitates a precisely formulated medium that controls the growth of the yeast while optimizing the growth of *H. pluvialis*. This balanced approach would facilitate controlled nutrient competition and the efficient utilization of synergistic gases (CO_2 and O_2) produced by each species, ultimately leading to a more productive co-culture system.

Dong and Zhao (2004) investigated the co-culture of *H. pluvialis* and *Phaffia rhodozyma* in BBM medium with varying glucose concentrations. They found that the biomass in the mixed cultures was higher than in pure cultures, particularly with glucose concentrations between 3-5 g/L, and it increased as glucose levels rose. *P. rhodozyma* also exhibited higher biomass at lower glucose concentrations, but saw a decline when concentrations exceeded 15 g/L, possibly due to the Crabtree effect. In contrast, *H. pluvialis* had low biomass concentrations, showing little variation across the tested glucose ranges (Dong and Zhao, 2004).

Recent studies have also shown that optimization of culture conditions, particularly the medium composition, plays a crucial role in the success of these co-culture systems. In a study conducted by Xu et al. (2024), it was shown that the addition of glucose and peptone significantly enhanced biomass production in *Chlorella-Saccharomyces* co-cultures, increasing it by approximately 2-fold compared to monocultures. Glucose supplementation alone led to a 3-fold increase in lipid content while restricting yeast growth. The combination of glucose and yeast extract benefited yeast monocultures but not the co-culture system (Xu et al., 2024). In another study, using food waste hydrolysate as a culture medium for *Rhodosporidium toruloides* and *Chlorella vulgaris* co-culture resulted in improved nitrogen utilization (23 % increase), reduced sugar utilization (17 % increase), and lipid production (12 % increase) compared with *R. toruloides* monoculture (Zeng et al., 2018). Additionally, utilizing different carbon and nitrogen sources can affect symbiotic relationships. Tian et al. (2020) demonstrated that a symbiotic yeast (*Cryptococcus sp*.) can hydrolyze sucrose, making it available for *Chlorella pyrenoidosa* under both heterotrophic and mixotrophic conditions.

Conclusion

This study investigated the optimal culture medium for the co-culture of *Haematococcus pluvialis* and *Xanthophyllomyces dendrorhous*, two microorganisms known for their ability to produce astaxanthin. Results showed that *H. pluvialis* exhibited the highest cell densities in BGM and BGY media, while *X. dendrorhous* growth was highest in BG medium. BGM demonstrated balanced growth for both species, while BGY resulted in the highest cell concentration for *H. pluvialis* and controlled growth of *X. dendrorhous*. The study highlights the importance of selecting a culture medium that balances the growth of both species, ensures controlled nutrient competition, and optimizes gas exchange for a productive co-culture system.

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Evaluation the Impact of Environmental Factors on the Bioremediation Efficiency of a Mono-azo Dye, Acid Blue 92 (AB92), by the Algae *Tetradesmus obliquus*

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Abstract

The Dyeing and textile industries are among the sectores that hold a significant global presence in Iran and worldwide. These industries are major water consumers; consequently, substantial quantities of wastewater containing toxic compounds, including synthetic dyes, are released into the environment at various stages of the process within these industries. The treatment and purification of wastewater generated by these industries is of great importance to reduce their associated risks, and a range of physical, chemical, and biological methods are employed to remove pollutants from industrial wastewater. Meanwhile, the use of microalgae for bioremediation is one of the primary eco-friendly treatment methods due to its low cost, relianced on natural processes, and reduced risk of toxic substance accumulation. In this study, the microalgae *Tetradesmus obliquus* were utilized to investigate the removal rate of Acid Blue 92 dye under various environmental conditions, including temperature, pH, and initial concentration of the dye, initial cell number, and reaction duration. The treatments encompassed temperature (25, 10, 5 ˚C), pH (8.5, 7.5, 6.5, 5.5, 4.5), initial dye concentration (5, 10, 20, and 50 mg/L), initial cell number (5, 10, 20, and 30 $\times 10^6$ cells. mL^{-1}), and reaction duration (every 24 hours for 4 days). Furthermore, the reusability of individual algal biomass in the continuous purification of the dyes was investigated through several consecutive decolorization experiments. Based on the results, the removal efficiency of Acid Blue 92 dye increased with increasing cell number and increasing temperature. However, the removal efficiency decreased with increasing initial concentration of the dye. In addition, the optimal pH for the dye decolorization process was determined to be in the pH range of 6-7. The results of the pollutant removal reproducibility tests showed that this alga can repeatedly remove the dye from the contaminated wastewater. Therefore, the algae probably absorb and degrade the pollutant from

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the environment to an acceptable extent by utilizing the biodegradation process. In conclusion, it can be stated that *T. obliquus* algae have a significant capacity for the removal and biotreatment of acid blue dye 92, particularly under optimal conditions, and may be considered as a viable option for eliminating colored pollutants in aquatic environments.

Keywords: Bioremediation, *Tetradesmus obliquus*, algae, Organic pollutants, mono-azo dye, Acid Blue 92

Introduction

The contamination of drinking water globally presents one of the main environmental challenges we face today. A significant portion of water pollution occurs due to the discharge of sewage containing waste as well as improper disposal of industrial wastes, which include dyes, heavy metals, pharmaceutical products, and more. These substances disrupt the ecological cycle of species and change the availability and quality of vital elements for living organisms. It is predicted that the world will face a 40% water shortage by 2030, which poses a serious challenge to sustainable development (Sun et al., 2016).

The dyeing and textile sectors are among the industries that have with extensive potential in Iran and globally. These industries are recognized as the largest consumers of water, consequently leading to the discharge of significant quantities of wastewater containing toxic compounds, including synthetic dyes, are released into the environment at different stages of the process in these industries. Currently, over one hundred thousand types of commercial dyes are utilized in various industries, with approximately 10-15% of these dyes being released into the environment. Furthermore, reports indicating that around 2.5×10^5 tons of dyes are discharged into the environment annually (Singh and Singh, 2017).

Due to their wide application and ease of production, synthetic dyes are widely used in industries such as paper printing, food products, pharmaceuticals, cosmetics, and as additives in petroleum products, in addition to the textile industry. The stability of these dyes is also much higher than that of natural dyes. Therefore, they can cause significant environmental problems by creating an imbalance in the chemical and organic content of aquatic ecosystems (Sarkar et al., 2017).

Discharging wastewater containing these compounds without treatment into aquatic environments will have harmful effects on aquatic life and subsequently on the health of aquatic consumers. Therefore, it seems necessary to take effective measures to treat water and wastewater contaminated with these dyes. Water sources contaminated with such toxic wastewater can, over time, contaminate the surrounding soil ecosystems and cause serious environmental problems for the growth of living organisms, both plants and animals. In this context, reports show that some azo dyes can severely affect plant growth by obstructing seed germination, diminishing seedling survival rates, reduc-

ing photosynthesis, and preventing branch and root elongation (Baena-Baldiris et al., 2020). Consequently, the treatment of these substantial volumes of wastewater is of great importance. At present, the management of water monitoring and treatment processes is dominated by physical and chemical treatment methods along with traditional infrastructures, which are characterized by classical control and treatment systems. Current techniques, such as physical and biological adsorption, membrane filtration, oxidation, ozonation, reverse osmosis, ion exchange, photocatalysis, and electrochemical oxidation are frequently used for the treatment of pollutants. These methods for treatment and removal do not always followed by the prevailing standards, which ultimately leads to serious pollution due to the production of new toxic intermediate compounds. These methods are also expensive, energy-intensive, and not affordable for small industries and low-income processors. This financial burden is likely the main reason for the deceleration in pollution control efforts, particularly in less developed and developing countries (Khandare et al., 2013; Ummalyma et al., 2018).

The technology of using algae to remove pollutants, called algae purification (Moradi et al., 2020), is superior to other technologies due to its low cost, being based on natural processes, and reducing the risk of toxic accumulation (Cepoi and Zinicovscaia, 2020). The process of bioremediation of pollutants can be carried out through biosorption or biodegradation. Biosorption refers to the removal of pollutants from the liquid phase, such as wastewater or culture medium and their transfer to the solid phase (biosorbent surface) (Rasolzadeh et al., 2019). Sometimes, the algae involved in bioremediation, after biodegrading the pollutant compounds, use them as a source of carbon, nitrogen, or energy (Moradi et al., 2020).

Bioremediation methods that utilize bacteria, fungi, yeast, and even their consortium designs have been effective in removing dyes, but they face implementation problems in the practical and field management of wastes. The use of plants and green algae to clean up pollutants in situ can be done with much lower remediation costs and also offers a carbon-neutral and therefore environmentally friendly approach to removing toxic pollutants from the environment (Dietz and Schnoor, 2001). The use of microalgae as a bioremediation agent for colored wastewater has attracted much attention because these microalgae play an important role in carbon dioxide fixation. In addition, the biomass produced by algae is very efficient as a raw material for the production of biofuels (Huang et al., 2018). Algae are considered potential biosorbents due to the diversity of functional groups, such as hydroxyl, carboxyl, amino, phosphate, and other groups present on the cell surface. In addition to the characteristics of the cell wall, the process of biosorption of pollutants into the algal cells is also of great importance (Moradi et al., 2020). The process of degradation and biodegradation of pollutants inside living cells is carried out through various enzymatic oxidation and reduction reactions (Zohoorian et al., 2020). In this study, the microalga *Tetradesmus obliquus* was utilized to investigate the removal rate of Acid Blue 92 dye

and the effect of some biotic and abiotic factors on bioremediation efficiency.

Material and methods

T. obliquus alga was obtained from the Artemia and Aquaculture Research Institute of Urmia University and cultured in BBM medium at 25 °C. This alga was identified by Asal Pishe et al. (2012) and registered in NCBI with the number (Accession Number OR393092).

T. obliquus was cultured in Bold's Basal Medium (BBM), a standard culture medium for freshwater algae. The culture medium was prepared according to standard protocols and sterilized by autoclaving at 121°C and 15 psi for 20 min (Asghari et al., 2023). To ensure uniformity and reproducibility, a fresh culture medium was used in all experiments (Torbati, 2019). Color removal experiments were performed in 250 mL Erlenmeyer flasks containing 100 mL of BBM culture medium by inoculating with *T. obliquus*.

Temperature (5, 10, and 25 °C), pH (4.5, 5.5, 6.5, 7.5, and 8.5), initial dye concentration (5, 10, 20, and 50 mg/L), initial cell number in the medium (5, 10, 20, 30 $\times 10^6$ cells. mL^{-1}), and reaction time (every 24 hours for 4 days) were adjusted and investigated as the main variables. Each time, the effect of just one factor on removal efficiency was determined, and other effective parameters were kept constant. The Erlenmeyer flasks were placed on a circular shaker at a speed of 150 rpm to provide uniform suspension of algal cells and proper aeration. Dilute KOH and H_2SO_4 solutions were also used to adjust the initial pH of the solution, and the pH was measured by a pH meter (Hanna Instrument Inc.).

The number of cells in the culture medium was counted using a hemocytometer slide (Kennari et al., 2008). These experiments were conducted over a 4-day period, with samples collected at specific time intervals (0, 24, 48, 72, and 96 hours). The samples were centrifuged at 4000 rpm for 10 minutes to separate the algal cells and leave a clear solution for analysis. The percentage of dye removal was calculated using the following equation at the maximum wavelength (λmax = 571 nm) using a UV-Vis spectrophotometer (Camspec M330 model, UK) (Torbati, 2019).

Dye Removal (%)

A_0: Initial absorption of dye solution

A: Solution absorption at the time of measurement.

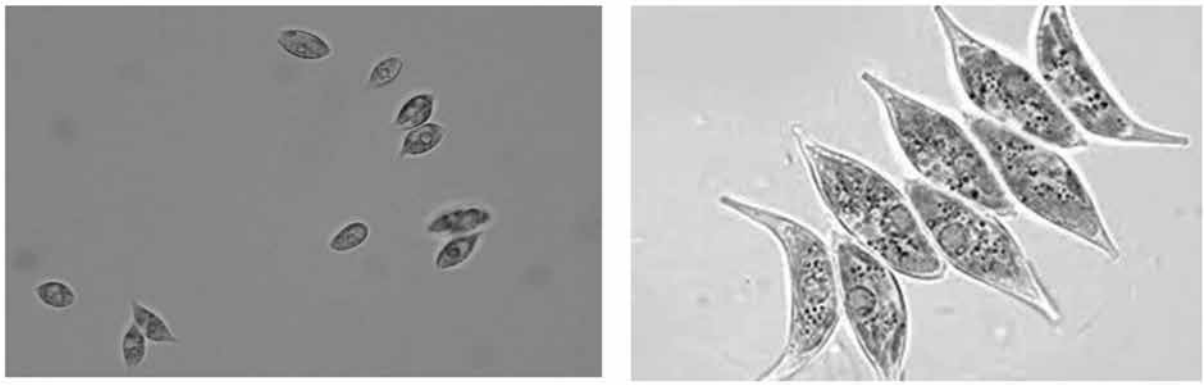

Fig. 1. Spindle cells of the algae *Tetradesmus obliquus*

Results

The results of the effect of the initial number of *T. obliquus* algae cells on the efficiency of AB92 dye removal over 4 days are shown in Figure 2. According to the results, with an increase in the number of algae cells, the percentage of dye removal from the medium also increased. So in the treatments of 5×10^6 to 30×10^6 cells mL^{-1}, respectively, 55 and 95 % of the dye with an initial amount of 10 mg/L was removed from the medium culture after 4 days. The initial concentration of the dye can play an important role in the efficiency of dye removal. The results of the effect of the initial concentration of the AB92 dye on the efficiency of dye removal in *T. obliquus* algae cells for 4 days are shown in Figure 3. According to the results, the efficiency of the dye removal process decreases with increasing initial concentration of the dye. However, the amount of dye removed per unit time increases with increasing initial concentration. These results probably indicate a complex relationship be-

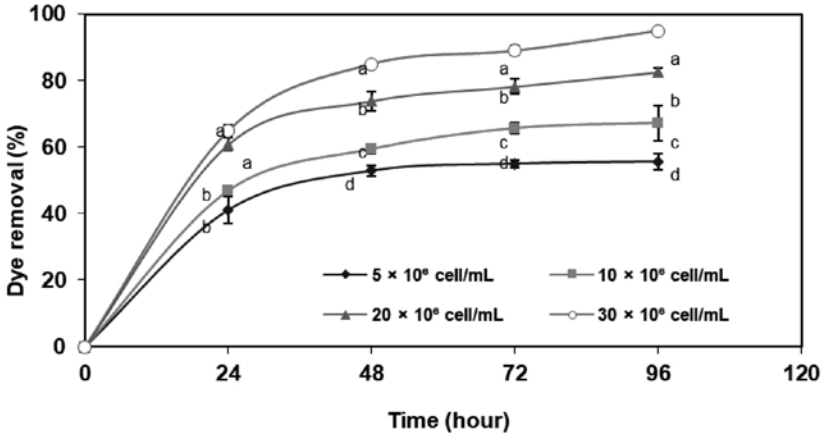

Fig. 2. Effect of cell number on the dye removal efficiency of T. *obliquus* algae for 4 days (data reported as Mean ± SD, T=25 ºC, pH=6.5, [AB92]0=10 mg/L; different letters in the statistical analysis for each 24 hours indicate significant differences in dye removal efficiency)

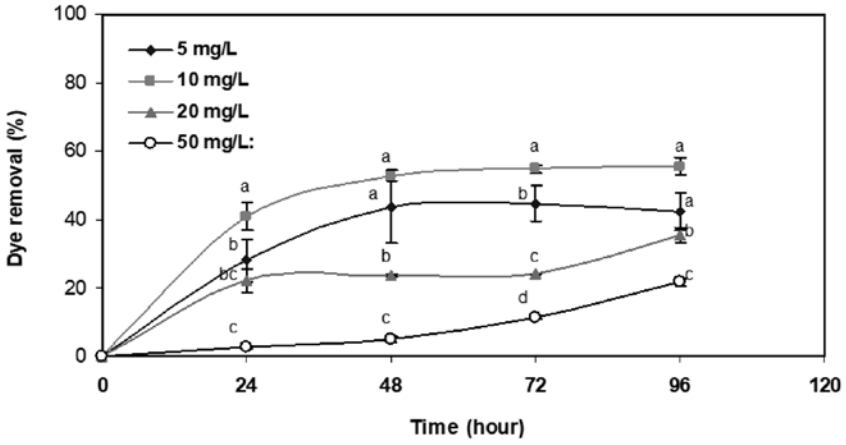

Fig. 3. Effect of initial dye concentration on dye removal efficiency of *T. obliquus* algae for 4 days (data reported as Mean ± SD, T=25 ºC, pH=6.5, Cell No.= 5×10^6 cell/mL; different letters in statistical analysis for each 24 hours indicate significant differences in dye removal efficiency)

tween the ability of the algae to decompose and the concentration of the dye.

The results of the effect of different pH levels of the *T. obliquus* algae culture medium on the efficiency of removal of AB92 dye by microalgae for 4 days are shown in Figure 4. The optimal efficiency of removal of AB92 dye was determined after 4 days of algae treatment at pH 6.5.

The results of the effect of different temperatures (25-5 ºC) of the *T. obliquus* algae culture medium on the efficiency of removing the AB92 dye in the microalgae for 4 days are shown in Figure 5. The results show that the efficiency of the dye removal percentage increases with increasing temperature.

