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Assessment of Wastewater Elements on Growth, Chlorophyll, and Reduction of Nitrate and Phosphate by the Marine Cyanobacterium *Fischerella muscicola* ISC123

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Abstract

Wastewater from different parts and industries have compounds, including nitrate and phosphate, that seriously affect ecosystems and environments. Remediation and treatment of these compounds are usually performed by physical, chemical, and biological methods. Since cyanobacteria have high nitrate and phosphate requirements, wastewater (sewage/ municipal mixed waters) with high loads of these nutrients can serve as a culture medium to enhance their growth and indirectly reduce the nutrient load, making it useful for other applications. So, in this research, the role of these elements in artificial wastewater as a culture medium on growth, chlorophyll contents, and removing potential of nitrate and phosphate by the marine cyanobacterium *Fischerella muscicola* ISC 123 is investigated. Our specimen was collected from the Caspian Sea, isolated, and identified according to the 16SrRNA. Wastewater treatments were designed in 15 runs using Design-Expert software, and the sample was cultured in BG110 medium with various amounts of NaCl, $NaNO_3$, and K_2HPO_4. Growth (O.D.), chlorophyll contents (methanolic extract), changes of nitrate and phosphate of cultures by standard methods, and effects of elements were analyzed by Response Surface Methods (RSM) of Design-Expert. The enhanced growth rate, chlorophyll content, and nitrate and phosphate removal were observed in runs 2, 6, and 14. NaCl reduced growth while most interactions of elements were compatible with the results of practical experiments. According to the results, run14 (NaCl 1%, $NaNO_3$ 350, K_2HPO_4 77 mg L^{-1}) had the optimum condition for the growth and removing nitrate and phosphate of *F.muscicola*. It can be concluded that removing nitrate and phosphate depends on the growth factors, and by studying and optimizing wastewater elements as culture media, microalgae can have the optimum growth and the most refining potential.

Keywords: *Fischerella muscicola*, Growth

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rate, Nitrate, Phosphate

Introduction

In recent years, the growth of population, development of cities, and industrialization have caused the overconsumption of energy, especially water resources. Cities mostly use environmental water resources that were already specialized for agriculture (Fasihi, 2014). Used water for various purposes in cities, cooling industrial instruments, and other applications inevitably form wastewater that after collecting in sewage systems guided around the cities. Transferring polluted water, especially their usage for watering farmlands, spreads pollution and affects each part of the environment, such as water, air, and soil (Fasihi, 2014).

Lack or insufficient refinement of these wastewaters usually have various compounds such as nitrogen and phosphorus in the forms of nitrate, nitrite, ammonia, and ammonium, causing eutrophication in ponds that are a severe threat to ecosystems and environments (Yang et al., 2008). Therefore, wastewater refinement is essential, but this process is costly. Today, various technologies like physicochemical, hybrid, and biological methods are used to solve this problem (Hoang Nhat et al., 2019). Phytoremediation is one of the most important bioremediation methods that, by use of plants or microorganisms (microalgae, cyanobacteria, bacteria, and fungi), remove pollutants (nutrients, organic compounds, and heavy metals) from wastewaters (Sood et al., 2015).

Microalgae have excellent viability in different environments with various polluted compounds. These microorganisms with oxygenic photosynthesis have an essential role in decreasing greenhouse gasses. Besides, these can be used for wastewater treatment and controlling environmental pollution. In recent years, biological treatments using microalgae have been widely investigated (Maity et al., 2014). Research showed that various microalgae species have great potential in removing nitrate and phosphate from media (McGinn et al., 2012; Su et al., 2012).

