Study of Wastewater Treatment by Microalga From Caspian Sea

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Abstract

In today's modern world, using water by man and different industries produces a large volume of effluents and wastewaters that are a serious threat to humans, environments, and ecosystems. Refining and treatment of the wastewaters are essential. This research investigated the potential of marine microalgae *Fischerella* sp. in refining nutrients from wastewater. *Fischerella* sp. was collected from the Caspian Sea. Artificial wastewater was prepared by adding different amounts of NaCl (1, 5%), $CaCl_2$ (35, 100 mg/L), $MgSO_4$ (75, 150 mg/L), $NaNO_3$ (50, 2000 mg/L) and K_2HPO_4 (6, 500 mg/L) to BG110 medium in 12 runs according to Design expert. The growth and chlorophyll contents in various treatments were measured, and nutrient analysis of the medium was performed on the 10th and 20th days after algal culture. Results showed that maximum growth, chlorophyll and decreasing of Ca^{2+}, Mg^{2+}, NO_3^- and PO_4^{3-} content were observed in 1% NaCl, 35 and 100 mg/L $CaCl_2$, 150 mg/L $MgSO_4$, 2000 mg/L $NaNO_3$ and 6, 500 mg/L K_2HPO_4. The most removing activity was shown in the stationary phase of algal growth. Also, in these conditions a decrease in TDS, TOC, and COD was observed. It can be concluded that *Fischerella* sp. is a suitable microalga decreasing nutrients in1% NaCl and the highest amount of N and P.

Keywords: Caspian Sea, Chlorophyll, *Fischerella* sp., Growth, Nutrients, Wastewater treatment

Introduction

Water is one of the most critical needs of humans worldwide. By increasing the population and man's activities like agriculture or industrialization, wastewater and effluents have proliferated, including saline wastewaters. However, water bodies and reservoirs are limited, and climatic changes have affected them directly.

Saline wastewater contains various types and inorganic salts such as NaCl, Na_2SO_4, $MgSO_4$, KNO_3, K_2HPO_4, and $NaHCO_3$ (Kester et al., 1967). There is no evidence or report about the exact amount or kinds of

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these elements because they depend on the sources that produce them. The significant sources of saline wastewaters are agriculture, industries, and secondary activities. (Hoang Nhat et al., 2018).

Excessive irrigation of saline lands and climatic changes such as lack or untimely rainfall, which are common in recent years, generate saline wastewater. In this case, changes in weather conditions (e.g., excessive heat) that cause more water exploitation should be considered. Mariculture is another activity that is responsible for producing saline wastewaters. Industrial activities such as tanneries, pharmaceutical, textile, petroleum, paper and pulp, and mining produce saline wastewaters with various organic, inorganic, heavy metals, and other contaminants. Another source of saline wastewater is related to the membrane and ion exchange technologies such as Reverse Osmosis (R.O.) in water desalination systems (Hoang Nhat et al., 2019).

Saline wastewater affects the environment seriously. It damages crop production by changing the osmotic balance, so the assimilation of nutrients is disturbed, and therefore growth rate and crop yields are affected. This result was later contradicted by Al-Jaloudet al. (1993). As this wastewater has various elements, it can pollute the drinking water, threatening the health of humans and other users. This wastewater affected the ecology directly and indirectly. It can instantly change the biological characters of flora and fauna of the environment (Alves et al., 2018), but in indirect form, as saline wastewater contains inorganic nutrients, primarily N and P; it is the main factor for eutrophication and is toxic to ecosystems and human beings (Anjuli et al., 2015).

By considering all above mentioned, the treatment of wastewater is essential. There are different methods for treating saline wastewater; biological (Ahmadi et al., 2017; Xiao & Robert, 2010), physicochemical (Aller, 2016; Wen et al., 2018), and hybrid technologies (Nguyen et al., 2018; Tomei et al., 2017). Among these, applying zeolite, using an anaerobic process, membrane process, and halophilic microorganisms (microalgae) process are most used. Most of these methods are costly because saline wastewaters contain a high degree of inorganic salts, but among them, as microalgal processes have no secondary pollutant and besides it can create profit; eco-friendly; they are considered as a cost-effective and green process (Hoang Nhat et al., 2019) (Anjuli et al., 2015).