Figure 6 shows the results of the evaluation of the repeated removal of dye (10 mg/L) by the same algal mass with an initial cell count of 5×10^6 alga during 4 consecutive trial pe-

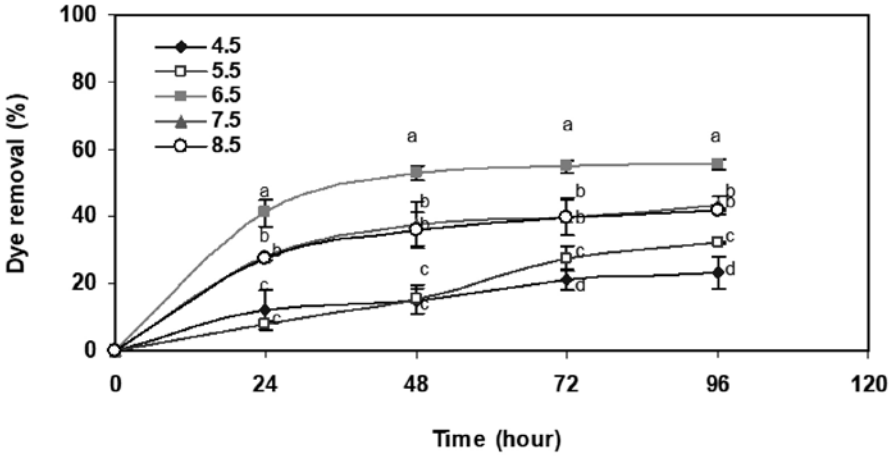

Fig. 4. Effect of different pH on the dye removal efficiency of *T. obliquus* algae for 4 days (data reported as Mean ± SD, T=25 ºC, Cell No. = 5×10^6 cell/mL, $[AB92]_0$=10 mg/L; different letters in the statistical analysis for each 24 hours indicate significant differences in dye removal efficiency)

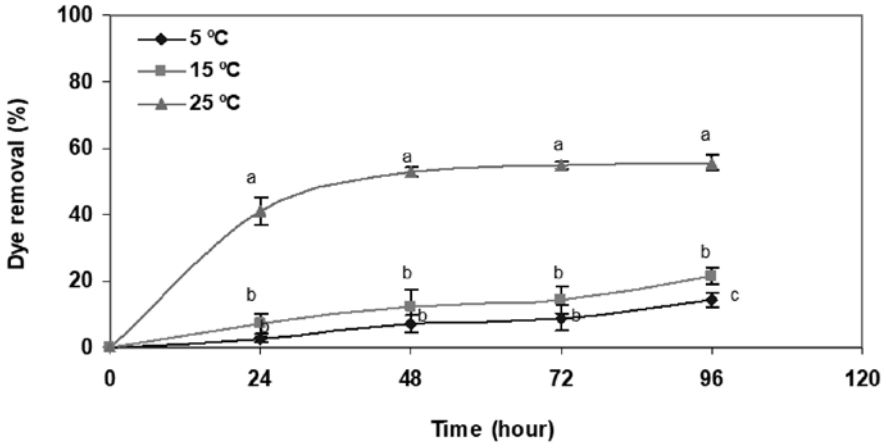

Fig. 5. Effect of different temperatures on the dye removal efficiency of *T. obliquus* algae for 4 days (data reported based on Mean ± SD, pH=6.5, Cell No. = 5×10^6 cell/mL, $[AB92]_0$=10 mg/L; different letters in the statistical analysis for each 24 hours indicate significant differences in dye removal efficiency)

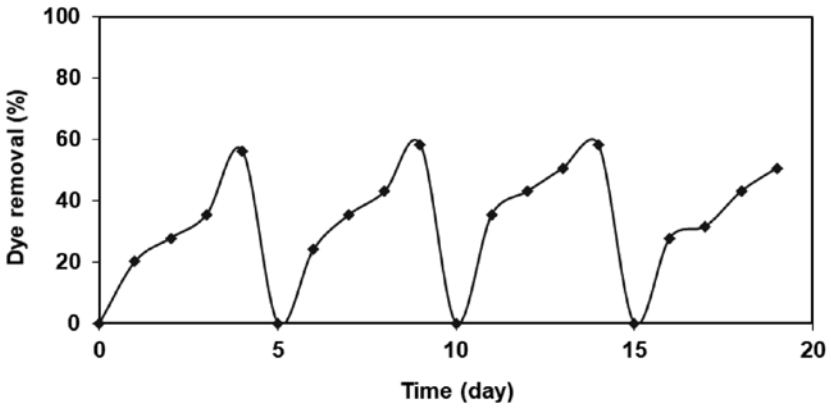

Fig. 6. Effect of successive iterations on the dye removal efficiency of AB92 in *T. obliquus* algae for 4 days (T=25 ºC, pH=6.5, Cell No. = 5×10^6 cell/mL, $[AB92]_0$=10 mg/L; average data reported per 24 hours)

riods. In this study, sampling was performed at intervals of every 24 hours, and the percentage of removal of this material was calculated. This process was repeated 4 times. According to Figure 5, the ability of the algal mass to repeatedly purify the dye was confirmed. The acceptable ability of the single algal mass to repeatedly remove the dye indicates that the biodegradation process is one of the main mechanisms involved in the removal of AB92 by algae.

Discussion

This study confirms the potential of *T. obliquus* as an effective bioremediation agent for synthetic dyes such as AB92. According to the results, the dye removal efficiency of this algae is significantly affected by environmental factors. The efficiency of the dye removal percentage increases with increasing initial algae number. According to the results of Torbati, 2019, the number of initial cells in the test medium also played a positive role in the efficiency of dye removal. It seems that with an increase in the number of algae cells in the medium, the number of pollutant biosorption sites increases, and subsequently, the pollutant removal rate increases (Ayele et al., 2021). Furthermore, according to the literature review, it has been determined that a threefold increase in the algal biomass of *Caulerpa scalpelliformis* and *Pithophora* sp. has resulted in a 60% and 33% increase in the removal efficiency of cationic dye, respectively (Aravindhan et al., 2007; Kumar et al., 2006).

The pH parameter is one of the most important factors affecting the efficiency of biosorption of dyes by algae (El-Naggar et al., 2018). In addition, the pH of the environment can affect the solubility of some dyes as well as the interaction between dye molecules and algae. The cell surface charge of algae depends on the pH of the environment due to the presence of functional groups such as carboxyl, hydroxyl, amine, etc. For example, at acidic pH, the surface of algae has a higher positive charge and is favorable for the absorption of anionic dyes (Sun et al., 2019). According to the literature review, the optimal pH for the growth of most microalgae has been determined to be in the

neutral range for the genus *Scenedesmus* (El-Sheekh et al., 2017). Based on our results, the optimal pH for the removal of AB92 is also in this range. This pH is probably ideal for the balance between algal health and dye absorption capacity. Also, the AB92 dye is an anionic dye compound, and the pH that is optimal for algal growth and activity, and also creates a more positive charge on the algal surface, which can be determined to be optimal for the absorption of this dye (Ayele et al., 2021).

Khataee et al. (2012) noted that as temperatures drop, the movement and absorption of water decline, and as a result of the reduced permeability of the algal membranes, the uptake of solvent molecules in water decreases, leading to a reduction in the effectiveness of the absorption process. In this study, temperatures higher than ambient temperature were not investigated due to the biological nature of the adsorbent and the negative role of high temperatures in the structure and activity of algae. The negative role of high temperatures in the bioremediation process has also been confirmed in previous reports. It has been found that the absorption of Golden Yellow C-2g dye in *C. scalpelliformis* algae decreases significantly with an increase in temperature from 20 to 60 ºC (Aravindhan et al., 2007). The viscosity of the dye-containing solution decreases, and its absorption by the biosorbent and subsequent removal at temperatures exceeding ambient levels is reduced. It is likely that at high temperatures, the rate of dye absorption decreases due to changes in the active sites of the biosorbent (Ayele et al., 2021).

This study confirms the effect of temperature (up to ambient temperature), while the efficiency of the removal process decreases with increasing initial dye concentration. Furthermore, the optimum pH for achieving the highest dye removal percentage was determined at pH 6.5. The integration of algal bioremediation strategies can play an important role in sustainable environmental management practices and, simultanously provide complementary benefits for the economic perspective through the utilization of biomass. These applications can help reduce the negative impacts of industrial pollution on the environment and lead to the development of sustainable and environmentally friendly solutions for the treatment of industrial wastewater.

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Regulation of *Dunaliella salina* Malate Dehydrogenase Gene Expression by Interfering Ribonucleotides

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Abstract

The microalga *Dunaliella salina* is one of the most resilient organisms adapted to harsh environments. Research indicates that the organisms, especially plants, respond to various environmental stresses differently. *D. salina* has emerged as a halotolerant model organism for studying stress adaptation due to its ability to thrive under extreme salinity, light, and nutrient-deficient conditions. It produces a vital carotenoid, 9-cis beta-carotene, which is utilized in medical industry. One of the significant interferences in stress responses is mediated by 21-24 nucleotide interfering RNAs. Malate dehydrogenase is a key enzyme involved in energy metabolism in both mitochondria and chloroplasts, and its transcription and activity regulation are highly significant. This study investigated the number of miRNA binding sites to the malate dehydrogenase transcript. The involvement of some miRNAs, including novel-m0533-3p, in energy-related metabolism has been identified. The results showed that the mitochondrial transcript had 5 binding sites and the chloroplast transcript had 1 binding site for novel-m0533-3p miRNA. The low number of miRNA binding sites to the chloroplast malate dehydrogenase mRNA sequence indicates that perhaps other gene expression regulation methods control the chloroplast malate dehydrogenase gene or probably, Chloroplastic Malat Dehydrogenase is regulated by enzyme activity, and also the 5 point of binding sites of the miRNA to the mitochondrial malate dehydrogenase mRNA, indicates that this type of gene expression regulation is more dominant. Our results suggest that miRNAs act as dynamic regulators that modulate MDH expression in a stress-type-dependent manner. These findings align with previous studies emphasizing post-transcriptional regulation as a key mechanism for microalgae adaptation to harsh environments.

Keywords: *Dunaliella salina*, Malate Dehydrogenase, Interfering Ribonucleotide, Gene regulation, transcription, Chloroplast, Mitochondria

Introduction

Dunaliella salina, a halotolerant microalga, has emerged as a model organism for studying stress adaptation due to its ability to thrive under extreme salinity, light, and nutrient-deficient conditions. This resilience

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is closely linked to its capacity for high-value metabolite biosynthesis, particularly 9-cis beta-carotene, a carotenoid of significant medical and industrial interest (Zarandi-Miandoab et al., 2019). Environmental stressors such as variation in light intensity, salinity fluctuations, and nitrogen deprivation trigger complex molecular responses in *D. salina*. These responses encompass differential gene expression and metabolic rewiring in prioritize protective compounds like beta-carotene over fatty acids (Zarandi-Miandoab et al., 2015; Barczak-Brzyżek et al., 2022).

Gene expression regulation (a process determining the type and quantity of proteins) plays a pivotal role in stress adaptation (Ni et al., 2009). Various methods of gene expression regulation are used to balance the concentration of enzyme proteins, and various other methods are used to regulate the activity of said enzyme proteins (Lackner and Bähler, 2008). In conditions of environmental stress, It is crucial to regulate and control the energy status of the cell (Kansal et al., 2021). Malate dehydrogenase is a key enzyme that plays a significant rolein energy metabolism and the production of nicotinamide adenine dinucleotide (NADH); thus, the regulation of its transcription and activity is vital. The enzyme malate dehydrogenase (MDH) is important in metabolic cycles occurring in two types of cellular organelles, mitochondria and chloroplasts (Xiao et al., 2018). Mitochondrial MDH (mMDH) is integral to the tricarboxylic acid (TCA) cycle, driving ATP production and maintaining the $NADH/NAD^+$ balance, while chloroplastic MDH (cMDH) facilitates carbon fixation and photoprotection (Ermakova et al., 2024; Fabian et al., 2009). Recent studies highlight the role of small non-coding RNAs, particularly microRNAs (miRNAs), in the post-transcriptional regulation of stress-responsive genes, including MDH isoforms (Fang and Rajewsky, 2011; Hurschler et al., 2010). For instance, miRNAs can silence target mRNAs via sequence complementarity, influencing metabolic pathways critical for stress survival (Infantino et al., 2021). However, the mechanisms underlying miRNA-MDH interactions in *D. salina* remain poorly characterized. This study investigates how miRNA-mediated regulation of MDH isoforms contributes to the alga's stress adaptability, with implications for biotechnological applications

Material and methods

Gene sequences were retrieved from the NCBI database, including mitochondrial (KT001001.1, KT001002.1) and chloroplastic MDH isoforms (AF522057.1, EU352600.1, EU352601.1). Putative miRNA binding sites were predicted using miRBase (v22) and MirGeneDB, followed by in silico interaction analysis with TargetScan and miRWalk under stringent criteria: minimum free energy (MFE $\leq$ -15 kcal/mol), seed region complementarity ($\geq$6 nucleotides), and evolutionary conservation across algal species. RNAhybrid, IntaRNA, and RNAfold were employed to validate miRNA-mRNA interactions, focusing on thermodynamic stability and structural accessibility. For physiological validation, VARNAv3.9 was utilized to model RNA secondary structures and assess the binding feasibility of

novel-m0533-3p under stress-mimicked conditions. This tool confirmed robust interactions between novel-m0533-3p and chloroplasts MDH transcripts, highlighting sequence-specific binding at conserved motifs within the 3'UTR, which likely modulates post-transcriptional repression under environmental stress.

Results and Discussion

The differential Targeting of miRNA on MDH Isoforms for *D. salina* malate dehydrogenase as identified in the NCBI database,) illustrated that mitochondrial MDH transcripts exhibited 44 predicted miRNA binding sites, whereas the fewer sites present in chloroplastic MDH (Table 1).

Four transcripts detailing the characteristics of *D. salina* malate dehydrogenase are presented in Table 1. As can be seen in the table, the first row corresponds to the mitochondrial enzyme (KT001001.1), which has the capacity to bind and interact with miRNA at 5 distinct points. The second, third, fourth rows pertain to the chloroplast enzymes, whose genes reside in the nucleus, while the resulting translation product is located in the chloroplast, contributing in the stroma and the Calvin cycle. All three transcripts of the chloroplast malate dehydrogenase gene (AF522057.1, EU352600.1, EU352601.1) can interact with miRNA at only a single point. The estimate of the number of points that can potentially bind to m0533-3p miRNA acts as a confirmation that interfering nucleotide affects the transcript of the nuclear malate dehydrogenase gene, which may alter the regulation of gene expression. The novel-m0533-3p sequence binds to the RNA of the malate dehydrogenase gene and alters its expression levels during stress (Lou et al., 2020).

The limited number of miRNA binding sites in the the mRNA sequence of chloroplast malate dehydrogenase suggests the presence of significant gene expression regulation pathways that control the chloroplast malate dehydrogenase gene. Conversly, the high abundance of the mRNA binding site within the mRNA sequence of mitochondrial malate dehydrogenaseimplies that this type of gene expression regulation is more dominant than other regulatory mechanisms of gene expression (Afonso-Grunz and Müller, 2015; Fang and Rajewsky, 2011; Wang et al., 2016). miRNA sequences are recognised as significant regulators of gene expression, however, their effects are typically varies based on the specific conditions of the plant and the nature of environmental stress. A study conducted by Brzyżek (2022) showed that miRNAs can affect the expression of certain chloroplast genes, yet their effects are often less than the effect of light (Barczak-Brzyżek et al., 2022).

The regulation of chloroplast malate dehydrogenase activity appears to be largely influenced by redox regulation via the thioredoxin system and is influenced by light (Yoshida et al., 2015; Miginiac-Maslow et al., 2000). It seems logical that the need for regulation of the function of such a key enzyme would require a high speed of action, mediated by light and at the post-translational level in the stroma. Certainly, regulation at the transcriptional level for a nuclear gene whose product is to function in the chloroplast requires more time. Generally, the

Table 1. Activity of gene characteristics, malate dehydrogenase transcripts, and number of predicted miRNA sites and m0533-3p miRNA binding sites in *D. salina*