Besides microalgal species, wastewater characteristics as culture media affect the efficiency of algal treatments. Wastewater pollution may be dangerous for phytoplanktons, but microalgae have high adaptability. More studies are needed to detect the mechanisms of microalgae used to tolerate these conditions. Usually, the ratio of N: P and hydraulic retention time (HRT) should be optimized to adapt specific microalgae to wastewater media and specific and main conditions such as nutritional load. According to the research of Rani et al. (2020), the rate of N: P in wastewater directly or indirectly affects the assimilation of nutrients by microalgae. The low molarity of N: P in municipal wastewater causes reduced biomass compared to the biomass produced in industrial wastewater with a high concentration of nutrients. According to Kim et al. (2017), 98% of removing nitrate and phosphate from anaerobic sewage is usually performed by microalgal species. Compared to other concentrations of N: P, the potential of removing phosphate by *Micractinium inermum* in 3:1 (N: P) increased. In this condition, 10.7 g/L biomass is produced, which is more than the printed results.

The capacity of absorbing nutrients by 3 benthic microalgae, *Cladophora, Klebsormium,* and *Pseudoanabaena* sp., in different amounts of N: P showed the rate of N: P has a significant effect on the growth and removing phosphate of these species. The appropriate amounts of N: P differ from species; for instance, the best amounts of N: P for *Cladophora* 5:15, *Klebsormidium* 7:10, and *Pseudoanabaena* 7:20 have been reported. In this condition, *Cladophora* sp. showed the most biomass; meanwhile, *Pseudoanabaena* sp. had the most changes in nitrogen and phosphorus. This research revealed that *Cladophora* sp. has a high capacity for removing phosphate from wastewater at the low rate of N: P, while *Pseudoanabaena* sp. is a more potent microalgae for removing nitrate at high rate of N:P (Rani et al., 2020).

Microalgal cultures are given more attention as probable biofuel production and lipid accumulation feedstock. These processes depend on diverse factors, such as microbial species used in the process and nutrients in the growth medium.

Heterocyst-forming filamentous cyanobacteria like *Anabaena siamensis* TISTR8012, *Nostoc* sp. CU2561 and *Fischerella muscicola* TISTR8215, which have both hydrogenase and nitrogenase enzymes, were characterized as a promising microorganism for high H_2 production (Wutthitien et al., 2019; Sukrachan and Incharoensakdi, 2020) in contrast to lower H_2 production in unicellular cyanobacteria such as *Aphanothece halophytica* (Taikhao et al., 2013). As for secondary and tertiary wastewater obtained after the algae-based treatment, the spent wastewater with a low N/P ratio may induce H_2 production. In this case, increased H_2 production was associated with increased heterocyst frequency in N-deprived cultures (Wutthitien et al., 2019).

Organic molecules can be utilized as a source of energy (ATP) to promote H_2 production through nitrogenase. It has been reported that glucose supplemented with *Fischerella* culture is a suitable substrate for H_2 production (Yodsang et al. 2018). Hence, secondary and tertiary wastewater with remaining organic matter can improve H2 yield in cyanobacteria (Wutthithien and Incharoensakdi, 2024).

In this research, the marine cyanobacterium *F. muscicola* ISC 123 has been cultured in BG110 medium supplemented with various amounts of nitrate, phosphate, and NaCl as wastewater condition and the effects of these elements on growth, chlorophyll synthesis, and removing the potential of nitrate and phosphate investigated to obtain the optimum condition for its growth and wastewater treatments.

Material and methods

Sampling, isolation, and culture

Sampling was performed from different parts of the Caspian Sea; Salmanshahr, Mahmoodabad, Khazarabad (Mazandaran province), and Geisome coastline (Gillan province); in northern Iran. Isolation was performed by solid agar plate (Belcher et al., 1982) in the petroleum microbiology lab of ACECR of Shahid Beheshti University. Dominant species were purified, and *Fischerella* sp. was selected as one of the most common specimens. Mass cultivation was performed in liquid culture in BG110 medium (Kaushik, 1987). Cultures were kept in the culture room of ACECR at 25 ± 2 ºC with LED lamps(2000 LUX) and a duration of 8/16 (L/D). Samples were aerated using an aquarium air pump, Artman HP-4000.