Microalgae are microscopic photoautotroph organisms. They can produce oxygen using atmospheric CO_2 and solar energy. Cyanobacteria (blue-green algae) are more suitable for wastewater treatment. Cyanobacteria are ubiquitous and, depending on their species, can tolerate various situations with different ranges like saline wastewaters (Anjuli et al., 2015).

These microorganisms have unique characteristics that make them suitable for application in biotechnology, agriculture, industry, and wastewater treatment. They can

grow in a variety type of mediums, even wastewaters. They are photoautotrophs and play an essential role in reducing the greenhouse effect on the environment. Besides oxygen production, some blue-green algae species have nitrogen fixation abilities that help them grow even in low N contents and make them a good candidate as biofertilizers.

Cyanobacterial biomass has many applications in, e.g., feed, pharmaceutical, industries, and biofuel. So cultivation of blue-green algae in wastewater is a safe method for treatment and producing biomass (Subramaniyan 2012; Anjuli 2015).

So in this research, the growth and chlorophyll contents of *Fischerella* sp. that isolated from the Caspian Sea of Iran in artificial wastewater (AWW) were investigated and its potential to remove nutrients in two-phase of growth (logarithmic and stationary phase) was studied.

Material and methods

Sampling and culture

Sampling was performed from different parts of the Caspian Sea; Salmanshahr, Mahmoodabad, Khzarabad (Mazandaran province), and Geisomcoastline (Gillan province) in the north of Iran from August-September 2019. Samples were transferred to the petroleum microbiology lab of ACECR of Shahid Beheshti University and cultured in F/2 medium (Guillard and Ryther,1962) by solidified agar plate (Belcher et al. 1982) and soil culture (Sardeshpande and Goyal, 1981) methods. Cultures were kept in the culture room of ACECR of Shahid Beheshti at 25± 2 ˚C with a Florence lamp and a duration of 8 L/16D.

After a month, the dominant specimens in both cultures were purified. The *Fischerella* sp. was selected as one of the most common specimens. Then mass cultivation is done in liquid cultures with BG110 medium (Kaushik, 1987). Liquid cultures also were kept in the culture room with the above condition. Aeration of samples was done by an aquarium air pump, Artman HP-4000.

Molecular identification

The DNA extraction was done by the Fermentas DNA extraction kit (K0512). Moreover, the molecular identification of specimens was performed by the PCR of the 16s ribosomal region and sequencing of the PCR product by Nubel et al. (2000).

Analysis of growth and chlorophyll contents

Analysis of growth was performed according to the biomass changes by optical density (OD) method in λ 750 nm (spectrophotometer, Light wave WPA) that was done every 2 days for 25 days (Soltani, 2006).

Chlorophyll contents were obtained by preparing methanol extracts of specimens and measuring their density in λ 665 nm according to (1) by Marker's (1972) method.

$$\text{Chl. } (\mu g/ml) = 13.14 \times \text{OD } 665 \text{ nm} \quad (1)$$

Experimental design

The artificial wastewater (AWW) used in this experiment was prepared by dissolving the common nutrients of wastewaters in BG110 medium with minimum and maxi-

Table 1. Designed runs as artificial wastewater in our experiments

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

mum levels as follows: NaCl (1, 5%), $CaCl_2$ (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 that were designed by Design expert (Table 1). Our blank was BG110 medium.

The *Fischerella* sp. is cultured under each run's specification and kept in the above culture room. The OD and chlorophyll contents of these 12 runs were also measured every 2 days for 25 days, as in section 2.3.

Analysis of growth medium

The growth medium was filtered with filter paper to study the changes in nutrients. It was analyzed in 3 phases: before adding algal specimens (preliminary study), at the logarithmic phase of algal growth (10^{th} day of cultivation), and during the stationary phase of algal development (20^{th} day of cultivation).

Analysis and removal percentage of cation and anions

In this research, cations: Na^+ (ppm), Ca^{2+} (ppm), and Mg^{2+} (ppm) were analyzed by ICP- OES method.

Cl^- (%) was measured by titration with $AgNO_3$; meanwhile, NO_3^-(ppm) was measured by spectrophotometry UV-visible (Shimadzu) in λ 220 and 270nm. Indeed, PO_4^{3-} (ppm) measured by Standard Method 4500-P-C.