Gene (Transcript ID)/ Organelle	mRNA	Predicted miRNA Sites	Number of adhesion points	miRNA novel-m0533-3p
Dunaliella salina putative Malate Dehydrogenase (MDH) gene, complete cds GenBank: KU001001.1 LOCUS KT001001 4445 bp DNA **353aa** linear PLN 31-OCT-2016 >ALY05463.1 putative malate dehydrogenase MLAYKRVAGSAAAQTSRGPSSRVTGLSPRVSRR KIVKCEAKKVALLGAGGGIGQPLSLLLKMNKLV TELSLYDIAGVTGVGADISHCNTPVKVSAFNGPE ELEGALKGAELIIIPAGVPRKPGMTRDDLFNVNA GIVKALVEAASKHCPEAIIQLVTNPVNSTVPIAAE VMKKAGTHNPAKIMGVSTLDVVRANTFVAEAK KLDVKDVDVPVVGGHAGATILPLLSQATPPVSFT DAEKKMMTEKIQNAGTVVVEAKAGKGSATLSM AYAAARMAESTLMGLNGEPNIYECSYVQSDVVP DCPFFASKLLLGPGGVAKVLPLGNMDAFEQACF DAMLPELKASIQKGIDFANA Mitochondria	[illegible]	44	5 point	UCCUGGACGCCCGGACUAUCAU
Dunaliella salina putative malate dehydrogenase (MDH) mRNA, complete cds. KT001002, VERSION KT001002.1 **352 aa** protein_id="ALY05464.1" >MLAYKRVAGSAAAQTSRGPSSRVTGLSPRVSR RKIVKCEAKKVALLGAGGGIGQPLSLLLKMNKL VTELSLYDIAGVTGVGADISHCNTPVKVSAFNGP EEEGALKGAELIIIPAGVPRKPGMTRDDLFNVNA GIVKALVEAASKHCPEAIIQLVTNPVNSTVPIAAE VMKKAGTHNPAKIMGVSTLDVVRANTFVAEAK KLDVKDVDVPVVGGHAGATILPLLSQATPPVSFT DAEKKMMTEKIQNAGTVVVEAKAGKGSATLSM AYAAARMAESTLMGLNGEPNIYECSYVQSDVVP DCPFFASKLLLGPGGVAKVLPLGNMDAFEQACF HAMLPELKASTQKGIDFANA Mitochondria	[illegible]	36	2 point	UCCUGGACGCCCGGACUA UCAU
Dunaliella salina cultivar CCAP 19/30 chloroplast Malate Dehydrogenase mRNA, partial cds; nuclear gene for chloroplast product GenBank: EU352601.1 LOCUS EU352601 **429aa** 1288 bp mRNA linear PLN 15-JAN-2008 >ABY61961.1 chloroplast malate dehydrogenase, partial MQLNLQKNVGLAQKQATVTPAFNVRGNVARKV QAGSNAAARRASSVVRAVDAPEKAAKQFGVFR LSYDVNNEDKEMMKNWKKTINV AVTGASGMIANHLLFMLASGEVYGKDQPIALQL LGSERSYEALEGVAMELEDSLYPLLREVSIGIDPY EIFAGADWALMVGAQPRGPGMERSDLIQKNGQI FQTQGRALNEVADRNCKVVVVGNPCCTNAFIA MENAPNLPRKNFHALTRLDENRAKSQLAMKAG KFYTSVSRVAVWGNHSTTQVPDFLNAKIGGLPA MNVIRDNQWFKDQFTPKVAMRGGALIKKWGRS SAASTAVSVADSIRSLITPTPPGDCFSSGVCSDGN LYGIQEGLNFSFPCRSKGDGDYEICNDFIIDDWLR MKIKAAEEELIQERDCVSHLIGREGGACAIGPNTP DTSVPGEK Chloroplast	[illegible]	44	1 point	UCCUGGACGCCCGGACUAUCAU
Dunaltella salina cultivar CCAP 19/25 chloroplast Malate Dehydrogenase mRNA, partial cds; nuclear gene for chloroplast product GenBank: EU352600.1 EU352600 **434aa** 1303 bp mRNA linear PLN 15-JAN-2008 >ABY61960.1 chloroplast malate dehydrogenase, partial MQLNLQKNVGLAQKQATAPSPAFSVRGNAVRS KLSAQLGSKAGFTRRAPVARAVAEAEDKASKQF GVFRLSYDVSNEDKEVMKNWKKTINVAVTGAS GTIANHLLFMLASGEVYGKDQPISLHLLGSERSY EALEGVAMELEDSLYPLLREVSIGIDPYEIFAGAD WALMVGAQPRGPGMERSDLIQKNGQIFQVQGR ALNEVADRNCKVIVVGNPCCTNALIAMENAPNL PRKNFHALTRLDENRAKSQLALKAGKFYTSVSR VAIWGNHSTTQVPDFLNAKIGNLPAMNVIRDNQ WFKEHFTPKVAMRGGALIKKWGRSSAASTAVS VADSIRSLITPTPPGDCFSSGVISDGNLYGIQEGLN FSFPCRSKGDGDYEICNDFIIDDWLRMKIKAAED ELIQERDCVSHLIGREGGACAIGPNTPDTSVPGEK Chloroplast	[illegible]	35	1 point	UCCUGGACGCCCGGACUAUCAU
Dunaliella salina Malate Dehydrogenase mRNA, partial cds; nuclear gene for chloroplast product GenBank: AF522057.1 LOCUS AF522057_1 **230 aa** >AAM83089.1 malate dehydrogenase, partial DRNCKVIVVGNPCCTNALIAMENAPNLPRKNFN ALTRLDENRAKSQLALKAGKFYTSVNRVAVWG NHSTTQVPDFLNAKIGGLPAMNVIRDNQWFKEE FTPKVALRGGALIKKWGRSSAASTAVSVADSIRS LITPTPPGDCFSSGVCSDGNLYGIQEGLNFSFPCR SKGDGDYEICNDFIIDDWLRMKIKAAEEELIQER DCVSHLIGREGGACQIGPGAPDTSVPGEN Chloroplast	[illegible]	34	1 point	UCCUGGACGCCCGG ACUAUCAU

differences in the expression of these two genes are related to the type of metabolism, environmental conditions, and the specific requirements of cells in response to both internal and external factors (Schwartzbach, 2017). This difference in expression allows cells to respond more effectively to their metabolic and energy demands. The higher miRNA targeting of mitochondrial MDH may reflect its central role in energy production and consumption during stress, requiring precise regulation to balance ATP production and redox homeostasis. In contrast, chloroplastic MDH suppression under stress (e.g., via novel-m0533-3p) could prioritize photoprotective carotenoid synthesis over carbon fixation, aligning with *D. salina's* stress-response strategy (Li et al., 2024).

The biological Implications of this variation in interaction with miRNA may be associated with the duration required to react and respond to environmental conditions. It appears that in response to environmental stimuli such as light, the chloroplast must adapt its energy state very quickly, which is why it employs the thioredoxin system to regulate MDH enzyme activity. However, to manage the energy state of the cell under various conditions, the cell nucleus has enough time to regulate at the transcriptional level through the intervention of the miRNA. This variation in performance leads to the dual regulatory role of miRNAs. A single miRNA can upregulate or downregulate gene expression, depending on the cellular context (Fabian et al., 2009). There is an emphasis on multi-miRNA targeting of miRNAs. Multiple miRNAs can target a single gene, and their combined activity determines the expression of a given gene. miRNA can act through binding to the 3'UTR of target mRNA (Fang and Rajewsky, 2011), mRNA Degradation, Translation Inhibition (Afonso-Grunz and Müller, 2015), Nascent protein degradation, mRNA storage in P-bodies (Fabian et al., 2009; Hurschler et al., 2010), and transcription inhibition (Fabian et al., 2009).

The findings of this study highlight the critical role of miRNAs in regulating the expression of mitochondrial and chloroplastic malate dehydrogenase (MDH) genes in the microalga *D. salina* under various environmental stresses. Validation using three bioinformatics tools, RNAhybrid, IntaRNA, and RNAfold, demonstrated that mitochondrial MDH exhibits significantly stronger constructive regulatory interactions compared to its chloroplastic counterpart, suggesting distinct miRNA-mediated post-transcriptional control mechanisms between the two organelles. Our results indicate that miRNAs act as dynamic regulators that modulate MDH expression in a stress-type-dependent manner. These findings align with previous studies highlighting posttranscriptional regulation as a crucial mechanism for microalgal adaptation to extreme environments (Wang et al., 2016).

A key discovery was the identification of novel-m0533-3p, which selectively binds to all three chloroplastic MDH transcripts (AF522057.1, EU352600.1, EU352601.1) and suppresses their expression (Li et al., 2023). The specificity of this miRNA for chloroplastic MDH suggests a compartmentalized regulatory strategy that may prioritize mitochondrial energy metabo-

lism during stress (Huang et al., 2018). Such compartmentalization is consistent with plant studies in which miRNAs regulate organelle functions to maintain cellular homeostasis (Nalawade and Singh, 2023). Notably, the mitochondrial MDH sequence (KT001001.1) showed minimal interaction with this miRNA, supporting the hypothesis of preferential activation of mitochondrial pathways for ATP production under stress. This mechanism enables stable β-carotene synthesis and osmotic balance.

The dual regulatory roles of miRNAs, both upregulating and downregulating gene expression, were evident in this study. For example, under hypoxic conditions, miR-206-like sequences likely enhance mMDH expression by binding to the 5'UTR region, a mechanism previously observed in animal systems (Rao et al., 2016). While speculative, this hypothesis could explain enhanced mitochondrial ATP output during stress, warranting validation via hypoxia-responsive miRNA profiling.

This contrasts with the canonical miRNA-mRNA interaction at the 3'UTR and underscores the tissue-specific nature of miRNA activity. Such functional flexibility may explain how *D. salina* dynamically regulates its metabolic network to balance energy demands, antioxidant production, and carotenoid synthesis under fluctuating environmental conditions.

Our findings reveal a compartment-specific miRNA regulatory network: mitochondrial MDH is spared from miRNA silencing, ensuring sustained energy production. At the same time, chloroplastic MDH is downregulated to redirect resources toward beta-carotene synthesis. This aligns with studies in Arabidopsis, where miRNAs fine-tune organellar functions under stress (Lou et al., 2020). For example, novel-m0533-3p-mediated cMDH suppression mirrors miR398-mediated silencing of Cu/Zn superoxide dismutase in plants under oxidative stress (Martinez-Vaz et al., 2024). The results align with *D. salina's* metabolic prioritization of beta-carotene under stress (Minarik et al., 2002), but reliance on in silico predictions introduces false positives. Tissue-specific RNA-binding proteins (RBPs) and alternative polyadenylation may further modulate miRNA accessibility factors unaddressed here. Comparative studies in *Chlamydomonas reinhardtii* could clarify the evolutionary conservation of these regulatory motifs (Musrati et al., 1998).

Conclusion

Gene expression regulation in *D. salina* is a complex process influenced by various environmental factors. The regulation of the malate dehydrogenase gene expression has a direct impact on cellular function and energy metabolism. Given the presence of two malate dehydrogenase isozymes in *D. salina*, regulation at different levels during transcription, post-transcription, translation, and post-translational modification and creating a proper balance is crucial. Once understood, these mechanisms go a long way in explaining how this small organism survives and resists.

This study elucidates a miRNA-driven regulatory framework enabling *D. salina* to balance energy metabolism and stress adaptation.

Significant findings highlight the specific targeting of MDH isoforms by miRNAs in different compartments. Notably, novel-m0533-3p acts as a suppressor of chloroplastic MDH during stress conditions, while mitochondrial MDH is emphasized for maintaining energy homeostasis.

Future studies should focus on integrating multi-omics approaches (e.g., transcriptomics and proteomics) to comprehensively map miRNA-MDH interactions. Techniques like CLIP-Seq can elucidate tissue-specific miRNA targeting, whereas CRISPR interference (CRISPRi) may reveal causal relationships between specific miRNAs and stress phenotypes. In addition, comparative analyses across microalga species identified conserved miRNA regulatory motifs, providing insights into universal stress adaptation mechanisms. These insights advance microalgal biotechnology, offering strategies to engineer high-beta-carotene strains resilient to environmental stressors. The carotenoids and other secondary metabolites produced under harsh living conditions in *D. salina* are useful and effective in metabolic diseases and cancer therapy.

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Role of Screening Elements on Quality of Wastewater by Marine Cyanobacterium *Fischerella muscicola*

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Abstract

Today, industrialization along with the growth of population has increased wastewater production, which has become one of the serious problems for ecosystems and the environment. Conventional wastewater treatments (physical or chemical methods) that consume a great deal of energy and cost are not so appropriate manner for removing nutrients like nitrate and phosphate,_so the application of biological methods, such as using microalgae, has been noticed. Wastewater usually contains various compounds such as nitrate and phosphate that can be used as culture medium for microalgae (cyanobacteria). Therefore, consumption and elimination of these elements from media not only helps to biological treatment of wastewaters but also lead to higher productivity of these organisms. In this research, screening artificial wastewater elements and their role in the physiological activities of the cyanobacterium *Fischerella muscicola* have been studied. Our specimen isolated from Caspian Sea and identified molecularly according to 16s rRNA. Artificial wastewater treatments were designed by Design-Expert software in 12 runs. Various amounts of NaCl, $CaCl_2$, $MgSO_4$, $NaNO_3$ and K_2HPO_4 was added to BG110 medium and microalgae were cultured. Analysis of treatments according to the changes of cations (Na^+, Ca^{2+}, Mg^{2+}), anions (Cl^-, NO_3^-, PO_4^{3-}), TDS and COD were performed on logarithmic phase (10th day of the culture). Screening of wastewater elements were done by parreto plot and normal plot charts. Results showed that among applied elements in wastewater, NaCl, $NaNO_3$ and K_2HPO_4 have the most effect on growth of *Fischerella muscicola* and changes of cations, anions, TDS, and COD. As removing nutrients from the media is related to the growth. Therefore, wastewater (especially with nitrate and phosphate) could be an appropriate medium for microalgae growth and the production of various bioactive compounds.

Keywords: Biological treatment, *Fischerella muscicola*, Elements, Screening, Wastewater

Introduction

In recent years, increasing population along with the process of urbanization and industrialization, resulted production and

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release of wastewater into water resources, which has finally become one of the major environmental problems (Spennati et al., 2021). So, by increasing the concentration of organic nitrogen and phosphorus in surface waters, eutrophication occurs (Goncalves, 2017; Benitez et al., 2019). In this situation, according to the high concentration of soluble nutrients, such as N and P, algal biomass increased naturally in water bodies, which causes to reduction of oxygen content and water quality. Therefore, some countries have set certain rules for the reuse or release of wastewater in water resources. Besides modern improvements in conventional wastewater treatment technologies, most of the existing treatment plants have some difficulties in effectively removing nitrogen and phosphorus in the standard format (Torres-Franco et al., 2021). Usually, common methods of wastewater treatment based on aerobic and anaerobic digestion that use activated sludge, coagulation, and sedimentation operations (Otondo et al., 2018), consume a great deal of energy and chemicals (Wang et al., 2016, Satpal & Khambete, 2016). Although these methods can significantly reduce COD (Chemical Oxygen Demand), but are not very effective in removing nitrogen and phosphorus. Therefore, the application of microalgae as a biological method has been proposed to remove these nutrients. There are various studies on the role of cyanobacteria in improving water quality which indicates some cyanobacterial species such as *Anabaena variabilis*, *Anabaena oryza*, *Tolypothrix ceylonica, Spirulina platens*is are efficient in reducing BOD (Biological Oxygen Demand), TDS (Total Dissolve Solid) and COD and improving quality of different types of wastewater. In this case, sewage wastewater treated with a cyanobacterial consortium of native strains including *Phormidium, Limnothrix, Anabaena, Westiellopsis, Fischerella,* and *Spirogyra* showed about 99 and 89% reduction of COD and BOD.

In recent years, biological wastewater treatment with microalgae has considered as an alternative to conventional methods. The potential of microalgae in removing nutrient from different wastewater has been shown in several studies (Al-Jabri et al., 2021; Yadav et al., 2019; Ziganshina et al., 2021). In this regard, according to high potential of microalgae to remove contaminants, they can be used in combination with activated sludge (as culture media) (Benitez et al., 2019; Kim et al., 2010). Microalgae can grow in wastewater effluents by consuming carbon, nitrogen, and phosphorus as the main growth nutrients (Chen, 2021) and produce a valuable biomass product. So, they can reduce energy consumption in comparison to conventional treatment methods (Otondo et al., 2018; Saptal & Khambete, 2016).

In several studies, microalgae have a complementary role in wastewater treatment, and their high efficiency in removing nutrients has been proven. Even in various projects, effluent from the secondary treatment stage (Kim, 2010), as well as in the stages of centrate (Mine, 2011) and also synthesis effluents (Benitez et al., 2019; Otondo et al., 2018), have been used. The proper selection of efficient microalgal species with considerable cell growth and high tolerance to wastewater is crucial to promote this meth-

od (Moondra et al., 2020). In this regard, applying a microalgae bacterial consortium is an important step to eliminate maximum nutrients and reduce the cost of wastewater treatment which has also been considered in recent years (Rada-Ariza, 2017; Ji & Liu, 2021). The microalgae-bacterial consortium is primarily suitable for wastewater treatment, particularly in systems with high levels of nutrients and low organic matter (Foladori et al., 2018; Krustok et al., 2016). In some studies, the microalgae-bacterial composition has been used to remove nutrients in both raw and artificial wastewater (Khaldi et al.,2017; Rada-Ariza et al., 2017). Wastewater (artificial or raw) can naturally be an optimum medium for microalga growth. Microalgae use light as an energy source and CO_2 as a carbon source for their photosynthesis and uptake nitrogen and phosphorus for their cellular functions. Thus, this process reduces the concentration of nutrients in wastewater and contributes to CO_2 mitigation. In addition, microalgae produce oxygen, which can be used by aerobic bacteria to biodegrade organic pollutants present in the wastewater (Otondo et al., 2018; Boonchai et al., 2012).

Among microalgae, cyanobacteria (blue-green algae) are better candidates for wastewater treatment, because of their wide distribution and viability in various environmental changes. The microalga species commonly employed in sewage treatment experiments are eukaryotic and prokaryotic blue-green species such as *Chlorella* sp., *Scenedesmus* sp., *Fischerella* sp., and *Oocystis* sp., which are more effective in purification and bioremediation processes (Rasoul-Amini et al., 2014). Although, other factors such as growth and resistance of these strains to wastewater conditions, their abilities to remove various pollutants (nitrogen, phosphorus ammonia, calcium, magnesium, sodium, potassium and heavy metals) have been considered for their selection (Mohammadi et al., 2018). Application of cyanobacteria in wastewater treatment is an eco-friendly method with no secondary pollution as their biomass can be reused. This technology, compared to other physical and chemical remediation processes, is also cost-effective. The high requirement of N and P for the growth of cyanobacteria is a good reason to consume these nutrients in wastewater for multiplication of these microorganisms. In this way, assimilated nitrogen and phosphorus can be recycled into their biomass as bioactive by-products (Sood et al., 2015). In this case, cyanobacterial species are effective microorganisms in improving the quality of different types of wastewater by changing their TDS and COD.

Fischerella muscicola a heterocystous cyanobacterium from Stigonamataceae, was one of the dominant species that were isolated according to the purification processes from the Caspian Sea and had the great ability to grow in wastewater conditions. So, screening elements of artificial wastewater is performed to select the most effective ones for its growth and to remove nutrients from wastewater.

Material and methods

Sample collection, isolation, and purification

Samples were collected from different parts of the Caspian Sea; Salmanshahr, Mahmoodabad, Khazarabad (Mazandarn Province), and the Geisom coastline (Gilan Province) in the north of Iran. Isolation was performed by solid agar plate (Belcher et al., 1982) in the Research Institute of Applied Science of ACECR. Dominant species were purified, and among them, *Fischerella* sp. was selected as one of the common. Mass cultivation was performed in liquid culture in BG110 culture medium (Kaushik, 1987). Samples were kept in the culture room of the ACECR at 25 ± 2 ºC using LED lamps (2000 LUX) with duration of 8/16 (L/D). Aeration of samples were performed by aquarium air pump, Artman HP-4000.

Sequence analysis

The DNA extraction of sample was performed using the Fermentas DNA extraction kit (K0512). According to the PCR of the 16S ribosomal region and sequencing of the PCR product by Nubel et al. (2000) sample was identified molecularly.

Wastewater treatments

Artificial wastewater (AWW) was prepared by dissolving NaCl (10000, 50000 mg/L), $CaCl_2$ (35, 100 mg/L), $MgSO_4$ (75, 150 mg/L), $NaNO_3$ (50, 2000 mg/L), and K_2HPO_4 (6,500 mg/L) in 12 runs which have modified by Design expert (Table 1). BG110 medium with no additives is considered as blank(control). The sample was cultured in modified runs in 2 L Erlenmeyer flasks and incubated in the culture room of ACECR for 23 days.