Molecular identification

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

Wastewater treatments

The artificial wastewater (AWW) was prepared by adding various amounts of NaCl (1–5%), $NaNO_3$ (350-2000 mg L^{-1}), and K_2HPO_4 (6-500 mg L^{-1}) to BG110 medium. Treatments were conducted by Design-Expert software V. 7.00 (RSM method) with 3 center points in 15 runs (Table 1). Samples were cultured in 2L Erlenmeyer flasks treatments and incubated in the culture room of the Research Institute of Applied Science of ACECR, at 25 ± 2 °C with LED lamps for 8L16D^{-1} duration for 25 days. An aquarium air pump, Artman HP-4000, was used for the aeration.

Analysis of Growth and Chlorophyll

Growth was analyzed according to the biomass changes by optical density (O.D.) method at λ 750 nm (spectrophotometer, light wave WPA) every 2 days for 20 days with three replicates (Soltani et al., 2006).

Chlorophyll contents were obtained by preparing methanol extract from specimens in three replicates and keeping them in the

Table 1. Artificial wastewater treatments designed by Design-Expert software

Run	NaCl (%)	$NaNO_3$ (mg L^{-1})	K_2HPO_4 (mg L^{-1})
1	3.00	2100.30	248.50
2	0.17	1075.00	248.50
3	5.00	350	420.00
4	5.83	1075.00	248.50
5	3.00	49.70	248.50
6	1.00	1800.00	420.00
7	5.00	1800.00	77.00
8	3.00	1075.00	248.50
9	3.00	1075.00	5.96
10	3.00	1075.00	248.50
11	3.00	1075.00	248.50
12	3.00	1075.00	248.50
13	3.00	1075.00	248.50
14	1.00	350	77.00
15	3.00	1075.00	491.04

refrigerator at 4 °C for 24 hours. Their density was measured at λ 665 nm according to (1) by Marker (1972).

Chl.(µg mL^{-1}) = 13.14 × OD 665 nm (1)

Removing nitrate and phosphate from the culture

The nitrate and phosphate removal capacity of cultures was measured on the first and 13^{th} day (logarithmic phase) of culture by filtering the cultures with filter paper and analyzing the nitrate by St. method 4500-NO_3-B and phosphate by St. method 4500 P-C. The nitrate and phosphate removal percentage was calculated by Do et al. (2019) as follows (2).

% Nutrient removal = C_1 (Initial concentration) – C_2 (Final concentration) / C_1 × 100 (2)

Effects of elements

Analysis of NaCl, $NaNO_3$, and K_2HPO_4 effects as one factor separately and their interaction on growth, chlorophyll contents, and removing the potential of nitrate and phosphate from culture were studied by Response Surface Method (RSM) in CCD form of Designe-Expert software by Morshedi and Akbarian (2014).

Statistical analysis

Statistical analysis was done using ANOVA (SPSS V.24) and Design - Expert V.7.0 software for three sample replications.

Results

Sequence analyzing

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

Growth and Chlorophyll contents in wastewater treatments

According to the analysis of the growth of *Fischerella muscicola* in wastewater treatments, the best growth was observed in runs 2, 6, and 14 with a significant difference ($P \leq 0.05$) with others from the third day of culture. The maximum growth rate was seen in run 2 (NaCl 0.17%, $NaNO_3$ 1075, K_2HPO_4 248.5 mg L^{-1}) (Fig 1).

