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 method 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 help in 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 were 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 method (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 contaid 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 viability 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 10^{th} 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

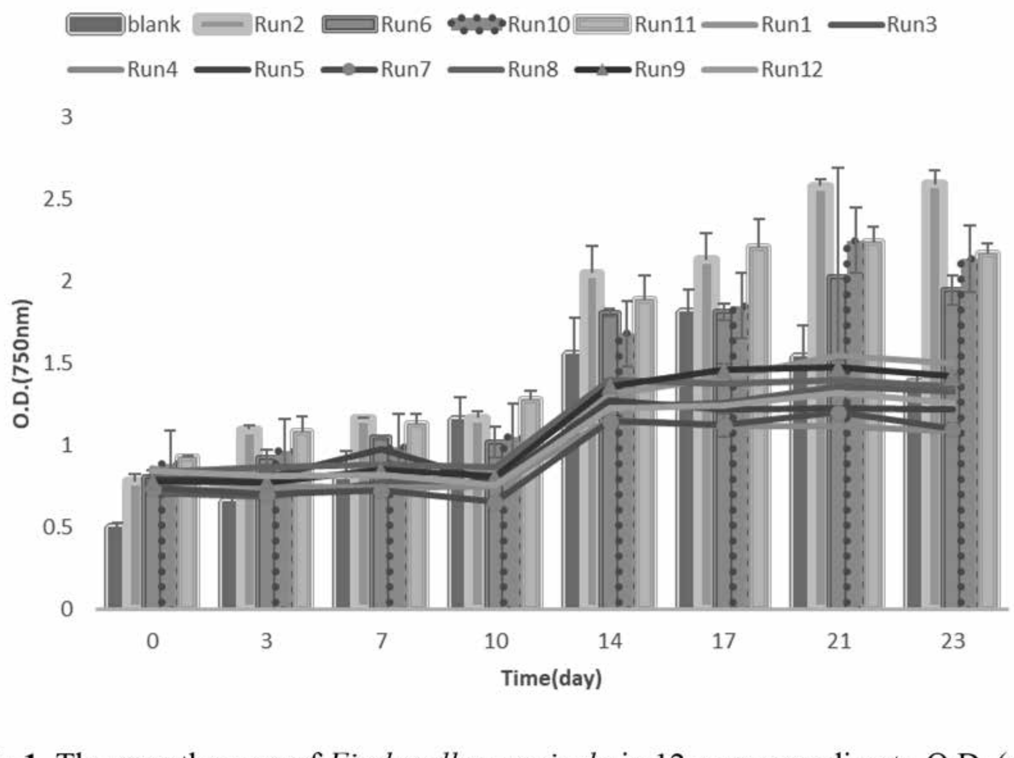

Fig.1. The growth curve of *Fischerella muscicola* in 12 runs according to O.D. ($p \leq 0.05$)

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)

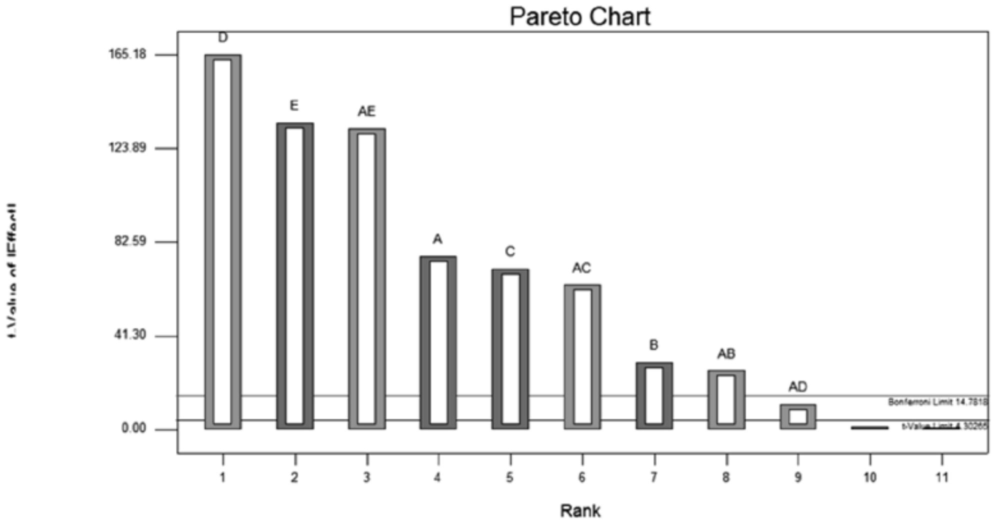

Fig. 2. Pareto Plot chart: effect of wastewater elements on nitrate amounts of medium after 10 days

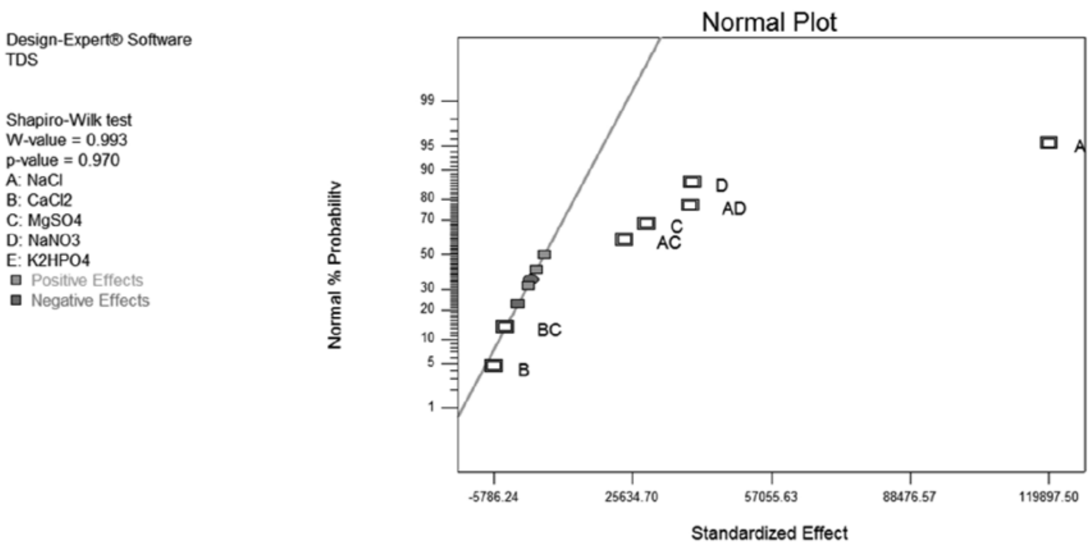

Fig. 3. Normal plot chart: effect of wastewater elements on TDS levels in the medium after the 10th day (p-value=0.970)

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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