Analysis of growth

Growth was analyzed by measuring biomass changes using an optical density (O.D.) method every 2 days with three replicates at λ 750 nm (spectrophotometer, WPA) for 20 days (Soltani et al., 2006). Before each test, samples were homogenized with an electrical homogenizer (Jenway) to obtain uniform

Table 1. Artificial wastewater treatments designed by Design-Expert

Run	NaCl (%)	$CaCl_2$ (mg/l)	$MgSO_4$ (mg/l)	$NaNO_3$ (mg/l)	K_2HPO_4 (mg/l)
1	1.00	35	150	50	6
2	1.00	100	150	2000	500
3	1.00	100	75	2000	6
4	5.00	100	75	50	500
5	5.00	35	75	50	6
6	1.00	35	75	2000	500
7	5.00	100	150	50	6
8	5.00	35	150	2000	6
9	5.00	100	75	2000	500
10	1.00	35	150	2000	6
11	1.00	100	75	50	500
12	5.00	35	150	50	500

cultures, and then sampling was performed.

Growth medium analysis

Growth medium changes were analyzed two times, before adding algal specimens (preliminary study) and at the logarithmic phase of algal growth (10th day of cultivation) by filtering culture media with filter paper.

Measuring removal changes of cations and anions

Cations; Na^{+} (ppm), Ca^{2+} (ppm), and Mg^{2+} (ppm), were analyzed using the ICP-OES method (Khan et al., 2022). Anions were analyzed by titration with $AgNO_3$ (ASTM, 2023) for Cl^{-}(%), UV-visible spectrophotometry (Shimadzu) at wavelengths of 220 and 270 nm for NO^{3-} (ppm) and Standard Method 4500-P-C (Kurniawati et al., 2025) for PO_4^{3-} (ppm). Analysis the nutrient removal percentage was calculated by Do et al. (2019) according to equation (1).

nutrient removal (%) = $C_1 - C_2 / C_1 \times 100$ (1)

C_1: initial concentration

C_2: final concentration

Analysis of TDS and COD

Measuring Total Dissolved Solid (TDS) (mg/l), were performed by electroconductivimeter, Chemical Oxygen Demand (COD) (mg/l as O_2) by Standard method 5220B in Kimiazi Analysis Research Lab.

Screening elements

Screening of wastewater elements, including NaCl (A), $CaCl_2$ (B), $MgSO_4$ (C), $NaNO_3$ (D), K_2HPO_4 (E), was performed according to the Parreto Plot and normal plot charts of Design-Expert.

Statistical analysis

Statistical analysis was performed by SPSS V.24 and Excel. Designing the experiments and studying their results was performed by Design-Expert V.7.0 software for screening the most effective factors in our experiments according to the factorial method. Statistical analysis with three sample replications in each test for measurement accuracy was done using one-way ANOVA, Post Hoc, and Dunken test for homogeneity of variances.

Results

Sequence analysis

The sequence of the 16S rRNA gene was identified as *Fischerella* sp. ISC 123. Results of the nucleotide sequences were submitted to NCBI under NCBI's accession number OK594059, and compared with the recorded sequence in the international gene bank, and the percentage of genetic similarity of samples was determined by BLAST. In this case, the identification accuracy of our sample was confirmed at the genus level with *Fischerella muscicola* with 99.9% similarity.

Growth measurement

According to the biomass changes of *Fischerella muscicola* in artificial wastewater and control runs 2, 11, 10, and 6 that containd 1% NaCl had the most growth with significant difference to control ($p \leq 0.05$) (Fig.1). According to the multiple comparison tests (Post Hoc) these runs had significant mean differences at logarithmic phase with others from 10^{th} day of culture till the 23th day. Among them, run 2; NaCl 1%, $CaCl_2$ 100, $MgSO_4$ 150, $NaNO_3$ 2000, K_2HPO_4 500 mg/L; showed the maximum growth, and its difference with runs 10, 11, and 6 was significant ($p \leq 0.05$). Runs with 5% NaCl had via-

bility in a stationary phase, but their growth decreased noticeably.

Analysis of cations, anions, TDS, and COD

According to the results (Table 2), runs 2, 6, 10, and 11 had the most effective in decreasing Ca^{+2} and Mg^{+2}. Furthermore, run 2 showed the highest percentage of removing anions, with 98% decrease of nitrate on the 10th day of the culture.

According to the analysis of TDS and COD, run 7 had the most removal of TDS (64.3%), meanwhile runs 10 and 11 had the highest removal of COD potential (Table 3).

Screening elements

Results of screening wastewater elements at the logarithmic phase (10th day) of culturing *F. muscicola* according to the Pareto plot (Fig. 2) and normal plot (Fig. 3) of each factor. The comparison showed that NaCl (A), $NaNO_3$ (D), K_2HPO_4 (E), and interactions had the most effect on the studied factors (Na^+, Ca^{2+}, Mg^{2+}, Cl^-, NO_3^-, PO_4^{3-}, TDS, COD) (Table 4).

Discussion

According to the results of the growth of *F. muscicola* in artificial wastewater, it can be concluded that this microalga has significant growth (more than the blank) in 1% NaCl at runs 2, 6, 10, 11. Evaluating other research for microalgal growth in different salinities was compatible with our results. In this way, Hoang Nhat et al. (2019) conducted a study on two marine microalgae, *Chlorella* sp., and *Stichococcus* sp., in different NaCl concentrations (0.1, 1, 3, and 5 %) and showed that the maximum growth and chlorophyll contents were observed in 0.1 M and 1% NaCl Furthemore, the results of Iranshahi et al. (2014) on *Nostoc* sp. and

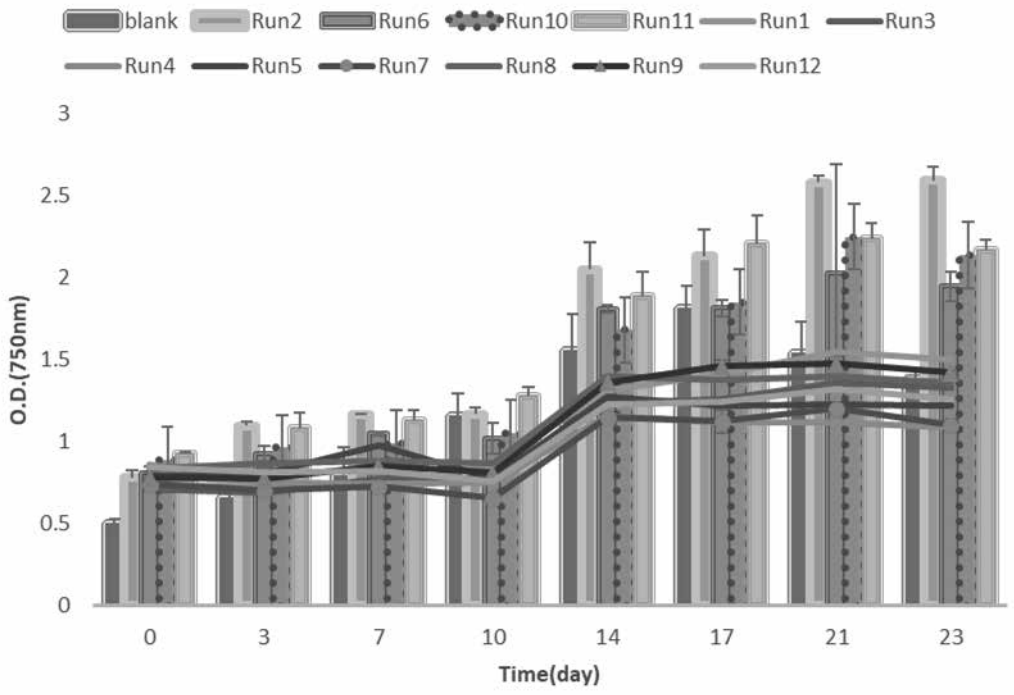

Fig.1. The growth curve of *Fischerella muscicola* in 12 runs according to O.D. ($p \leq 0.05$)

Table 2. Removing percentage of cations and anions from culture by *F. muscicola* on the 10th day of culture ($p \leq 0.05$)

Runs	Ca^{2+} (%)	Mg^{2+} (%)	Cl^- (%)	NO_3^- (%)	PO_4^{3-} (%)
1	1.7	-	27	-	32
2	49.7	4.6	30	98	-
3	5	-	30.5	-	65
4	-	-	73.3	-	-
5	-	-	-	-	-
6	47.4	8.2	-	-	-
7	-	2.7	-	-	6
8	1.9	-	-	-	-
9	-	-	-	3	.43
10	-	-	-	-	42
11	63.8	10.6	-	21	10
12	-	-	-	-	5.8

(-: no effect on removing cations and anions)

Table 3. The removal percentage of TDS and COD from culture by *F. muscicola* on the 10th day of culture ($p \leq 0.05$)

Runs	TDS(%)	COD(%)
1	-	-
2	52	-
3	55	65.7
4	-	66.1
5	35.4	57.7
6	48.4	75
7	64.3	12.5
8	-	75
9	57	81.5
10	-	92.1
11	47.5	85
12	7	75

Table 4. Effect of wastewater elements on cations, anions, TDS, and COD of the medium on the 10th day of culture

Factors	Wastewater elements
Na^+	A, D
Ca^{2+}	A, B
Mg^{2+}	C, A
Cl^-	A, D, AD
NO_3^-	D, E, AE
PO_4^{3-}	E
TDS	A, AD, D
COD	A, AB, D
growth	E

(A: NaCl, B: $CaCl_2$, C: $MgSO_4$, D: $NaNO_3$, E: K_2HPO_4)

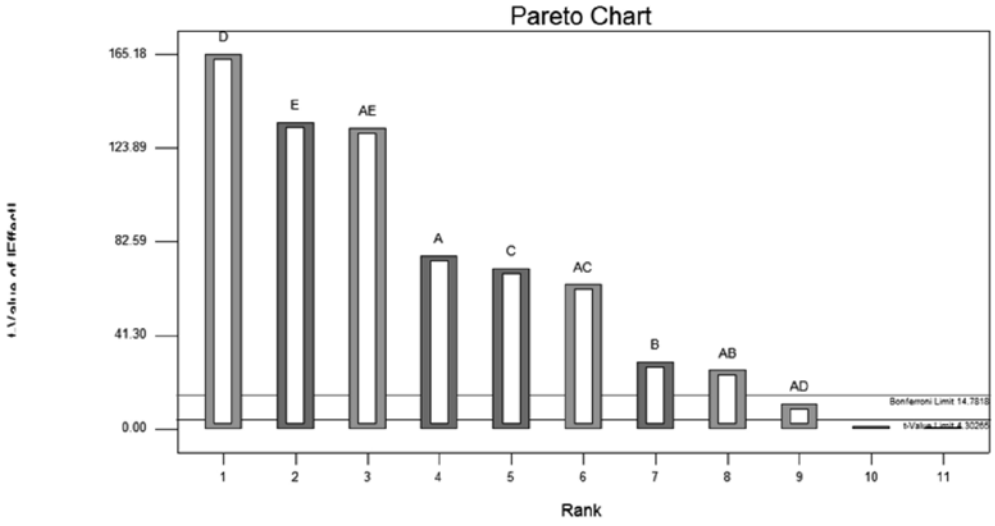

Fig. 2. Pareto Plot chart: effect of wastewater elements on nitrate amounts of medium after 10 days

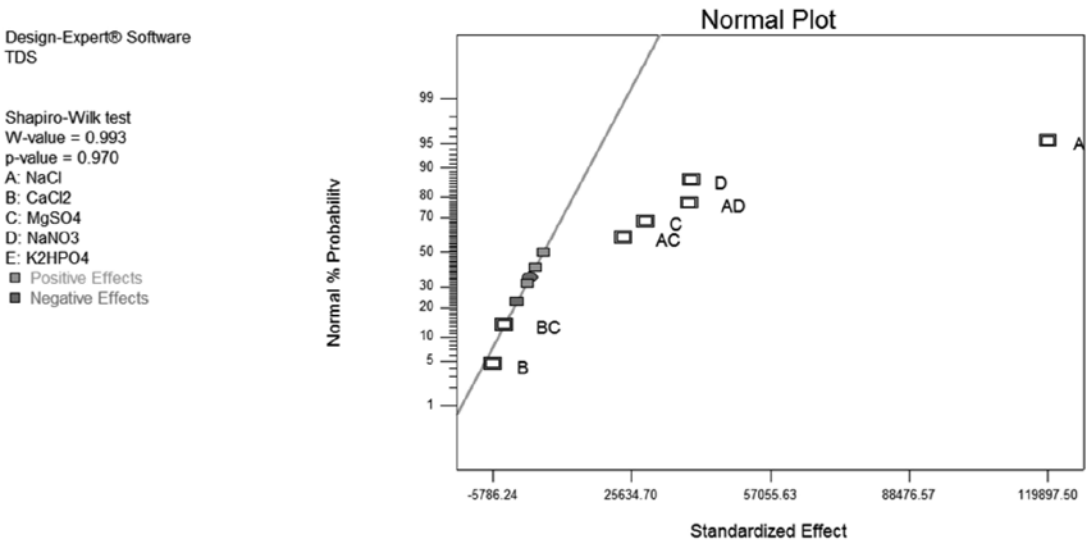

Fig. 3. Normal plot chart: effect of wastewater elements on TDS levels in the medium after the 10th day (p-value=0.970)

Anabaena sp. in various salinities indicated that the maximum growth of both species was observed in NaCl 1 %.

Besides NaCl, the common points of these treatments were maximum amounts of $NaNO_3$(2000 mg/L) and K_2HPO_4(500 mg/L) together in runs 2 and 6 or each of them in runs 10 and 11. As N and P are the main factors for the growth of microalgae, increasing the growth in these treatments can be related to these elements. These findings align with the research of Sood et al. (2015). They particularly studied the growth rate of cyanobacterial strains in municipal wastewater and their ability to remove nutrients (N, P) from these media.

Results of wastewater quality (as culture media) revealed that the maximum quantity of decreasing cations and nitrate occurred at the runs with the highest growth rate, such as runs 2 and 11. Therefore, it can be concluded that decreasing these elements relates to the growth condition. Studying the result of screening elements of wastewater was also compatible with these findings. Among various elements, NaCl, $NaNO_3$, and K_2HPO_4 showed the most effect on the physiological activities of the cyanobacterium *Fischerella* in wastewater, therefore affecting its quality. Ajala et al. (2020) investigated the relationship between phosphate assimilation and the growth rate on the first day of microalgae cultivation in wastewater. They found a significant correlation, particularly at the logarithmic phase, where rapid phosphate removal coincided with the exponential growth rate.

Research by Mostafaei et al. (2023) on *Chlorella vulgaris* showed that this microalga can remove and decrease nitrate, nitrite, phosphate, COD, and ammonium ions from raw municipal wastewater.Thus, it can be concluded that wastewater due to its high nutrient content could be an appropriate medium for the growth of this strain. According to the researches, microalgal poential to remove over 90% of contaminants without using bacteria or filtration, making them an effective alternative for the biological treatment of raw municipal wastewater.

As microalgae and cyanobacteria have a high demand for nutrients, particularly Nitrogen and Phosphate, for their growth, wastewater with high levels of these nutrients can be an applicable medium. In this way, by consuming and removing these elements from wastewater beside removing contaminants, the microalgae have opportunity to produce biomass with a concentration of nearly 2.03 g/l and also effective potential to reduce the COD and bacterial content of the wastewater.

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Investigation of phycobiliproteins in *Osmundea caspica (Laurencia)* (Zinova & Zaberzhinskaya) Maggs & L.M.McIvor Collected from the Coastal Waters of Nowshahr, Caspian Sea

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Abstract

Phycobiliproteins, including phycocyanin, allophycocyanin, and phycoerythrin, have diverse applications in food, cosmetics, and biomedical industries. Consequently, optimizing extraction conditions and identifying high-yielding species remain critical areas of study. The genus Osmundea is recognized for its significant phycobilin content. This study examines the macroalga *Osmundea caspica*, a member of the phylum Rhodophyta. The specimens of *O. caspica* were collected from the Caspian coasts of Sisangan in Mazandaran Province (Iran). The samples were lyophilized and subsequently ground after washing and removal of impurities. The extraction of phycobilins was evaluated using three solvents: distilled water, 100 mM phosphate-buffered saline (PBS), and 150 mM PBS (all adjusted to pH 7). Two distinct protocols: freeze-thaw at –20 °C for 24 hours and ultrasonication at a power of 70 W for 10 minutes. The results demonstrated that phycoerythrin exhibited the highest concentration among the extracted phycobilins, with an average of 0.0453 mg/mL, followed by phycocyanin (0.0067 mg/mL) and allophycocyanin (0.0018 mg/mL). Conversely, utilizing distilled water as the extraction solvent in conjunction with the Freeze-thaw Pre-treatment resulted in a greater extraction efficiency when compared to alternative methods. The results of one-way ANOVA showed that the differences in the mean concentrations and purity levels of phycobiliproteins among the extraction methods were statistically significant at the 0.05 level. For concentrations of phycocyanin ($F:3.551$, df: 5, $P< 0.05$), allophycocyanin ($F: 23.984$, df: 5, $P< 0.05$), phycoerythrin ($F: 23.685$, df: 5, $P< 0.05$), total phycobiliproteins yield ($F: 18.489$, df: 5, $P< 0.05$), purity of phycocyanin ($F: 16.109$, df: 5, $P< 0.05$), allophycocyanin ($F: 34.155$, df: 5, $P< 0.05$) and phycoerythrin ($F: 25.353$, df: 5, $P< 0.05$). This study presents promising results, particularly regarding the potential of phycoerythrin among the phycobiliproteins of the red alga *Osmundea caspica*, and offers a clear perspective for further exploitation of this species.