The results of chlorophyll changes in

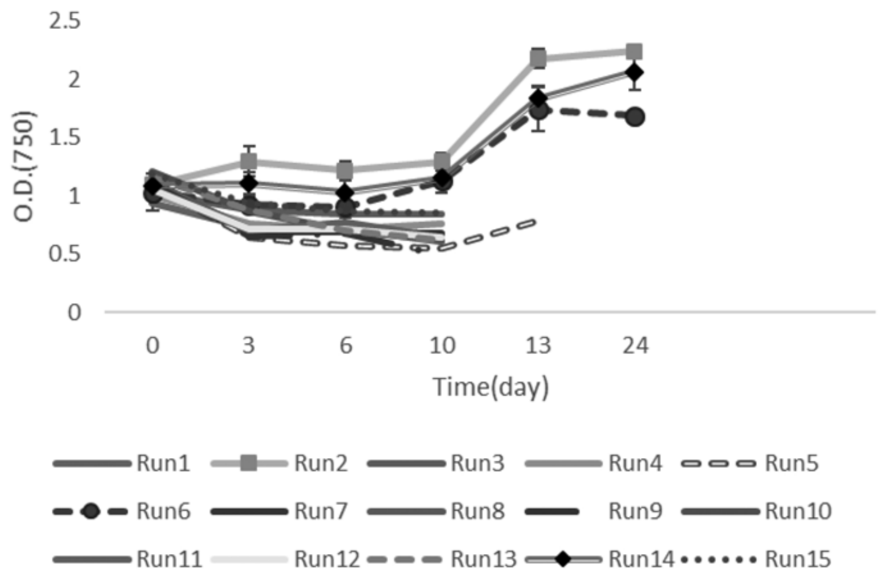

Fig 1. Study the growth of *Fischerella muscicola* in wastewater treatments

wastewater treatments were the same as growth. So, the maximum chlorophyll contents were observed in runs 2, 6, and 14.

Effect of wastewater elements on growth and chlorophyll contents

The results of the factor effect of NaCl, $NaNO_3$, and K_2HPO_4 on growth and chlorophyll contents of *Fischerella muscicola* showed the negative effect of NaCl on growth (increasing NaCl concentration caused the decrease of growth). Maximum growth was observed in NaCl 1% (Figure 2). $NaNO_3$ had little effect, whereas K_2HPO_4 did not affect growth. The exact manner was also seen in chlorophyll contents.

Analysis of the interaction of NaCl-$NaNO_3$, NaCl-K_2HPO_4, and $NaNO_3$-K_2HPO_4 by Design-Expert software, showed the maximum growth in NaCl 1% $NaNO_3$ 350 mg L^{-1} (14) (Figure 3), NaCl 1% K_2HPO_4 420 mg L^{-1} (6), NaCl 1% K_2HPO_4 77 mg L^{-1} (14), $NaNO_3$ 350 K_2HPO_4 77 mg L^{-1} (14). The interaction of elements on chlorophyll contents was the same as growth (Figure 4).

Removing nitrate and phosphate in wastewater treatments

A study on the removal potential of nitrate from culture showed that all the treatments except runs 1, 4, and 6 had this ability, and the maximum removal rate of nitrate was observed in run 7(40%) and run 14 (37%).

Phosphate concentration decreased in runs 2, 6, and 14 with a maximum removal rate of 17% (14) on the 13^{th} day of culture (Table 2).

Effects of wastewater elements on removing nitrate and phosphate

Results of a one-factor analysis of NaCl and K_2HPO_4 on removing nitrate from the medium showed these factors did not affect the decrease of nitrate.

Studying the interaction of NaCl-K_2HPO_4 and $NaNO_3$-K_2HPO_4 by ANOVA analysis showed their significant effect ($P \leq 0.05$) on removing nitrate; however, NaCl-$NaNO_3$ had no interaction for reducing nitrate. Analysis of Design-Expert were the same as these results, so the most nitrate reduction was seen in NaCl 1% with K_2HPO_4 420 mg L-1 (6) and NaCl 5% with K_2HPO_4 77 mg L^{-1}.