The nutrient removal percentage was calculated by Do et al. (2019) according to equation (2):

$$\% \text{ nutrient removal} = C_1(\text{initial concentration}) - C_2(\text{final concentration})/C_1 \times 100 \quad (2)$$

Other analysis

Other analyses, including Total Dissolved Solid (TDS) (mg/l), was performed by electroconductivitimeter, Total Organic Carbon (TOC) (mg/l as C) by Standard method 5310 B; Chemical Oxygen Demand (COD)

(mg/l as O_2) by Standard method 5220B.

Statistical analysis

Statistical analysis of growth and chlorophyll contents were done for three replication of each samples by ANOVA (SPSS V.24) and also removing nutrients were done by and ANOVA (Design-Expert V.7.0 Softwares).

Results

Growth and chlorophyll contents

Growth curves of *Fischerella* sp. according to the biomass change in 12 run and blank are shown in Fig.1. According to these results, runs 2, 11, 10, and 6 with 1% NaCl had more growth and also had significant difference with the blank sample($p \leq 0.05$). Among them run 2; NaCl 1%, $CaCl_2$ 100, $MgSO_4$ 150, $NaNO_3$ 2000, K_2HPO_4 500 mg/L; had the most growth and its difference with runs 10, 11, 6 was significant($p \leq 0.05$).

Runs with 5% NaCl kept their viability in stationary form, but their growth was noticeably less than the others.

Measuring of chlorophyll contents in 12 runs and blank also showed that the blank (BG110 with no additive nutrients) had the most chlorophyll with significant difference ($p \leq 0.05$) with other samples. Runs with 1% NaCl (10, 1, 11, 6, 2, 3) had the same content of chlorophyll, but among them, the most chlorophyll contents were observed in run 10; NaCl 1%, $CaCl_2$ 35, $MgSO_4$ 150, $NaNO_3$ 2000, K_2HPO_4 6 mg/L. The chlorophyll contents in 5% NaCl decreased significantly.

Sequence analysis

The sequence of the 16S rRNA gene was determined for *Fischerella* sp. ISC 123. The nucleotide sequences described in this study were submitted to NCBI under NCBI's accession number OK594059.

Analysis of nutrients in growth medium

Analysis of cations and anions

Analysis of cations (Na^+, Ca^{2+}, and Mg^{2+}) and anions (Cl^-, NO_3^- and PO_4^{3-}) and removing percent of them from growth media were shown in Table 2. According to the results, it can be concluded that runs 2,

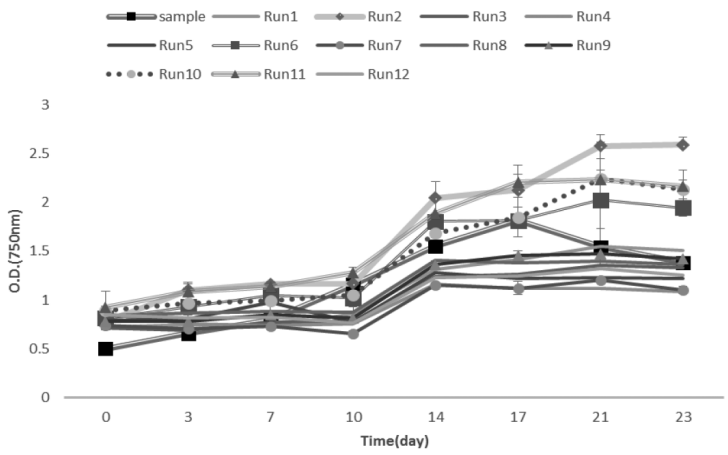

Fig. 1. Growth curve of *Fischerella* sp. in 12 runs according to OD

6, 10, and 11 had the most effective in decreasing cations, especially Ca^{2+} and Mg^{2+}. Results also showed that the most percent of removing anions were observed in run 2. This run removed 98% nitrate from growth media on the 10th day of culture. Runs 1 and 11 could remove significant amounts of nitrate(95,94%) on the 20th day of culture. Run 4 could remove 73.3% Cl^- on the 10th day.