Keywords: Phycobiliproteins**,** Phycoerythrin, Algae, Rhodophyta, Caspian Sea

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Introduction

Marine biomass is recognized worldwide as a valuable carbon source, which can be used for food, feed, chemicals, and biopharmaceuticals of paramount industrial relevance (Merlo et al. 2021). Macroalgae comprise a group of marine algae classified into three major groups: green algae (*Chlorophyta*), brown algae (*Phaeophyta*), and red algae (*Rhodophyta*). The species diversity of macroalgae in the Caspian Sea is lower compared to the Persian Gulf. However, 13 species of brown algae and 25 species of red algae have been reported from this sea, which are typically distributed along rocky-coastal areas (Eshaghzadeh et al., 2023; Stepanian, 2016). In the Iranian coasts of the Caspian Sea, *O. caspica* is the only confirmed species of red algae. Previously, in past classifications, this species was categorized under the genus *Laurencia* (Eshaghzadeh et al., 2023). Red algae, along with cyanobacteria and cryptophytes, are among the primary sources of phycobiliproteins (PBPs).

PBPs are water-soluble pigments. They are organized into complexes known as phycobilisomes, which are located on the outer surface of the thylakoid membrane (Kovaleski et al., 2022). This complex functions in light energy harvesting, as chlorophyll a exhibits maximum absorption at wavelengths of 430 nm and 660 nm (Glazer, 1994). Thus allowing the photosynthesis and the survival of living organisms even at low light intensities (Dumay et al., 2014). Phycobilisome captures light energy through its phycobilin chromophores and directs it towards reaction centers where it is converted into chemical energy (Roy et al., 2011). Based on their structure and properties, specifically on their radiation absorption ability, PBPs are divided into four main types (Pagels et al., 2019), include phycoerythrin (PE), which exhibits a pink-purple color and λmax = 540-570 nm; phycocyanin (PC), with a blue color and λmax = 610-620 nm; allophycocyanin (APC), with a blue-green hue and λmax = 650-655 nm (Lijassi et al., 2024) and Phycoerythrocyanin (PEC), with a magenta color and λmax = 560–600 nm (Munier et al., 2014). PE, with a total molecular weight around 240 kDa, can be classified into four classes: B-PE (Bangiophyceae PE, containing PEB only or containing PEB and phycourobilin), C-PE (cyanobacterial-PE), and R-PE (Rhodophyta-PE). Indeed, R-PE is recognized for its stability towards several denaturant agents, namely temperature and pH (Galland-Irmouli et al., 2000). As the main light-harvesting complexes, phycobilisomes represent one of the crucial factors of algae and cyanobacteria mass cultures' productivity. It has been demonstrated that phycobilisome truncation can enhance biomass accumulation under strong light (Kirst et al., 2014). On the other hand, under modest or low irradiance, the antenna truncation resulted in growth rates and biomass accumulation reduction (Kirst et al., 2014; Page et al., 2012). These proteins are utilized in the food industry as natural colorants (soft candy, jellies, and ice sherbets), as well as in cosmetic and biomedical applications (e.g., as fluorescent labels for flow cytometry, immunoassays, and more). Additionally, they exhibit a wide range of biological activities, including antioxidant, antibacterial, anticancer (Lijassi et al., 2024), anti-inflammatory,

neuroprotective, and immunomodulatory (Lauceri et al., 2019). PBPs aqueous extracts obtained from *Arthrospira platensis* (*Spirulina*) are approved by EFSA (Regulation (EU) No. 1333/2008 and No. 231/2012) as coloring foodstuff. The US FDA classifies PC (21CFR73.1530) as a food natural color additive (Lauceri et al., 2019). Various methods are available for disrupting the cell wall to extract phycobilins. Early protocols typically employed physical or chemical methods to destroy trichomes and extract PBPs by using water as a major solvent (Doke, 2005; Eriksen, 2008). These include repeated freeze-thaw cycles, ultrasonication, pressurized distilled water, microwave treatment, pulsed electric field, homogenization, and others. The solvents mentioned (distilled water, PPB, PBS, Tris-Cl buffer with sodium azide, and sodium chloride) are often used as buffers and diluents in various scientific techniques, particularly in biological and biochemical research. These solvents help maintain a stable pH, which is crucial for the stability and activity of biological molecules like proteins and nucleic acids. (Kovaleski et al., 2022). More recent works combined chemical and physical methods for cell wall disruption and introduced other methods like enzymatic cell wall digestion or supercritical CO_2 extraction (Marzorati et al., 2020; Berrouane et al., 2022). The cell wall of macroalgae consists of polysaccharides (agar and cellulose), which are an obstacle to cell rupture during the extraction of their bioactive compounds (Mittal et al., 2017). The concentration and quality of phycobilins depend on key extraction parameters, such as the method of cell wall disruption, the solvent used, extraction time, and separation conditions. The present study aimed to investigate the extraction of phycobilins, particularly phycoerythrin, from the red macroalga *O. caspica*, which is native to the southern coast of the Caspian Sea. Given the biological significance and commercial potential of phycobilins as natural pigments and fluorescent markers, this research sought to evaluate the efficiency of the extraction process and to explore the feasibility of utilizing *O. caspica* as a novel and sustainable source of high-value phycobiliproteins.

Material and methods

Sampling

The red macroalga *O. Caspica* was collected from shallow areas (approximately 20 to 40 centimeters deep) of the rocky

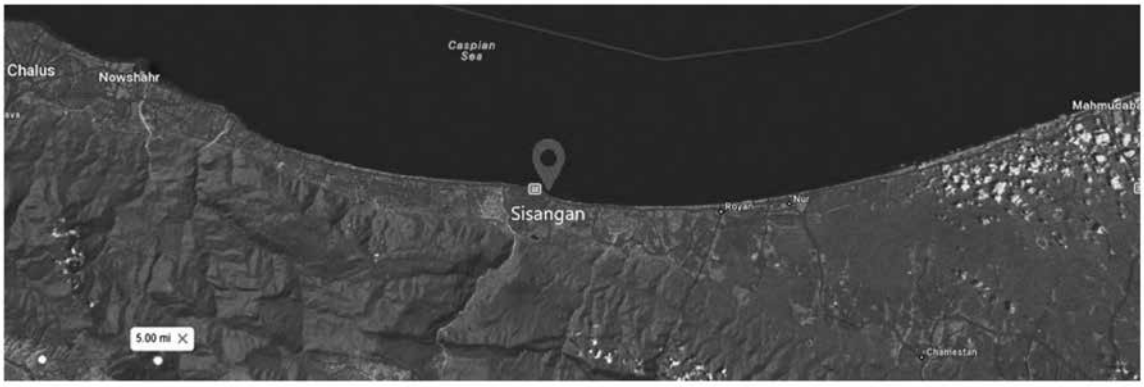

Fig. 1. Sampling location

shores in Sisangan, Nowshahr, Mazandaran Province, Iran, in mid-March (36.579133 N, 51.828014 E) (Figure 1). Sampling was carried out.

Preparation and Processing

The samples were washed with marine water and then distilled water to remove impurities, and then lyophilized in the dark for 24 hours. The samples were then ground using a porcelain mortar, and their weight was measured.

Phycoerythrin extraction using ultrasonication

To compare the effects of solvents in the extraction process, three solvents were used: distilled water, 100 mM PBS, and 150 mM PBS, all adjusted to pH 7, with a weight-to-volume ratio (W/V) of 1:25. Additionally, for cell disruption, the performance of intracellular content release was compared using sequential freeze-thaw cycles and ultrasonication methods. Accordingly, one set of samples underwent the freeze-thaw process, in which the samples were frozen at –20 °C for 24 hours and subsequently thawed at room temperature. while the second set was subjected to ultrasonication. During the ultrasonication process, the samples were placed in an ultrasonic device (Tosee Fanavari, 220-Iran) at a power of 70 W for 10 minutes. The freeze-thaw and ultrasonication procedures were repeated for three cycles. Between each cycle, the samples were vortexed for 2 minutes to enhance cell disruption and extraction efficiency. The solution was then filtered using filter paper (Fig. 2) and centrifuged (Universal 320R Hettich-Germany) at 8,000 rpm for 10 minutes at 4 °C. The supernatant was collected for spectrophotometric analysis.

Quantification of Extracted Phycobilins

The quantification of phycobilins was performed using a spectrophotometer (Lambda 25-Singapur) and modified equations (Lijassi et al., 2024). Thus, the amount of each of the PC, APC, and PE compounds was calculated using the following equations.

Phycocyanin *(*mg⁄ml*)*

$$= \frac{[(A620 - A720) - 0.474 \times (A652 - A720)]}{5.34}$$

Allophycocyanin *(*mg⁄ml*)*

$$= \frac{[(A652 - A720) - 0.208 \times (A620 - A720)]}{5.09}$$

phycoerythrin (*mg⁄ml*)

$$= \frac{[A562 - 2.41(PC) - 0.849(APC)]}{9.62}$$

The extraction yield of PBPs was estimated following the equation of (Silveira et al. 2007):

PBPs(mg/g)

$$= \frac{(PC + ACP + PE) * V}{DB}$$

V is the solvent volume (ml), and DB is dry biomass (g).

Purity was determined by using the formula below (Minkova et al., 2003):

PC Purity = $\frac{A620}{A280}$

APC Purity = $\frac{A652}{A280}$

PE Purity = $\frac{A562}{A280}$

Statistical analysis

The significant differences between mean values were evaluated using one-way analysis of variance (ANOVA). Tukey's test was performed with SPSS software (version 26.0) to determine whether there were any statistically significant differences at the $p< 0.05$ level.

Fig. 2. Initial purification of the algal extract was performed using ordinary filter paper

Results

The macroalga *O. caspica* (Fig. 3) was identified based on its morphological characteristics using microscopic images of internal structures and external morphological features (Rousseau et al., 2017) (Fig. 4).

The quantitative comparison of phycobilins indicates that PE had the highest concentration, with an average of 0.0453 mg/mL, followed by PC at 0.0067 mg/mL and APC at 0.0018 mg/mL, respectively (Figure 5). The yield of PBPs varied according to the extraction conditions: 1.05–1.76 mg/g (Table 1).

The results of one-way ANOVA showed that the differences in the mean concentrations (Table 1) and purity levels (Table 2) of phycobiliproteins among the extraction methods were statistically significant at the 0.05 level. For concentrations of phycocyanin (F:3.551, df: 5, P< 0.05), allophycocyanin (F: 23.984, df: 5, P< 0.05), phycoerythrin (F: 23.685, df: 5, P< 0.05), total phycobiliproteins yield (F: 18.489, df: 5, P< 0.05), purity of phycocyanin (F: 16.109, df: 5, P< 0.05), allophycocyanin (F: 34.155, df: 5, P< 0.05) and phycoerythrin (F: 25.353, df: 5, P< 0.05).

According to the results presented in Table 1, the combination of the freeze–thaw pretreatment and 150 mM PBS as the extraction solvent was significantly more effective for phycocyanin (PC) compared to other methods. In the case of allophycocyanin (APC), the freeze–thaw pretreatment combined with distilled water yielded the highest recovery. For phycoerythrin (PE), the freeze–thaw pretreatment in combination with both distilled water and 150 mM PBS demonstrated superior performance relative to the other

extraction approaches. Overall, the freeze–thaw pretreatment coupled with distilled water—and to a lesser extent with 150 mM PBS—proved to be more efficient than other extraction methods for the total recovery of phycobiliproteins.

According to Table 2, the purity of phycoerythrin (PE), consistent with the concentration results, was significantly higher when the freeze–thaw pretreatment was combined with either distilled water or 150 mM PBS, compared to the other extraction methods.

Fig. 3. *Osmundea caspica* habitat collected from Nowshahr, Iran

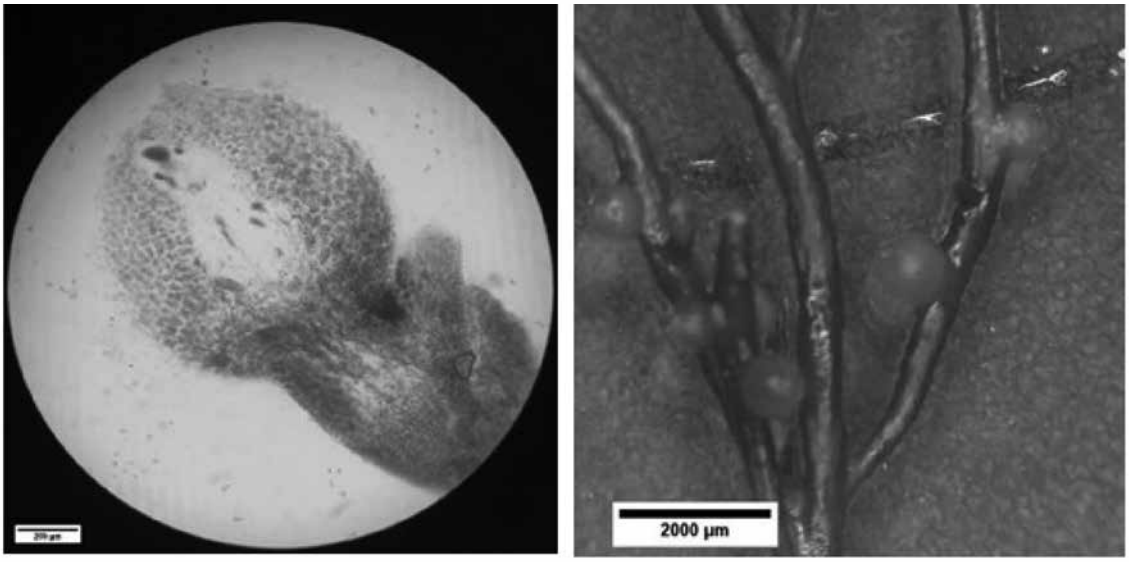

Fig. 4. Internal structure (left) and external morphology (right) of the cystocarp.

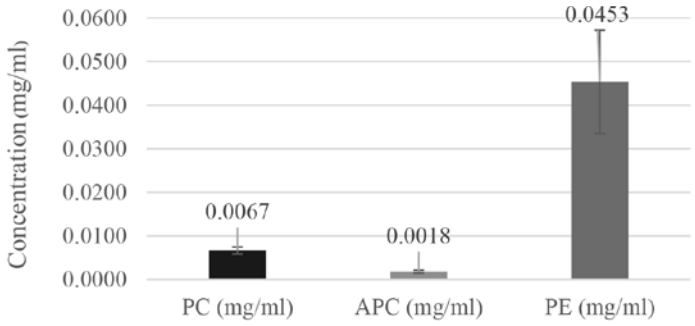

Fig. 5. Comparison of the concentrations of extracted phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE) from *Osmundea caspica*

Table 1. Effect of pretreatment and solvent on PBPs extraction yield and concentration

Pre-treatment	solvent	PBPs concentration (mg/ml)			Ratio	Yield (mg/g)
		PC	APC	PE	PC:APC:PE	PBPs
Freeze-thaw	distilled water	0.0070^{ab}±0.0002	0.0023^{a}±0.0001	0.0611^{a}±0.0006	0.11:0.04:1	1.7596^{a}±0.0178
	PBS 100 mM	0.0055^{b}±0.0001	0.0012^{d}±0.0000	0.0437^{b}±0.0000	0.13:0.03:1	1.2594^{b}±0.0032
	PBS 150 mM	0.0075^{a}±0.0012	0.0017^{bc}±0.0002	0.0579^{a}±0.0077	0.13:0.03:1	1.6783^{a}±0.2290
ultrasonication	distilled water	0.0066^{ab}±0.0006	0.0021^{ab}±0.0000	0.0332^{b}±0.0047	0.20:0.06:1	1.0462^{b}±0.1312
	PBS 100 mM	0.0069^{ab}±0.0001	0.0018^{bc}±0.0000	0.0371^{b}±0.0009	0.19:0.05:1	1.1455^{b}±0.0268
	PBS 150 mM	0.0067^{ab}±0.0006	0.0016^{c}±0.0002	0.0370^{b}±0.0048	0.18:0.04:1	1.1337^{b}±0.1400

Data (calculated from triplicate experimental values ± standard deviation) in the same column with different letters are significantly different ($p < 0.05$)

Table 2. Effect of the extraction process on purity

Pre-treatment	solvent	Purity		
		PC	APC	PE
Freeze-thaw	distilled water	0.0167^{a}±0.0006	0.0092^{bc}±0.0005	0.1755^{a}±0.0043
	PBS 100 mM	0.0108^{b}±0.0007	0.0052^{d}±0.0004	0.1086^{b}±0.0064
	PBS 150 mM	0.0167^{a}±0.0027	0.0080^{c}±0.0013	0.1670^{a}±0.0212
Ultrasonication	distilled water	0.0193^{a}±0.0010	0.0122^{a}±0.0008	0.0978^{b}±0.0131
	PBS 100 mM	0.0184^{a}±0.0005	0.0105^{ab}±0.0005	0.1099^{b}±0.0023
	PBS 150 mM	0.0162^{a}±0.0008	0.0086^{c}±0.0004	0.1059^{b}±0.0122

Data (calculated from triplicate experimental values ± standard deviation) in the same column with different letters are significantly different ($p < 0.05$)

In contrast, the highest purity was achieved for allophycocyanin (APC) using ultrasonic pretreatment in combination with distilled water. A comparative evaluation of the obtained results with previous studies on red macroalgae is presented in Table 3.

Discussion and Conclusion

According to existing scientific reports, *O. caspica* is the only confirmed species of red macroalgae along the southern coasts of the Caspian Sea (Eshaghzadeh et al., 2023). For many years, the red macroalga of the Southern Caspian Coast was considered a species of the genus Laurencia. However, a molecular study conducted in Azerbaijan led to the reassignment of this species from *Laurencia* to *Osmundea* (Rousseau et al., 2017). Despite this taxonomic revision, no molecular studies have been conducted on this species along the Iranian coasts of the Caspian Sea. This highlights a research gap, suggesting the necessity of molecular investigations to confirm its classification in Iranian waters. Furthermore, there is significant potential for broader research efforts aimed to exploring additional species of red macroalgae along the Southern Caspian Coastline.