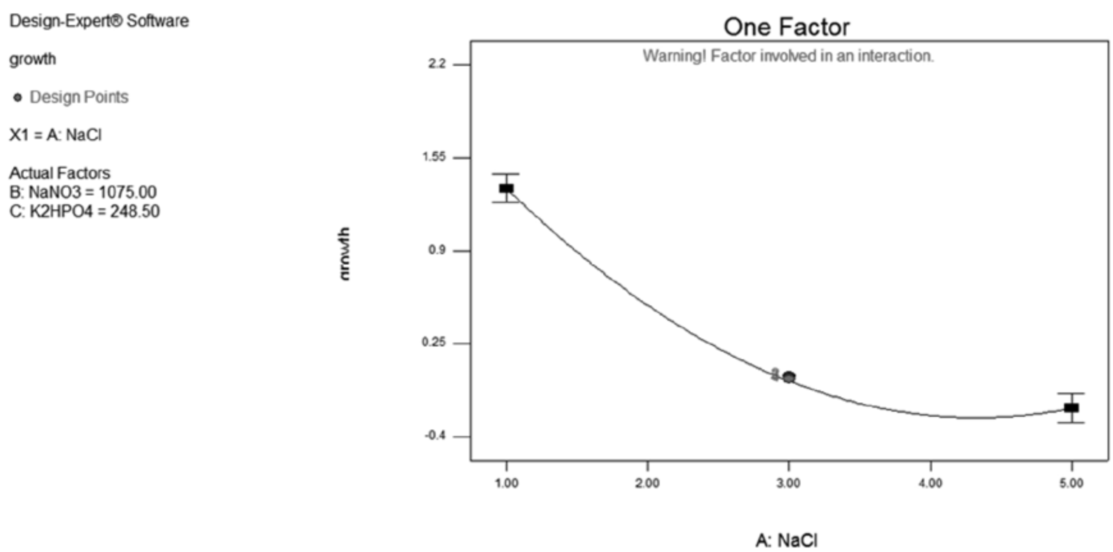

Fig 2. The effect of NaCl on the growth of *Fischerella muscicola*

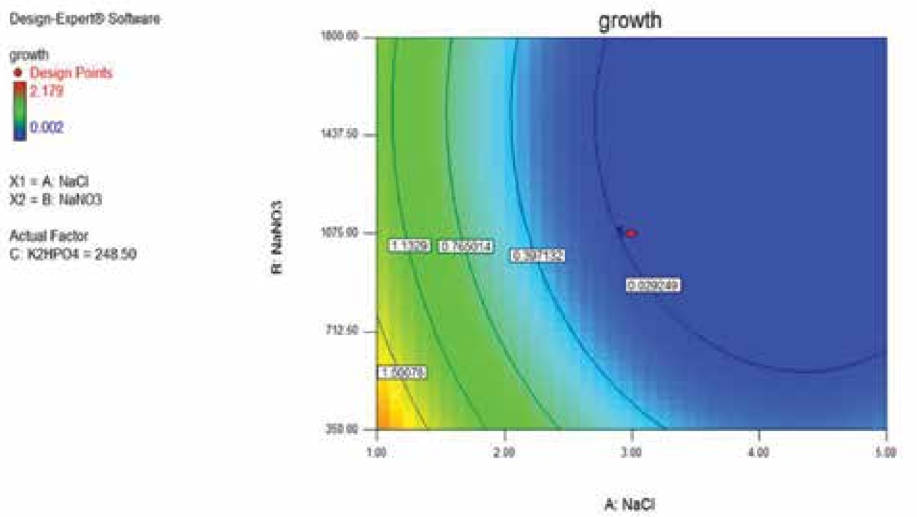

Fig. 3. Counter curve: interaction of NaCl- $NaNO_3$ on the growth of *F.muscicola*