Analysis of TDS, TOC and COD

Analysis of TDS, TOC and COD and their removing percentage from growth media were shown in Table 3. According to the results it can concluded that runs run 7 had the most removal of TDS (69%) and runs 10 and 11 had the most potential in removing TOC and COD.

Discussion

According to the growth results, it can be concluded that salinity is a critical factor in controlling the growth. In this case, *Fischerella* sp. had a noticeable growth in 1% NaCl, and even in some cases, its development was more than the blank (with no added NaCl). Although NaCl 5% was unsuitable for growth, this microalga could tolerate it for two weeks and had a steady growth phase. Our specimen was a marine sample, so it adapted to this condition better.

These results were similar to the research on *Chlorella vulgaris* (freshwater microalgae), *Chlorella* sp., and *Stichococcus* sp. (marine microalgae) with 0.1, 1, 3. 5, and 5% NaCl. In this case, *C. vulgaris*,

Table 2. The removing percentage of cations (Na^+, Ca^{2+}, Mg^{2+}) and anions (Cl^-, NO_3^-, PO_4^{3-}) from growth media in 12 runs during the10th and the 20th day of microalga culture

	Na^+ %		Ca^{2+} %		Mg^2+ %		Cl^- %		NO_3^- %		PO_4^{3-} %	
Runs	10th	20th	10th	20th	10th	20th	10th	20th	10th	20th	10th	20th
1	-	-	1.7	-	-	-	27	5.8	-	95	32	3.57
2	-	-	49.7	73	4.6	4	30	4.48	98	-	-	-
3	-	-	5	7.6	-	-	30.5	18.6	-	-	65	68
4	-	-	-	-	-	-	73.3	14.5	-	37	-	-
5	-	-	-	-	-	-	-	12.8	-	-	-	-
6	-	-	47.4	62	8.2	7.3	-	1.56	-	25	-	-
7	-	-	-	-	2.7	-	-	-	-	-	6	-
8	-	-	1.9	-	-	-	-	-	-	20	-	-
9	-	-	-	-	-	-	-	-	3	7	0.43	-
10	-	24.3	-	-	-	-	-	-	-	15	42	-
11	-	-	63.8	85	10.6	7	-	-	21	94	10	-
12	-	-	-	-	-	-	-	13.35	-	28	5.8	-

Table 3. The removing percentage of TDS, TOC and COD from growth media in 12 runs during 10th and 20th day of microalga culture

	TDS %		TOC %		COD %	
Runs	10th	20th	10th	20th	10th	20th
1	-	-	-	-	-	-
2	52	-	-	-	-	-
3	55	45	80	62	65.7	71.4
4	-	-	50.2	35.3	66.1	70.7
5	35.4	29	79	74.5	57.7	61.5
6	48.4	47.5	73.3	71.2	75	65
7	64.3	69.2	-	-	12.5	-
8	-	34	41.5	36.1	75	37.5
9	57	66.5	81.2	74	81.5	75
10	-	-	94.6	82.6	92.1	89.5
11	47.5	52	89.6	56	85	60
12	7	34	50.2	20.2	75	56.2

the freshwater microalgae, has maximum growth and biomass yield in 0.1 salinity. Still, the other marine ones had the most growth and biomass yields at 1%, and by increasing the salinity(3.5 and 5%), their biomass reduced significantly (Hoang Nhat et al., 2018).

Although there are some exceptions depending on the species and the medium conditions, for example, Zhou et al. (2017) explored that *Spirulina platensis* could produce noticeable biomass in 2.24% salinity. It should be considered that other culture conditions such as nutrient concentration, pH, and different light: dark cycles are also responsible in this case. In another study, Kim et al.(2016) also showed that *Acutodesmus obliquus* have suitable biomass in 5.2% salinity. Church et al.(2017), which researched the characteristics of *C. vulgaris* in synthetic saline wastewater, also remarked that higher salinities decrease the biomass content, similar to our findings. Another study on *C. vulgaris* and the effect of NaCl and KCl on its growth by Church et al.(2017) also showed that algal growth would be decreased even lower biomass by increasing salt concentration for both salts production was observed with KCl than NaCl.