The absorption spectra of PBPs may vary significantly among different species of algae and cyanobacteria, and even between strains of the same cyanobacterial genus. Therefore, specific wavelengths and absorption coefficients used to determine phycobilins for particular strains are generally not applicable to other strains (Zavřel et al., 2018). This study is no exception in this regard; however, it utilizes the standard methods from previous studies, with an awareness of the potential errors specific to the species *O. caspica*. The optimal extraction method depends on the type of phycobiliprotein, the type of algae, and the operational conditions. One common approach for extracting molecules is the use of solvents. PBPs are hydrophilic proteins. Therefore, common solvents used for their extraction are water or buffers, which also serve to control the pH of

Table 3. Comparison of the methods and results of the present study with conventional studies

solvent	APC (mg/g)	PC (mg/g)	PE (mg/g)	Reference
distilled water	0.06	0.17	1.53	(Data obtained from the Freeze-thaw Pre-treatment)
PBS 100 mM	0.03	0.14	1.09	
PBS 150 mM	0.04	0.19	1.45	
PBS 100 mM	0.27	0.25	0.37	(Sudhakar et al. 2015)
Distilled water	0.34	0.28	0.5	
Sea water	0.18	0.14	0.36	
PBS 25 mM	*	*	0.84	(Eshaghzadeh et al. 2023)
PBS 50 mM	*	*	0.86	
PBS 100 mM	*	*	0.97	
PBS 20 mM	0.04	0.027	1.57	(Karuppannan et al. 2024)

*: (No data)

the environment. These solvents include sodium phosphate buffer, acetate buffer, citrate buffer, carbonate buffer, Tris-HCl buffer, and ethylenediaminetetraacetic acid (EDTA) (Kovaleski et al., 2022). Sharmila et al. (2017) tested extraction methods using various buffers and pH levels, demonstrating that phosphate buffer (pH 7.2) combined with freeze-thawing at temperatures between -20 °C and -25 °C yielded the best results. Sintra et al. (2021) used phosphate buffer for C-PC extraction and reported a 90% recovery rate. Nguyen et al. (2016) compared different concentrations of phosphate buffer (20 mM, 50 mM, and 0.1 M) with tap water and distilled water, finding that the 20 mM phosphate buffer with pH 7.1 showed the best results for PE in *Mastocarpus stellatus*. Sudhakar et al. (2015) investigated the extraction of PE and PC from *Gracilaria crassa* using distilled water, seawater, and phosphate buffer (0.1 M). The results demonstrated that distilled water performed best for extracting PE (0.35 mg/g) and PC (0.18 mg/g). Based on these studies, one of the objectives of the present study was to investigate the effect of the solvent on the extraction process. The difference in the amount of extracted PE compared to other phycobilins, as well as the relatively higher efficiency of distilled water as a solvent for extraction, aligns with the findings of similar studies (Sudhakar et al., 2015; Karuppannan et al., 2024). The superior performance of distilled water in extracting phycobilins compared to PBS solutions can be analyzed from several perspectives. One key factor is the difference in osmotic pressure between the extracellular environment and the intracellular space, which is higher in distilled water than in saline solutions. This increased osmotic pressure can lead to greater cell turgescence, facilitating the release of intracellular components. Additionally, PBPs are hydrophilic and exhibit higher solubility in pure aqueous environments like distilled water compared to saline solutions. Furthermore, extraction techniques such as ultrasonication and freeze-thaw cycles may be more effective in distilled water, as its salt-free and purer nature prevents interference from ionic interactions, thereby enhancing the efficiency of the extraction process. On the other hand, the freeze–thaw pretreatment, which has been employed in most similar studies, has proven to be a more efficient method compared to ultrasonication. Although Pereira et al. (2020) and Mittal et al. (2019) reported favorable outcomes for ultrasonication or its combination with maceration, their findings focused on specific red algae species and may not be generalized to all biomass types. Moreover, in studies such as that by Sharmila et al. (2017), the freeze–thaw method—particularly at lower temperatures (−20 °C to −25 °C)—showed comparable or superior performance in terms of pigment recovery. In our experimental conditions, the freeze–thaw method not only provided higher purity and yield of phycobiliproteins but also preserved their structural integrity more effectively. Additionally, it required no special equipment and maintained a gentle processing environment, minimizing the risk of denaturation. Accordingly, the present study identifies the freeze–thaw pretreatment combined with distilled water

as the most effective overall approach for phycobiliprotein extraction. Factors influencing the extraction of phycobilins include species potential, initial preparation, and the type of solvent-extraction protocol. Numerous studies have examined and confirmed the significance of each of these factors (Lijassi et al., 2024; Eshaghzadeh et al., 2023; Sudhakar et al., 2015; Karuppannan et al., 2024). The findings of the present study are no exception to these three factors. Hence, to improve the extraction of phycobilins, broader comparative studies across different species, initial preparation processes, and more refined modifications in the choice of solvent or protocol are suggested. Given the high demand for PBPs in various industries, research and development in improving extraction and purification methods continues to ensure the sustainable and economic utilization of these natural resources.

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Innovative Biosorption Techniques for Heavy Metal Removal Using Microalgae: A Comprehensive Review

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Abstract

Water and soil pollution represents a fundamental human issue globally. Heavy metals are one of the basic pollutants of water and soil, which can be caused and intensified by anthropogenic activities, including mining, transportation, and various industries. Due to the toxic effects of these metals on the environment, organisms, and human health, the removal or recovery of these elements from polluted environments is of particular importance. Different methods and techniques have been applied to remove these pollutants, among which, bioremediation has received considerable attention due to its eco-friendly and cost-effectiveness. Bioremediation uses the ability of varoius organisms to decrease or remove pollutants. Algae are among the organisms that show significant capabilities in removing different types of contaminants, especially heavy metal ions. Phycoremediation is an application of algae as bio-remediate agents, and depends on factors such as light, temperature, pH, type of pollutant, and type of taxon. Various strains are known for their ability to remediation of heavy metals. The most basic methods in removing pollutants using algae are biosorption into the cell (absorption) and surface biosorption (adsorption), which uses the living or non-living mass of algae. New techniques, such as using transgenic microalgae, are among the effective detoxifying and rapidly growing methods. Genetic engineering for algae gene editing and gene silencing benefits various technologies and tools such as reporter genes, Cre-lox recombination, and CRISPR-Cas systems, modular cloning toolkits, regulatory elements, promoters, vectors, restriction enzymes, and post-transcriptional gene silencing technologies. Other novel techniques whose future on an industrial scale seems promising are the combined use of microalgae and bacteria, biochar addition, and biogenic nanomaterials generated from algae. These innovative methods offer sustainable and cost-effective solutions for environmental pollution, therefore boosting

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public health. Studies on the development and implementation of these techniques are ongoing in the world. In this paper, the ability of 78 algae species to remove 18 heavy metals has been reviewed.

Keywords: Absorption, Bioremediation, Heavy metals, Phycoremediation, Transgenic microalgae

Introduction

Environmental pollution is considered one of the most critical issues around the world. Due to the rapid expansion of urbanization, industrialization, manufacturing, and production of hazardous by-products, this problem is getting worse by the day and ultimately endangers environmental sustainability and human health (Rahman et al., 2021). Heavy metals (HMs) are regarded as one of the most considerable pollutants in soil and aquatic ecosystems (Ahmad et al., 2021). The natural weathering of minerals, as well as recent industrial and anthropogenic activities, led to the discharge of significant levels of HMs into the environment (Malik and Kaur Sandhu, 2023). Mining, smelting, and refining processes produce enormous amounts of contaminants and HMs, which could be distributed through the air and negatively affect the nearby areas (Izydorczyk et al., 2021). Furthermore, industrial emissions and automotive industries, fossil fuels, sewage sludge, household activities, and excessive use of pesticides and insecticides significantly contribute to polluting the environment with HMs (Briffa et al., 2020) (Fig. 1). Contamination of the terrestrial and aquatic ecosystems with HMs poses a significant hazard to the environment and therefore human health as a result of direct toxic impacts on living organisms and further potential for increased exposure along the food chain (Huang et al., 2018). Numerous severe health conditions, including cancer, lung adenomas, kidney failure, neurological disorders, inhibition of enzyme activity, and infertility, are among the ailments caused by HM exposure (Alengebawy et al., 2021; Żukowska and Biziuk, 2008).

The rising content of HMs, their persistence in the environment, and potentially deleterious effects on ecological and human health demand effective remediation technologies. There are several methods for removing HMs from contaminated environments, including water and soil. Traditional methods such as ion exchange, chemical precipitation, coagulation, conventional and advanced oxidation, ultrafiltration, and electrochemical removal have some limitations like usability for limited metal ions, consuming higher energy and chemicals, and producing a considerable amount of sludge/solid waste (Razzak et al., 2022). Therefore, developing more effective and environmentally friendly solutions is very important. Today, the emergence of affordable methods in which no cutting-edge technology is required have attracted substantial attention as these methods are economically feasible for developed and developing countries. Among the new technologies used to reduce HMs is bioremediation, which has received greater attention from various communities

because of its low-cost, simple technology, and availability.

Bioremediation is a process that applies organisms' potential to clean up environmental contamination, such as wastewater, ground or surface waters, sediments, and soils (Boopathy, 2000). The bioremediation technique uses bacteria, fungi, plants, and algae to break down, remove, change, immobilize, or detoxify different chemicals and physical pollutants from the ecosystem (Bala et al., 2022). When biological agents interact with pollutants, bioremediation occurs spontaneously without the aid of any chemical catalysts. To facilitate and speed up the bioremediation process, it is vital to generate the optimum environmental conditions (Verma and Jaiswal, 2016). The characteristics of the contaminated site have a great impact on the bioremediation process. The bioremediation process is influenced by various factors, including soil texture, permeability, pH, water-holding capacity, temperature, nutrients, and oxygen content (Boopathy, 2000).

Phycoremediation, the application of algae to remove contaminants from the environment, is recognized as an effective and affordable bioremediation technique. Microalgae are recognized as effective bioremediation agents in soil due to their rapid growth, large surface area, strong affinity

Fig. 1. Main sources of soil and water pollutants, including pesticides and insecticides, refining and smelting industries, automotive industries, mining, fossil fuels, sewage sludge, and household activities. Pictures used in this figure are adopted from (Di Capua, 2013; Khan, 2022), (https://commons.wikimedia.org/), (https://www.brainhealth.scot/), (https://iglobalpunjab.com/), and (https://truthout.org/).

for metal binding, high tolerance for various contaminants, and eco-friendly nature (Chugh et al., 2022; Yeheyo et al., 2024). They utilize natural metabolic processes through techniques such as bioconcentration and volatilization to detoxify and remediate polluted soils effectively. Their effectiveness lies in their ability to accumulate and degrade contaminants within their cellular structures. Furthermore, microalgae release exudates that support the growth of beneficial microorganisms in the soil, enhancing soil health and resilience (Yeheyo et al., 2024). Phycoremediation of water systems is primarily recognized for its ability to purify water contaminated with HMs and/ or other pollutants; however, it can be integrated into a broader bioremediation strategy that benefits both aquatic and terrestrial environments. Since the biomass produced by algae following the phycoremediation is used as feedstock to generate biofuel and other valuable products, algal-based bioremediation is strongly favored (Razaviarani et al., 2022)such as wastewater treatment and bioenergy industries. Microalgae are mixotrophic microorganisms that have potential to utilize nitrogen and phosphate (nutrients. Therefore, this review encompases the current research, advancements, and modern approaches in the phycoremediation of heavy-metal-polluted environments.

Phycoremediation of HMs

Algae are considered a new biological step that is a permanent, environmentally friendly, and affordable procedure for environmental protection (Touliabah et al., 2022). The merits of bioremediation based on algae are better production of biomass and higher aggregation ability, detoxification, and degrading xenobiotics and contaminants. Moreover, the produced biomass during bioremediation is cost-efficient in the field of clean energy.

Different species of algae, including macro- and micro-algae, diatoms, and cyanobacteria, can remove pollutants from soil and water. As an example, the ability of *Neochloris aquatica* in removing HMs including chromium (Cr) (88.7%), lead (Pb) (75.9%), nickel (Ni) (87.6%), cadmium (Cd) (60.4%), cobalt (Co) (52.9%), zinc (Zn) (84.9%), and copper (Cu) (54.4%) is considerable (Tamil Selvan et al., 2020). The study conducted by Ajayan et al. (2015) revealed the important effect of *Scenedesmus* sp. on reducing the HM pollution of Zn (65–98%), Pb (75–98%), Cu (73.2–98%), Cr (81.2–96%), and nutrients such as phosphate (>95%) and nitrate (>44.3%). Marine macroalgae such as *Caulerpa lentillifera* can also be used as inexpensive adsorbents to remove Cd, Cu, Zn, and Pb from aqueous solutions (Apiratikul and Pavasant, 2006). Table 1 lists 78 microalgae strains with the ability to bioremediate 18 HMs.

Diatoms, another group of microalgae, can bioremediate diverse forms of effluents due to their cellular structure and adaptive techniques. It may absorb and use different micro- and macro-elements (Marella et al., 2020). *Cylindrotheca closterium* be able to remove phthalate acid esters (PAEs) from surface sediments (Gao and Chi, 2015). Moreover, the herbicide mesotrione (an aromatic ketone) can be absorbed by *Halamphora* (*Amphora) coffeiformis* (Valiente Moro et al., 2012). *Nitzschia* sp., another

diatom genus, causes fast decomposition of organic matter by enhancing aerobic bacterial activity (Yamamoto et al., 2008). Besides, *Nitzschia* sp. and *Skeletonema costatum* can degrade the highly toxic polyaromatic hydrocarbons (PAHs) from sediments (Hong et al., 2008).

Some reports on the biodegradation of pesticides by algae can be found in the literature (Megharaj et al., 2000, 1994, 1987). El-Bestawy et al. (2007) illustrated the ability of the strains *Synechococcus*, *Oscillatoria*, *Nostoc*, *Cyanothece,* and *Nodularia* in degrading of the pesticide Lindane (chlorinated aliphatic pesticide). Kuritz and Wolk (1995) also reported the ability of *Nostoc ellipsosporum* and *Anabaena* sp. to degrade Lindane. Moreover, soil isolates of *Chlorella vulgaris, Synechococcus elongatus, Tetradesmus obliquus* (*Scenedesmus bijugatus*), *Leptolyngbya tenuis* (*Phormidium tenue*), *Leptolyngbya* (*Phormidium*) *foveolarum, Kamptonema animale* (*Oscillatoria animalis*), *Desmonostoc* (*Nostoc*) *muscorum,* and *Nostoc linckia* can detoxify and break down the organophosphate insecticides (Megharaj et al., 1994, 1987).

Acid mine drainage bioremediation using algae

Acid Mine Drainage (AMD) is considered an important source of HM pollution around the world that endangers species of plants, animals, and human life (Samal et al., 2020). Different strains of algae, especially microalgae, are used as a cost-efficient way of removing HMs these days. Some genera and species, such as *Spirulina*, *Scenedesmus*, *Chlorella*, *Cladophora*, *Anabaena*, *Oscillatoria*, *Stigeoclonium*, *Phaeodactylum tricornutum,* non-living *Caulerpa lentillifera*, *Ulothrix zonata*, and *Turbinaria ornate*, are among the hyper-accumulator and hyper-adsorbent microalgae from AMD. They also produce a lot of alkalinities, which is important during HM precipitation treatment (Apiratikul and Pavasant, 2006; Bwapwa et al., 2017; Kandasamy et al., 2021). The lifeless biomass of *Spirulina* sp. can absorb Zn (86–98%), iron (Fe) (100%), Cu (38–76%), and Pb (40–78%) and decrease the acidity of AMD by enhancing the pH, as AMD has the acidic nature (Bwapwa et al., 2017). *Stigeoclonium* spp. are freshwater algae that can thrive in mine water containing high levels of HMs, particularly Zn, and are recognized for their effectiveness in removing Zn from the environment (Pawlik-Skowrońska, 2001).