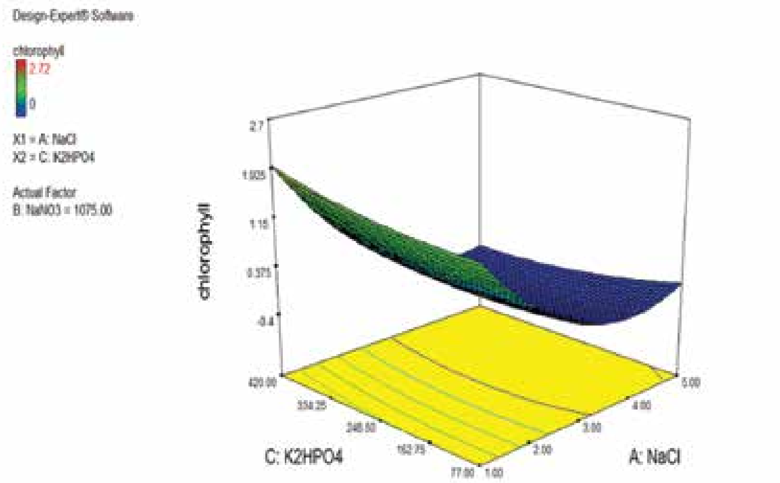

Fig. 4. Interaction of NaCl-K_2HPO_4 on chlorophyll content of *F.m uscicola*

Table 2. Study removing percentage of nitrate and phosphate by *F. muscicola* in wastewater treatments on the 13th day of culture

Runs	NO_3^-(%)	PO_4^{3-}(%)
1		
2	13	15
3	33	
4		
5	6	
6		14
7	40	
8	15	
9	19	
10	20	
11	13	
12	20	
13	12	
14	37	17
15	6	

$NaNO_3$-K_2HPO_4 had the most interaction on nitrate reduction. The most nitrate reduction was observed in $NaNO_3$ 350 K_2HPO_4 420 mg L^{-1} and $NaNO_3$ 350 K_2HPO_4 77 (14) (Figure 5).

Phosphate

Results of a one-factor analysis of NaCl and $NaNO_3$ on removing phosphate from the medium showed these factors did not affect the decrease of phosphate.

According to the statistical analysis, the interaction of NaCl-$NaNO_3$ had a significant effect ($P \leq 0.05$) on phosphate reduction. This way, the most phosphate reduction was seen in NaCl 1% $NaNO_3$ 1800 mg L^{-1} (6) and NaCl 5% $NaNO_3$ 350 mg L^{-1} (Figure 6).

According to the results, interactions of NaCl-K_2HPO_4 (Figure 7) and $NaNO_3$-K_2HPO_4 did not remove phosphate.

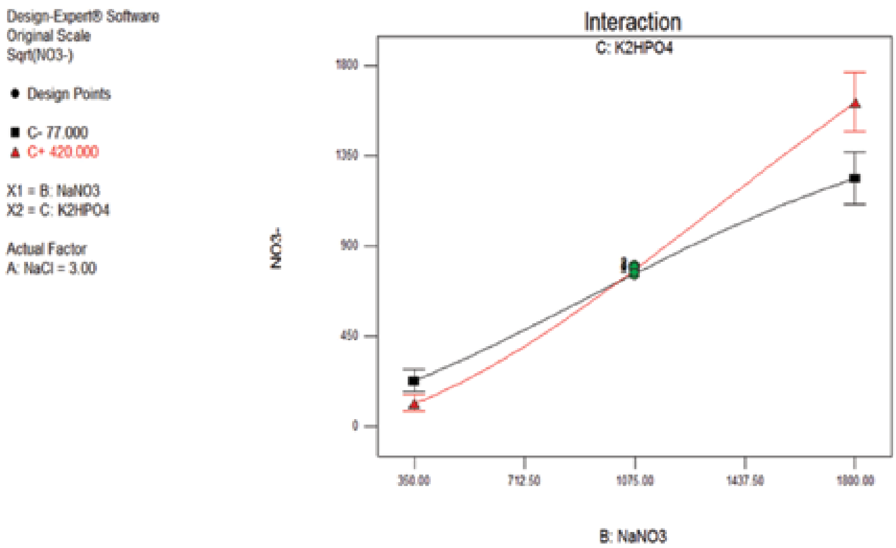

Fig. 5. The interaction of $NaNO_3$-K_2HPO_4 in removing nitrate by *F.muscicola*