Photosynthesis activities include photosystem I (PSI) and PSII in microalgae (Kebede, 1997). PSI relates directly to the chlorophyll contents and their changes. So

studying the chlorophyll contents, especially in stress conditions like salinity and wastewater, is essential. The chlorophyll contents showed that this pigment is too sensitive to increasing salinity because all the runs in 5% NaCl had a significant decrease in chlorophyll. It means that *Fischerella* sp. had low nutrient consumption in this salinity. In 1% NaCl, after a week, chlorophyll production increased, and the difference among the runs related to the other nutrients in the culture medium. These results were similar to *Stichococcus* sp., which produces a significant amount of chlorophyll in 0.1 and 1% salinity, but its contents at 3.5 and 5% were very low (Hoang Nhat et al., 2018). It should be noticed that marine algae usually adapt more to saline stress because their physiological systems are compatible with these conditions. Guetet al.(2012) reported that the increased salinity in *Nanochloropsis oculata* causes culture to decrease the growth rate and pigment contents (e.g., chlorophyll and carotenoids).

Eliminating cations and anions in different runs showed that the highest potential of removing nutrients was observed in runs with high growth and chlorophyll contents (2, 6, 10, 11) in 1% NaCl. So it can be concluded that the amount of elimination relates to the growth of samples.

Studies on *C. vulgaris* in various amounts of NaCl and KCl (0- 1.5- 3 and 4.5%) shows that lower salt concentration causes faster nutrient to remove (ammonia) for both salt type (Church et al., 2017).

Considering the removing percentage in 2 phases (the10^{th} and 20^{th} day) revealed that although both steps were active in removing, the most removing activities were performed at the end of the growth phase (20^{th} day) in most runs.

In this case, research on *Phormidium* sp. showed that this microalga could remove 48 and 30% of PO_4-P and NO_3-N from anaerobically treated swine wastewater (Su et al., 2012). Kamilya et al. (2006) revealed that *Nostoc muscorum* and *Spirulina platensis* have nutrient removal potential from fish culture effluent. In this case, *N. muscorum* can remove 14.17 and 41.8 % of NO_3-N and PO_4-P while *S. platensis* remove 50.39 and 47.76% of NO_3-N and PO_4-P, respectively, in 7 days. Besides microalgal monocultures, Silva-Benavides and Torzillo (2011) use the co-culture of *Planktothrix* sp. and *Chlorella* sp. to treat municipal wastewater. This co-culture can remove 100 and 88% of PO_4-P and NO_3-N. Also, a consortium of filamentous strains (*Phormidium*, *Limnothrix*, *Anabaena*, *Westiellopsis*, *Fischerella*, and *Spirogyra*) was studied and reported to remove 100 and 97 % of nitrate and phosphate (Anjuliet al., 2015). These results indicate that cyanobacterial species can significantly remove nutrients (mainly N and P) from various types of wastewater.

Research on *C. vulgaris*, *Scenedesmus obliquus*, and *Oocystis minuta* by Ajala and Alexander (2019) removing nutrients in wastewater revealed that *C. vulgaris* could reduce 93% nitrate after 14 days of culti-

vation; meanwhile, *O. minute* can consume phosphate up to 95%.

The removal of TOC and COD was also related to growth. Total Organic Carbon (TOC) is a crucial nutrient for microalgae. Hoang Nhat et al. (2018) showed that the highest TOC removal is at 0.1 and 1% salinities. In this case, *C. vulgaris* can remove 92% TOC on the 10th day, and *Stichococcus* sp. can remove more than it at 1% NaCl. El-Bestawy (2008) researched *Anabaena variabilis*, *A. oryzae* and *Tolypothrix ceylonica* about their role in improving water quality in domestic industrial wastewater. They showed that *A. variabilis* had the highest BOD and TDS reduction (89.3 and 39%) while can reduce COD up to 73.6%. Another research by Nagasathya and Thajuddin (2008) on wastewater treatment potential and water quality improvement of paper mill effluent by *Phormidium tenue* was performed, removing 14.5, 17.6, and 45.26% of salinity, BOD, and COD in 20 days.

By considering the results, it can be concluded that marine species have more adaptability to saline conditions. In this case, *Fischerella* sp. is a suitable microalga with good growth and chlorophyll in wastewaters with 1% NaCl and high amounts of nitrate and phosphate. Besides this, its ability to the treatment of wastewater elements is considerable.

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