The bioremediation mechanism of algae

Algae from various species can be used to break down organic contaminants. HM removal from the environment can also be accomplished through bioremediation. It is worth mentioning that the terms bioremediation and biodegradation are increasingly interchangeable (Singh, 2019). However, biodegradation is considered a natural process in nature, while bioremediation is commonly controlled to optimize the conditions for microorganisms. This process can take a few to several months to finish and is carried out in situ or ex-situ. In-situ bioremediation includes the remediation of pollutants at the site, while ex-situ involves the removal of the pollutants in another site (Gavrilescu, 2010). Ex-situ bioremediation can be employed if the environmental conditions are unfavorable for the growth of microorganisms (Al-

Table 1. List of micro- and macroalgae used for phycoremediation of HMs

Number	Algae species	Family	Metal	References
1	*Halamphora (Amphora) subtropica* (Wachnicka & E.E.Gaiser) Rimet & R.Jahn	Amphipleuraceae	Ni	(Dahmen-Ben Moussa et al., 2018)
2	*Dolichospermum affine (Anabaena affinis)* (Lemmermann) Wacklin, L.Hoffmann & Komárek	Aphanizomenonaceae	As	(Huang et al., 2014)
3	*Anabaena cylindrica (subcylindrica)* Lemmermann	Aphanizomenonaceae	Cu, Co, Pb, Mn	(El-Sheekh et al., 2005)
4	*Trichormus (Anabaena) variabilis* (Kützing ex Bornet & Flahault) Komárek & Anagnostidis	Aphanizomenonaceae	Pb, Zn, Ca, Mn, Cr	(Abd El-Hameed et al., 2018; Ahammed et al., 2023)
5	*Ascophyllum nodosum* (Linnaeus) Le Jolis	Fucaceae	Cu, Cd, Zn, Ni, Pb	(Leusch et al., 1995; Medeiros et al., 2017)
6	*Chlamydomonas reinhardtii* P.A.Dangeard	Chlamydomonadaceae	Pb, Cd, Cu, Hg	(Bayramoğlu et al., 2006; Flouty and Estephane, 2012; Li et al., 2021; Tüzün et al., 2005)
7	*Chlorella sorokiniana* Shihira & R.W.Krauss	Chlorellaceae	Cu, Ni, Cd, Pb, Cr	(Akhtar, 2004; Akhtar et al., 2008; Husien et al., 2019; Liang et al., 2017; Petrovič and Simonič, 2016)
8	*Mychonastes homosphaera (Chlorella minutissima)*	Mychonastaceae	As, Cd, Cu, Zn, Mn, Cr	(Arora et al., 2017; Singh et al., 2012; Yang et al., 2015)

	(Skuja) Kalina & Puncochárová			
9	*Auxenochlorella (Chlorella) pyrenoidosa* (H.Chick) Molinari & Calvo-Pérez	Chlorellaceae	Mn, Cu, Zn, Pb, Cd, Cr, Ni	(Kothari et al., 2022; P.S et al., 2021; Purushanahalli Shivagangaiah et al., 2021; Zhou et al., 2012)
10	*Chlorella miniata (Kützing)* Oltmanns	Chlorellaceae	Ni, Cr	(Han et al., 2014, 2007; Wong et al., 2000)
11	*Chlorella* sp. Beyerinck [Beijerinck]	Chlorellaceae	Cr	(Shukla et al., 2012)
12	*Chlorella vulgaris* Beijerinck	Chlorellaceae	Ca, Mn, Pb, Cu, Cd, Ni, Zn, Cr, Fe	(Ahammed et al., 2023; Atoku et al., 2021; Manzoor et al., 2020)
13	*Pleurastrum (Chlorococcum) aquaticum* (P.A.Archibald) Sciuto, M.A.Wolf, Mistri & Moro	Pleurastraceae	Pb	(Liyanage et al., 2020)
14	*Chlorococcum infusionum* (Schrank) Meneghini	Chlorococcaceae	Fe, Mn	(Gomes et al., 2021)
15	*Chlorococcum* sp. Meneghini	Chlorococcaceae	Cu, Cd, As	(Qiu et al., 2006; Upadhyay et al., 2022)
16	*Cladophora vagabunda (fascicularis)* (Linnaeus) C.Hoek	Cladophoraceae	Cu, Pb, Cd	(Deng et al., 2008, 2007b, 2007a, 2006)
17	*Cladophora fracta* (O.F.Müller ex Vahl) Kützing	Cladophoraceae	Cu, Cd, Zn, Hg	(Ji et al., 2012)

18	*Cladophora glomerata* (Linnaeus) Kützing	Cladophoraceae	Cr, Zn, Cu, Pb, Cd, Co, Ni, Fe, Mn	(Çeleklı and Bulut, 2020; Khan et al., 2023; Vymazal, 1990, 1984)
19	*Cladophora parriaudii* C.Hoek	Cladophoraceae	Cu, Al, Pb, Mn	(Ross et al., 2021)
20	*Cladophora rivularis* (Linnaeus) Kuntze	Cladophoraceae	Pb	(Jafari and Senobari, 2012)
21	*Cladophora* sp. Kützing	Cladophoraceae	Pb, Cu, Cr, Cd, Zn, Ni, As	(Abioye et al., 2020; Sargın et al., 2016)
22	*Closterium lunula* Ehrenberg & Hemprich ex Ralfs	Closteriaceae	Cu	(Yan and Pan, 2002)
23	*Dunaliella salina* (bardawil) (Dunal) Teodoresco	Dunaliellaceae	Al, Cd, Cu, Cr, Fe, Zn, Mn, Ni, Pb	(Akbarzadeh and Shariati, 2014)
24	*Dunaliella* sp. Teodoresco	Dunaliellaceae	Zn, Ni	(Dahmen-Ben Moussa et al., 2018; Elleuch et al., 2021)
25	*Ecklonia radiata* (C.Agardh) J.Agardh	Lessoniaceae	Zn, Pb	(Matheickal and Yu, 1996; Zhang et al., 2022)
26	*Euglena gracilis G.A.Klebs*	Euglenaceae	Cu, Ni, As	(Tahira et al., 2019; Winters et al., 2017)
27	*Fucus distichus* ssp. Evanescens (C.Agardh) H.T.Powell	Fucaceae	Cu, Cd, Pb, Zn	(Medeiros et al., 2017)
28	*Fucus vesiculosus* Linnaeus	Fucaceae	Cu, Cd, Ni, Pb, Hg, Zn	(Brinza et al., 2020, 2009; El-Naggar et al., 2021; Henriques et al., 2017; Mata et al., 2008; V.R. et al., 2019)

29	*Gloeocapsa* sp. Kützing	Aliterellaceae	Pb	(Raungsomboon et al., 2008)
30	*Hydrodictyon reticulatum* (Linnaeus) Bory	Hydrodictyaceae	U	(Zhang and Luo, 2022)
31	*Laminaria digitata* (Hudson) J.V.Lamouroux	Laminariaceae	Cd, Cu	(Anacleto et al., 2017; Papageorgiou et al., 2008)
32	*Saccharina (Laminaria) japonica* (J.E.Areschoug) C.E.Lane, C.Mayes, Druehl & G.W.Saunders	Laminariaceae	Cd, Pb, Zn, Fe	(Ghimire et al., 2008; Luo et al., 2006; Xiao et al., 2012; Yin et al., 2001)
33	*Limnoraphis (Lyngbya) hieronymusii* (Lemmermann) J.Komárek, E.Zapomelová, J.Smarda, J.Kopecký, E.Rejmánková, J.Woodhouse, B.A.Neilan & Komárková	Microcoleaceae	Cd, Pb, Hg	(Inthorn et al., 2002)
34	*Phormidium (Lyngbya) taylorii* (Drouet & Strickland) Anagnostidis	Oscillatoriaceae	Cd, Pb, Ni, Zn	(Klimmek et al., 2001)
35	*Micrasterias denticulata* Brébisson ex Ralfs	Desmidiaceae	Cr	(Volland et al., 2012)
36	*Microcystis aeruginosa* (Kützing) Kützing	Microcystaceae	Pb, Cu, Zn, Cd, Ni, Hg, As	(Chen et al., 2005; Deng et al., 2020; Huang et al., 2014; Pradhan and Rai, 2001; Rzymski et al., 2014; Zeng et al., 2022)
37	*Nostoc commune* Vaucher ex Bornet & Flahault	Nostocaceae	Cu, Cd, Fe, Ni, Pb, Zn	(Atoku et al., 2021; Morsy et al., 2011)

38	*Desmonostoc (Nostoc) muscorum* (Bornet & Flahault) Hrouzek & S.Ventura	Nostocaceae	Pb, Cu, Co, Mn, Zn, Cd	(Abd El-Hameed et al., 2018; Dixit and Singh, 2014; El-Hameed et al., 2021; El-Sheekh et al., 2005; Hazarika et al., 2015; Roy et al., 2015)
39	*Nostoc* sp. Vaucher ex Bornet & Flahault	Nostocaceae	Pb, Cu, Cd, Zn, Ni	(Ahad et al., 2021; Rakic et al., 2023)
40	*Oedogonium hatei* N.D.Kamat	Oedogoniaceae	Cr, Ni	(Gupta et al., 2010; Gupta and Rastogi, 2009)
41	*Oedogonium rivulare* A.Braun ex Hirn	Oedogoniaceae	Cu, Cr, Pb, Cd, Co , Ni, Zn, Fe, Mn	(Vymazal, 1984)
42	*Oedogonium* sp. Link ex Hirn	Oedogoniaceae	Cr, Cu, Co, Fe, Hg, Ni, Zn, U	(Bakatula et al., 2014; Rai et al., 2008)
43	*Oocystis* sp. Nägeli ex A.Braun	Oocystaceae	Cd, Ni, Pb	(Karaca, 2008)
44	*Jaaginema angustissimum (Oscillatoria angustissima)* (West & G.S.West) Anagnostidis & Komárek	Synechococcales familia incertae sedis	Zn	(Ahuja et al., 1999)
45	*Jaaginema quadripunctulatum (Oscillatoria quadripunctulata)* (Brühl & Biswas) Anagnostidis & Komárek	Synechococcales familia incertae sedis	Cu, Pb, Co, Zn	(Ajayan et al., 2011)
46	*Oscillatoria limosa* C.Agardh ex Gomont	Oscillatoriaceae	Cu, Cd, Fe, Zn, Ni, Pb	(Atoku et al., 2021)

47	*Phormidium nigrum (Oscillatoria nigra)* (Vaucher ex Gomont) Anagnostidis & Komárek	Oscillatoriaceae	Cr, Fe, Ni	(Rai et al., 2008)
48	*Oscillatoria* sp. Vaucher ex Gomont	Oscillatoriaceae	Cd, Cr, Ca	(Bon et al., 2021; Jayashree et al., 2012; Mathimani et al., 2024; Shukla et al., 2012)
49	*Oscillatoria tenuis* C.Agardh ex Gomont	Oscillatoriaceae	As	(Huang et al., 2014)
50	*Padina* sp. Adanson	Dictyotaceae	U	(Khani, 2011)
51	*Parachlorella kessleri* (Fott & Nováková) Krienitz, E.H.Hegewald, Hepperle, V.Huss, T.Rohr & M.Wolf	Chlorellaceae	Cd, Cr	(Bauenova et al., 2021)
52	*Phormidium bohneri* Schmidle	Oscillatoriaceae	Cr, Fe, Ni	(Rai et al., 2008)
53	*Phormidium* sp. Kützing ex Gomont	Oscillatoriaceae	Cr, Ca	(Mathimani et al., 2024; Shukla et al., 2012)
54	*Pylaiella littoralis* (Linnaeus) Kjellman	Acinetosporaceae	Fe, Cu, Co, Cd, Cr, Zn, Al	(Carrilho and Gilbert, 2000)
55	*Tetraselmis (Platymonas) subcordiformis* (Wille) Butcher	Chlorodendraceae	Sr	(Mei et al., 2006)
56	*Porphyridium purpureum (cruentum)* (Bory) K.M.Drew & R.Ross	Porphyridiaceae	Cu, Cd, Cr, Pb, Hg, Ni	(Karaca, 2008; Soeprobowati and Hariyati, 2013; Zaib et al., 2016)
57	*Sargassum filipendula* C.Agardh	Sargassaceae	Cu, Cr, Cd, Ni, Pb, Zn, Ag, Fe, Mn	(Cardoso et al., 2016; Davis et al., 2000; Seepersaud et

				al., 2018; Verma et al., 2018, 2016)
58	*Sargassum fluitans* (Børgesen) Børgesen	Sargassaceae	Cd, Pb, Cu, Zn, Ni	(Fourest and Volesky, 1996; Kratochvil et al., 1995; Leusch et al., 1995; López-Miranda et al., 2020)
59	*Sargassum natans* (Linnaeus) Gaillon	Sargassaceae	Pb	(López-Miranda et al., 2020)
60	*Sargassum* sp. C.Agardh	Sargassaceae	Cd, Zn	(Esteves et al., 2000; Mahmood et al., 2017)
61	*Sargassum vulgare* C.Agardh	Sargassaceae	Cu, Cd, Zn, Pb, Ni, Mn, Fe	(Seepersaud et al., 2018)
62	*Tetradesmus obliquus (Scenedesmus acutus)* (Turpin) M.J.Wynne	Scenedesmaceae	Cd, Cu, Zn, Cr, Pb	(Alayi et al., 2021; Monteiro et al., 2011, 2009; Omar, 2002; P.S et al., 2021; Purushanahalli Shivagangaiah et al., 2021; Zhang et al., 2016; Zhou et al., 2012)
63	*Desmodesmus (Scenedesmus) protuberans* (F.E.Fritsch & M.F.Rich) E.Hegewald	Scenedesmaceae	Cd, Pb, Ni	(Karaca, 2008)
64	*Desmodesmus communis (Scenedesmus quadricauda)* (E.Hegewald) E.Hegewald	Scenedesmaceae	Zn, Cr, Cu, Pb	(Kafil et al., 2022; Omar, 2002)

65	*Scenedesmus* sp. Meyen	Scenedesmaceae	As, Fe, Zn	(Ajayan et al., 2015; Arora et al., 2017; Bte Jais et al., 2015)
66	*Spirogyra hyalina* Cleve	Spirogyraceae	Cd, Hg, Pb, As, Co	(Kumar and Oommen, 2012)
67	*Spirogyra* sp. Link	Spirogyraceae	Fe, Cr, Cu, Ni, As, Cd, Pb, Mn, Se, Zn	(Abioye et al., 2020; Mane and Bhosle, 2012; Rai et al., 2008)
68	*Limnospira (Spirulina) platensis* (Gomont) K.R.S.Santos & Hentschke	Microcoleaceae	Cd, Cr, Cu, Co, Ni, Pb, Al, Fe, Zn, Sr, Ba	(Balaji et al., 2015, 2014; Diaconu et al., 2023; Kumar et al., 2020; Rangsayatorn et al., 2002; Zinicovscaia et al., 2018)
69	*Limnospira (Arthrospira) indica* (Desikachary & Jeeji-bai) Nowicka- Krawczyk, Mühlsteinová & Hauer	Microcoleaceae	Pb, Cr, Cd	(Balaji et al., 2014)
70	*Limnospira (Arthrospira) maxima* (Setchell & N.L.Gardner) Nowicka-Krawczyk, Mühlsteinová & Hauer	Microcoleaceae	Pb, Cr, Cd	(Balaji et al., 2014)
71	*Spirulina* sp. Turpin ex Gomont	Spirulinaceae	Cr, Cd, Pb,Mn, Se, Fe, Cu, Zn	(Hernández and Olguín, 2002; Mane and Bhosle, 2012)
72	*Synechococcus elongatus* (Nägeli) Nägeli	Synechococcaceae	Fe, Mn	(Gomes et al., 2021)
73	*Tetradesmus (Scenedesmus) incrassatulus* (Bohlin) M.J.Wynne	Scenedesmaceae	Cr, Cd, Cu	(Alayi et al., 2021; Peña-Castro et al., 2004)

74	*Tetraselmis indica* Arora & Anil	Chlorodendraceae	Mn, Pb, Al, Ca, Cd, Cu	(Amit et al., 2017)
75	*Trichormus variabilis* (Kützing ex Bornet & Flahault) Komárek & Anagnostidis	Aphanizomenonaceae	Cd	(El-Hameed et al., 2021)
76	*Ulothrix* sp. Kützing	Ulotrichaceae	Ni	(Rai et al., 2008)
77	*Ulva lactuca* Linnaeus	Ulvaceae	Cd, Cu, Cr, Fe, Zn, Mn, Ni, Pb	(Mofeed, 2017)
78	*Vaucheria cruciata (debaryana)* (Vaucher) De Candolle	Vaucheriaceae	Cd, Pb	(Khan et al., 2023)

ori et al., 2022)chlorosis, growth inhibition, root tips browning, and death of plant. Soil pollutants such as hydrocarbon and heavy metals are absorbed by crops and such ends up being consumed by human posing health risk like cancer and respiratory abnormally. Conventional methods of remediation such as chemical and physical methods are very expensive and not sustainable. Excavation, which is a type of physical method, merely shifts the pollutant from one site to another. Bioremediation is a biological method of reclaiming polluted soils. Bioremediation is less expensive and more sustainable and safer when compared to the conventional methods of reclamation of polluted environment. This biological method of remediation is an extremely attractive, important, and productive alternative for cleaning, debugging, managing, and rehabilitating and consequently ameliorating contaminated environments judicious utilization of microbial activities. The rate, at which the waste substances are degraded, is usually dictated by competitiveness among biological agents, sub-optimal supply of essential nutrients, unconducive abiotic conditions (in forms of temperature, aeration, pH, and moisture. It usually included biological augmentation (bioaugmentation), during which some selected strains of microorganisms are added to the process to accelerate the breakdown of a pollutant (Herrero and Stuckey, 2015).

Algae species take the HMs by biosorption and bioaccumulation (Singhal et al., 2021). During biosorption, certain living/non-living microorganisms or biomass can passively concentrate and bind pollutants onto their cellular structure through the physiochemical process and immobilize them (Volesky and Holan, 1995). In other words, biosorption is the term used to describe the capacity of biological materials to ingest HMs physically or chemically from wastewater (Fard et al., 2011). While bioaccumulation is carried out in the following stages of biosorption and involves living organisms (Hlihor et al., 2017). Bioaccumulation and biosorption

are subcategories of bioremediation. During biosorption, metals are retained through interactions with functional groups on the cell surface (*e.g.*, adsorption, ion exchange). This process can be affected by variables, including ionic strength, environmental acidity, biomass concentration, temperature, particle size, and other ions (Pagnanelli et al., 2003; Vilar et al., 2005). It can occur with both living and non-living biomass, as it does not depend on cell metabolism. In contrast, bioaccumulation involves both intra- and extracellular processes. Therefore, only live biomass can perform bioaccumulation (Coelho et al., 2015). Algae species often filter nutrients, heavy metals (depending on the species), and other minerals from wastewater through a combination of biosorption and their ability to absorb, adsorb, and bioaccumulate. Since these species need nutrients to grow, algae growth occurs as these elements are removed from wastewater. Some are absorbed by outer cells, while others are absorbed by inner cells (Bwapwa et al., 2017) (Fig. 2).

Most algae species (*e.g.*, *Euglena* sp., *Scenedesmus* sp., *Oscillatoria* sp., *Chlorella* sp.) absorb contaminants and immobilize them within their cell structure; these microalgae biomass can later be used as energy-enriched biomass for biofuel generation (Kandasamy et al., 2021).