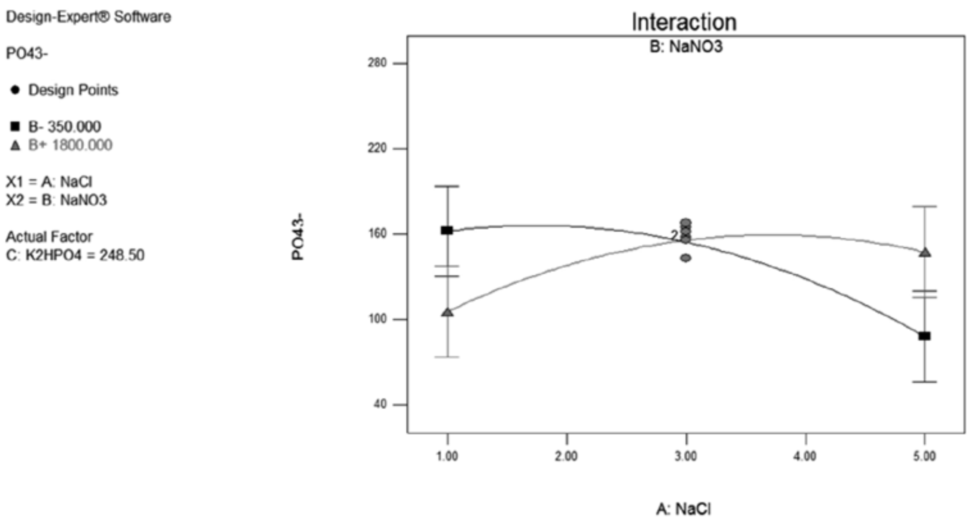

Fig. 6. The interaction of NaCl-$NaNO_3$ in removing nitrate by *F.muscicola*

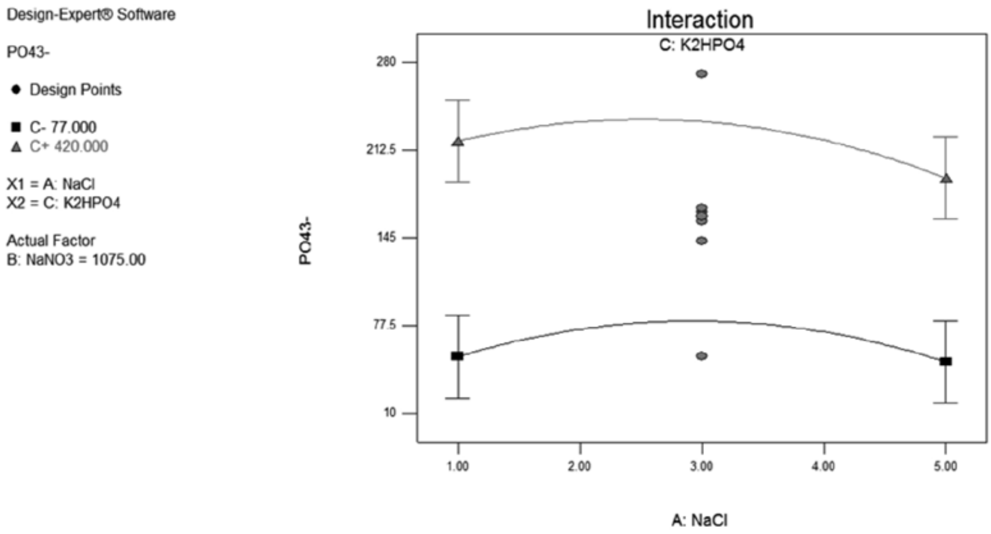

Fig.7. The interaction of NaCl-K_2HPO_4 in removing phosphate by *F.muscicola*

Discussion

Results of growth and chlorophyll contents of *F.muscicola* in wastewater treatments and analysis of Design-Expert revealed the critical role of NaCl for this specimen's growth and chlorophyll production. Because among the tested factors, only NaCl negatively affected these parameters.

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 the maximum growth and chlorophyll were observed in NaCl 0.1 and 1 %. Also, the results of Iranshahi et al. (2014) on *Nostoc* and *Anabaena* sp. in various salinities indicated the maximum growth of both specimens in NaCl 1%. Therefore, it can be concluded that except for some specimens like *Spirulina platensis* (Zhou et al., 2017) and *Actodesmus obliquus* (Kim et al., 2016), most microalgae such as *Fischerella* sp. had their maximum growth and chlorophyll contents in NaCl 1 % or less.

As elements of cultural media usually interact, their study in terms of their effectiveness for desired results is essential. According to the results, the interaction of NaCl-K_2HPO_4 and $NaNO_3$-K_2HPO_4 had an influential role in the growth of *F.muscicola*. Research by Ajala and Alexander(2020) on *Chlorella vulgaris*, *Scenedesmus obliquus,* and *Oocystis minuta* in wastewater supplemented with various amounts of $MgSO_4$, $NaNO_3$, and K_2HPO_4 (Case1, Case2, Case3, Case4) showed that all 3 species had maximum growth in Case3 ($NaNO_3$ 377, K_2HPO_4 104, $MgSO_4$ 793 mg L^{-1}) in the 14^{th} day of culture which is similar with run 14. So, it can concluded that increased nitrate and phosphate concentration in growth media up to a specific limit has resulted in a corresponding increase in the growth rate of microalgae. However, the increase in sulfate concentration was probably responsible for the negative effect on microalgal growth. As nitrate and phosphate had no effect separately on growth, it can concluded that the presence of three elements (NaCl, $NaNO_3$, and K_2HPO_4) together with particular concentrations in culture media have a significant impact on growth and synthesis of pigments like chlorophyll.

Results of removing nitrate from the culture showed that most runs, even with high concentrations of NaCl (3 and 5 %), could decrease nitrate. This result was compatible with the results of the interaction of NaCl-K_2HPO_4 and $NaNO_3$-K_2HPO_4 that showed the most reduction potential ($NaNO_3$ 350, K_2HPO_4 77 mg L^{-1}, run 14) and ($NaNO_3$ 350 K_2HPO_4 420, run 3). Results of nitrate removal by *C. vulgaris*, *S. obliquus,* and *O. minuta* in wastewater supplemented with various amounts of $MgSO_4$, $NaNO_3$, and K_2HPO_4 (Case1, Case2, Case3, Case4) showed that *C. vulgaris* had the most nitrate reduction of culture in Case 3. In this case, Ahmed et al. (2019) reported 100 % nitrate reduction by *S.obliquus* in municipal wastewater. Also, Guldhe et al. (2017) reported that 76% of nitrate removal with *C. sorokiniana* was done in aquaculture wastewater. Therefore, the importance of NaCl, $NaNO_3$, and K_2HPO_4 interaction in nitrate removal was defined. According to the results, the potential of nitrate removal relates to growth in the same manner.

Phosphate reduction was also observed in runs with the highest growth rate (2, 6, and 14) and the maximum rate (17%) observed in run 14, which was compatible with the results of interaction elements in culture. Research by Ajala and Alexander (2020) in phosphate reduction by *C. vulgaris*, *S. obliquus,* and *O. minuta* in wastewater supplemented with various amounts of $MgSO_4$, $NaNO_3$, and K_2HPO_4 (Case1, Case2, Case3, Case4) showed that *O. minuta* had the maximum reduction of phosphate in Case 3(the same condition as run14). In this case, research by Yadav et al.(2018) showed that *C.vulgaris* had phosphate consumption of about 55% initial concentration after 15 days of culture in sewage wastewater. Also, the findings of Ahmed et al. (2019) showed that *S.obliquus* could remove 94% of phosphate from municipal wastewater.

According to the research, microalgae can potentially remove nitrate and phosphate from wastewater. This ability depends on the wastewater condition, such as culture media and specimens. Our results showed that *F.muscicola* ISC123 is a potent cyanobacterium that can grow in this media and consume elements. The presence of 3 elements, NaCl, $NaNO_3$, and K_2HPO_4, have a critical role in this case. As eliminating nitrate and phosphate depends on the growth condition, it can be concluded that studying wastewater conditions, their supplement with critical elements, and optimizing them are essential for microalgal growth and activities as treatment agents. In this case, run 14 is the optimum culture condition for growth, chlorophyll contents, and removing nitrate and phosphate for *Fischerella muscicola* ISC 123.

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