In some strains, HMs or other nutrients with positive charges are clasped negatively charged groups (*e.g.*, —OH/ hydroxyl, —COOH/ carboxyl, —SH/ sulphydryl, —NH_2/ amino, —PO_3H_2/ phosphoryl) on the surface layer of the cell wall (adsorption) (Spain et al., 2021). While in several microalgae, these pollutants are taken into the algae cell (absorption) (Gündoğdu and Türk Çulha, 2023). These algae accumulate HMs in their intercellular regions or their vacuoles (Torres, 2016). *Spirogyra* algal species had a removal efficiency of 20 mg/L Cu (II) (58-85%) at 30 minutes (Bishnoi et al., 2004). *Cladophora glomerata* and *Oedogonium rivulare* are among the species with the abil-

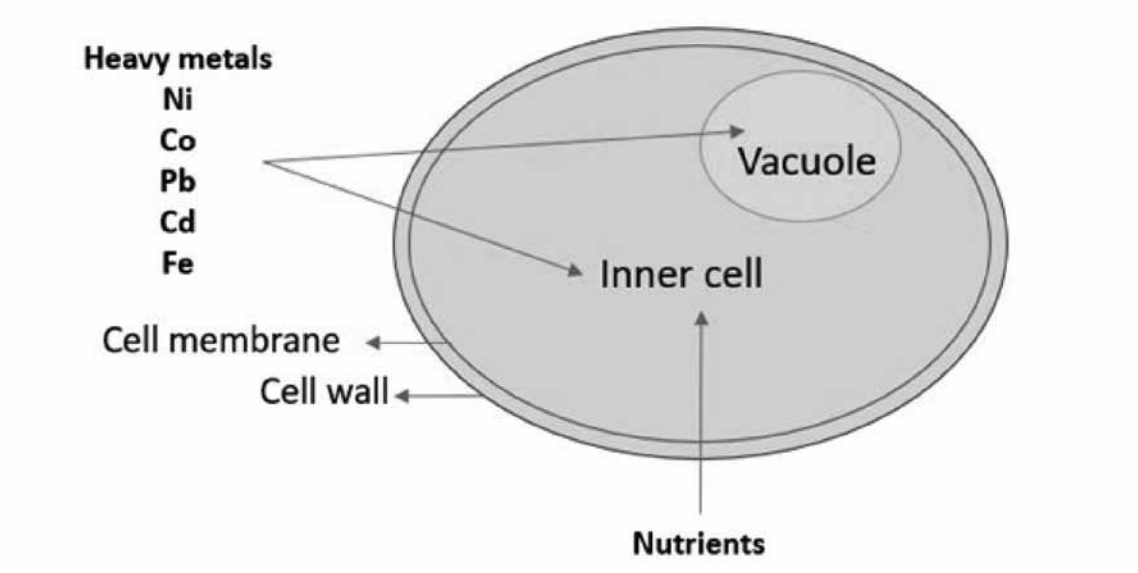

Fig. 2. A microalgae cell's absorption and adsorption scheme (modified from Kaplan, 2013)

ity to remove Co, Pb, Ni, Mn, Cd, Cr, Cu, and Fe from contaminated water (Vymazal, 1984). *Ulothrix zonata* and *Turbinaria ornata* are also considered great adsorbents of HMs (Nuhoglu et al., 2002; Vijayaraghavan et al., 2005).

Factors affecting phycoremediation

Algae can remove HMs in a variety of ways. This process depends on the metal type, taxon, pH, light, and temperature (Mehta and Gaur, 2005; Novis and Harding, 2007). As algae are sensitive to light and temperature, the efficiency of phycoremediation can also be affected through different seasons. For example, the best time to remove HM sand contamination by algae is variable depending on the season (Brake et al., 2004; Elbaz-Poulichet, 2000). The strain of algae is also important in the process of phycoremediation. Some strains are more resistant to pollutants and have a higher ability to detoxify the HMs. An ecological study of soil in polluted sites with insecticides shows the replacement of sensitive species with resistant species (Megharaj et al., 1999). As previously mentioned, the non-living biomass of microalgae has the ability to adsorb pollutants. It was revealed that the biomass of non-living algae adsorbs a higher amount of metals than that of living algae (Mehta and Gaur, 2005).

Methods to increase the efficiency of HM remediation by algae

To increase the efficiency of phycoremediation, some modern technologies have been developed in recent years. Chemical and molecular techniques are among the methods being used in this regard to manage algae to boost their productivity.

Transgenic algae to improve bioremediation

Genetic engineering for algae gene editing and gene silencing benefits various technologies and tools such as reporter genes, Cre-lox recombination, and CRISPR-Cas systems, modular cloning toolkits, regulatory elements, promoters, and vectors, restriction enzymes, and post-transcriptional gene silencing (PTGS) technologies (Fajardo et al., 2020). Data required for finding the appropriate genes to manipulate genetically is supplied by multi-omics approaches, including data of proteomics, transcriptomics, genomics, interactomics, and metabolomics, for various strains of algae, and is freely accessible on different online platforms (Ranjbar and Malcata, 2022)due to accelerated anthropogenic activities, and is nowadays, a matter of serious global concern. Removal of such inorganic pollutants from aquatic environments via biological processes has earned great popularity, for its cost-effectiveness and high efficiency, compared to conventional physicochemical methods. Among candidate organisms, microalgae offer several competitive advantages; phycoremediation has even been claimed as the next generation of wastewater treatment technologies. Furthermore, integration of microalgae-mediated wastewater treatment and bioenergy production adds favorably to the economic feasibility of the former process—with energy security coming along with environmental sustainability. However, poor biomass productivity under abiotic stress conditions has hindered the large-scale deployment of microalgae. Recent advances encompassing molecular tools for genome editing, together with the advent of

multiomics technologies and computational approaches, have permitted the design of tailor-made microalgal cell factories, which encompass multiple beneficial traits, while circumventing those associated with the bioaccumulation of unfavorable chemicals. Previous studies unfolded several routes through which genetic engineering-mediated improvements appear feasible (encompassing sequestration/uptake capacity and specificity for heavy metals.

Transporters of HMs in algae cell membranes are important options in genetic engineering. These membrane proteins, which are responsible for the transportation and tolerance of metals, basically Co, Cd, Fe, Ni, Mn, and Zn, are known as metal-tolerance proteins (MTP) (Ram et al., 2019). Some species of microalgae, including *Microcystis aeruginosa*, *Spirulina* sp., *Synechococcus* sp., *Nostoc* sp., *Anabaena flos-aquae*, and *Fischerella,* carry MTP genes. These genes, which are involved in the regulation of metal ion storage, are expressed in response to higher concentrations of HMs like Cu (Kandasamy et al., 2021). Different families of MTP genes are known in *Chlamydomonas*. More than eleven gene families are responsible for encoding the metal ion transporters (Rajamani et al., 2007). Upregulation of *CRMTP4*, which encodes the metal transporter, enhances the tolerance of *Chlamydomonas reinhardtii* to the toxicity of Cd; these microalgae strains with upregulated *CRMTP4* had 2.81-3.06 times higher ability in bioaccumulation of Cd in comparison to wild *Chlamydomonas reinhardtii* (Ibuot et al., 2017).

However, the authors indicated that wastewater-adapted strains, *Parachlorella kessleri*, *Parachlorella hussii*, and *Jaagichlorella* (*Chlorella*) *luteoviridis*, had also higher tolerance to Cd, Zn, Al, and Cu than the wild strain of *Chlamydomonas reinhardtii*. These three microalgae also revealed higher tolerance and bioaccumulation of Cd than the upregulated *CRMTP4 Chlamydomonas reinhardtii*. This indicated clearly that the mechanisms of adapted strains, which can be attributed to their oxidative stress tolerance and upregulation of several genes, overcome the upregulation of a single MTP gene (Ibuot et al., 2017), and therefore, producing transgenic microalgae with multi-metal tolerance and absorption should be prioritized in genetic engineering of algae.

In *Auxenochlorella protothecoides*, high expression of metal transporter genes, the Nramp family, has been shown under Cd stress (Lu et al., 2019). These genes also play a role in Cd tolerance of *Chlamydomonas acidophila* (Puente-Sánchez et al., 2018). In *Chlamydomonas reinhardtii*, a member of the Nramp family, *DMT1*, is responsible for the transportation of Cd, Cu, Fe, and Mn (Rosakis and Köster, 2005). Moreover, it seems that *MTP1* in *Chlamydomonas reinhardtii* encodes the vacuolar membrane protein which plays a critical role in detoxification of Cd and homeostasis of Zn (Blaby-Haas and Merchant, 2012). Phosphate transporters in *Microcystis aeruginosa* play a role in accumulation of arsenate (As) due to similar chemical structure of organic phosphate and As, lead to indiscrimination between these two elements (Wang et al., 2019).

Expression of *acr3* gene, which encodes

protein ACR3 present in vacuole membrane of *Pteris vittata* and involved in bioaccumulation of As, in *Chlamydomonas reinhardtii* resulted in 1.5-3 times enhancement of As accumulation; the ability of this recombinant strain in bioaccumulation of As was even higher in environment with reduced phosphate (Ramírez-Rodríguez et al., 2019). Microalga *Euglena gracilis* exposed to Cd, Hg, and Pb revealed an enhancement in membrane transporter Major Facilitator Superfamily, P(1B)-type ATPases, Cd/Zn-transporting ATPase, as well as proteins participating in microalgae stress response and thiol-rich proteins which play an important role in metal chelation, at proteome level (Khatiwada et al., 2020). Cell surface engineering can also be employed to enhance the algae-based bioremediation of HMs. Transgenic *Chlamydomonas reinhardtii* due to plasma membrane-anchored metallothionein polymer expression revealed enhanced capacityfor Hg (II) binding compared to wild strains (He et al., 2011).

It has also been shown that microalgae under HM stress upregulate particular HM-binding organic molecules in order to reduce the HM toxicity through the formation of chelated forms (Balzano et al., 2020). Transformed *Chlamydomonas reinhardtii* with increased synthesis of cysteine (*HAL2* gene) revealed 5-times enhancement in metal binding capacity (Rajamani et al., 2007; Ranjbar and Malcata, 2022)due to accelerated anthropogenic activities, and is nowadays, a matter of serious global concern. Removal of such inorganic pollutants from aquatic environments via biological processes has earned great popularity, for its cost-effectiveness and high efficiency, compared to conventional physicochemical methods. Among candidate organisms, microalgae offer several competitive advantages; phycoremediation has even been claimed as the next generation of wastewater treatment technologies. Furthermore, integration of microalgae-mediated wastewater treatment and bioenergy production adds favorably to the economic feasibility of the former process—with energy security coming along with environmental sustainability. However, poor biomass productivity under abiotic stress conditions has hindered the large-scale deployment of microalgae. Recent advances encompassing molecular tools for genome editing, together with the advent of multiomics technologies and computational approaches, have permitted the design of tailor-made microalgal cell factories, which encompass multiple beneficial traits, while circumventing those associated with the bioaccumulation of unfavorable chemicals. Previous studies unfolded several routes through which genetic engineering-mediated improvements appear feasible (encompassing sequestration/uptake capacity and specificity for heavy metals. Moreover, the engineering of microalgae to enhance the activity of particular enzymes to tolerate HM can be effective. For example, in *Chlorella vulgaris* chromate reductase play a role in the reducing toxic of Cr (VI) to the less dangerous trivalent chromium (Cr (III)). Therefore, it enhances the tolerance of microalgae cells against Cr toxicity (Yen et al., 2017).

Biochar addition for optimizing the phycoremediation

As biochar is enriched with nutritional com-

ponents, a combination of biochar made from plant biomass with microalgae could aid in the cleanup of HMs and other hazardous materials. Microalgae may use biochar nutrients to boost their biomass. The bioremediation process is carried out simultaneously by biochar and metal-tolerant algae. This promising and long-term technique could result in more efficient phycoremediation with energy-containing biomass of microalgae. With the appropriate energy conversion method, this energy-enriched biomass of microalgae is able to generate a greater volume of ethanol (Anae et al., 2021).

Biogenic nanomaterials generated from algae

Biogenic nanoparticles are those that are produced using biological organisms. Biologically produced nanoparticles have emerged as a viable substitute for chemically synthesized nanoparticles due to their nontoxicity. Several biogenic nanoparticles have been produced in recent years with possible applications in medicine and environmental cleanup. Biogenic nanoparticles like palladium nanocrystals, nano-magnets, biogenic manganese oxide (BioMnOx), and biogenic iron species have been shown to be successful at removing a variety of micropollutants, HMs, refractory pollutants, and halogenated chemicals. Nano-bioremediation has the potential to be a more effective, safer, environmentally friendly, and cost-efficient technology, with a significant long-term impact on the field of environmental remediation (Kumari et al., 2019).

Algal nanocomposites reveal novel materials that mix algae-based polymers with nanoparticles. One of the main applications of these nanocomposites is in the remediation of wastewater. The application of alginate, derived from algae, as the base material in wastewater treatment is a green alternative to conventional fossil-fuel-based treatment methods (Lakshmi et al., 2023). Researchers have developed a *Fucus vesiculosus*-based sorbent for the effective removal of HMs, including Pb (II), Cd (II), Cu (II), and Zn (II) from polluted waters (Demey et al., 2018). Moreover, the ability of algal-made nanocomposites for the removal of Cr (VI) and iron compounds has been approved (Wu et al., 2018). In another research, a higher ability of *Sargassum glaucescens* and chitosan/polyvinyl alcohol (PVA) nano-fiber membrane at pH 6 for biosorption of Ni in a continuous system has been shown (Esmaeili and Aghababai Beni, 2018). The world nanomaterials market, including algal nanocomposites, reached 10.88 billion US dollars in 2022 and is projected to show a 14.8% growth by 2030 (Yuan et al., 2023).

Algae and bacterial consortia

Microalgae combine with other aerobic or anaerobic microorganisms to form a microbial community. Compared with a single microorganism, a combination of algae and bacteria can work together to eliminate organic and inorganic pollutants. The combined use of microalgae and bacteria can be complementary and synergistic to obtain better pollutant degradation efficiency (Fu and Secundo, 2016). For instance, consortia of algae and bacteria mix revealed a significant removal rate of 92.6% for 1,2-dichloroethane from the petroleum industry (Alhajeri et al., 2024). On the one hand, algae

photosynthesis produces oxygen, which is a key electron acceptor for heterotrophic bacteria to break down pollutants into organic matter. On the other side, bacteria provide carbon dioxide and other stimulating media to support the photosynthetic autotrophic growth of their partners (Subashchandrabose et al., 2011). The mixing of different strains, *i.e*., algae-bacteria, can produce a synergistic effect, and the microbial population usually acts more effectively than a single strain or species. Some advantages of co-cultivation are the robustness to environmental fluctuations, the stability of the limbs, the ability to survive periods of nutrient limitation, to share metabolites, and resistance against other species. The self-oxidation of these natural systems that have been tested is beneficially used to remediate many pollutants (Muñoz and Guieysse, 2006). Compared with traditional engineering technology, it is more economically and technologically superior (Subashchandrabose et al., 2013). Contemporary molecular technology, combined with the careful selection of specific members of the microbial community, will enable the creation of autonomous systems that serve the dual purpose of contaminant removal and metabolite production.

The bacteria-algae complex is effective in dealing with harmful pollutants, and their efficiency in the bioremediation of HMs has been established (Boivin et al., 2007). The normal growth and metabolism of algae require small amounts of various metals, but higher levels of the same metals are toxic. In this way, algae communities in symbiotic interactions can absorb and detoxify the metals. The process of detoxification involves physical or chemical adsorption, active absorption into the cell for a small amount, covalent bonding, ion exchange, surface precipitation, redox reaction, or cell surface crystallization (Muñoz and Guieysse, 2006; Subashchandrabose et al., 2013). Besides the HMs, the mentioned methods can be used by microalgae to degrade organic contaminants such as black oil, naphthalene, acetonitrile, phenol, thiocyanate, benzopyrene, azo compounds (Mahdavi et al., 2015; Muñoz and Guieysse, 2006; Ryu et al., 2015; Subashchandrabose et al., 2013), and toxic pesticides including methion, quinophos, methyl parathion, DDT, atrazine, and α-endosulfan (Subashchandrabose et al., 2013, 2011).

Compared with individual microorganisms, microalgae and bacterial consortia can effectively detoxify inorganic and organic contaminants and remove nutrients from wastewater. The resource competition and pollutant reduction cooperation between the two microbial associations will determine the success of the consortium project while harnessing the biotechnology potential of the partners (Subashchandrabose et al., 2011).

Conclusion and perspectives

Bioremediation of polluted environments has attracted much attention during the last decades. As it is considered an eco-friendly and cost-effective method of treating contaminated water and soil, it has some advantages over other known existing techniques. Microalgae with an excessive tolerance to HMs and a high capacity for metal ion binding are the best accumulators of metals. Algae species such as Chlorella, Spirulina,

Spirogyra, Scenedesmus, and many others are applied for the disposal of Cr, Cu, Ni, Cd, Hg, Sp, Pb, and other HM ions. Although using algae for bioremediation of HMs could encounter some problems, such as poor adaptability of exogenous microalgae with contaminated sites, and is affected by the intensity of light, operation time, and temperature, yet using various techniques, including ex-situ bioremediation and bioaugmentation, can help to manage these limitations. Moreover, some new methods and technologies have been employed to enhance the efficiency of phycoremediation. Biochar addition, applying genetically engineered and transgenic microalgae with MTP genes, and consortia of microalgae together or with other microorganisms, are among the new techniques that are rapidly growing to provide a greener world.

In spite of numerous advantages, some novel techniques encounter challenges, including scaling up. Most phycoremediation processes employing genetically engineered algae are still confined to laboratory settings (Pradhan et al., 2022). The main limitations include low product yields and high cultivation costs (Wang et al., 2024). In order to address these challenges, it is necessary for future studies to focus on the expansion and development of universal cloning toolkits and rapid expression kits, which enable gene editing tools to be applicable to a broad range of microalgae (Webster et al., 2024).

Algae-bacteria consortia have a notable advantage over other monoculture techniques in resistance to contamination (Naseema Rasheed et al., 2023). This feature makes them suitable for application in open ponds (Su et al., 2022). Moreover, the partnership offers practical benefits in harvesting the biomass due to enhanced flocculation efficiency when certain strains of bacteria are co-cultured with algae (Ravindran et al., 2016). Recent developments have focused on creating optimized consortia through careful selection of species and engineering. It has been shown that identification of the most effective combination, with some engineered consortia, achieves over 90% removal for various pollutants (Cai et al., 2024). These systems not only perform better in terms of pollutant removal but also generate valuable biomass that can be used for various applications (Navarro and Caipang, 2024; Torres et al., 2024).

The implementation of advanced algal bioremediation techniques remains primarily in developmental stages, with most successful applications in controlled conditions. A key challenge in scaling up these technologies is a requirement for a better understanding of how microalgae-microalgae or microalgae-bacteria co-culture perform in open systems over a long time (Al-Jabri et al., 2020). The future success of these applications will depend on continued research to optimize performance and validate long-term effectiveness, particularly in outdoor conditions where environmental factors can significantly impact system performance (Al-Jabri et al., 2020). Natural symbiotic relationships between algae and native microorganisms show promise, particularly for water treatment applications, as these partnerships can effectively utilize carbon dioxide and minerals while producing oxygen

without generating waste products (Touliabah et al., 2022). In Iran, there is inadequate information about the implementation of these methods. However, these approaches could be applicable in local environments.

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