Caspian J. Env. Sci. 2010, Vol. 8 No.2 pp. 109~114

CJES
Caspian Journal of Environmental Sciences

[Research]

The independent effects of ferrous and phosphorus on growth and development of *Tetraselmis suecica*; an *in vitro* study

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ABSTRACT
Five treatments including Conway medium, media containing 0.1, 0.17, 0.3 and 0.5 mg l^{-1} ferrous (Fe; in the first experiment), media with 1, 1.26, 1.59, 2 mg l^{-1} concentrations of phosphorous (P; in the second experiment) and a pure sample of *Tetraselmis suecica* were cultured under laboratory conditions. Growth rate of the algae was determined using the optical density method at 750 nm and number of algal cells were counted with a hemocytometer. The results of the first experiment showed that the amount of Fe for maximum growth of this species was 0.3 mg l^{-1}, while Fe concentration in Conway medium was 0.27 mg l^{-1} ($P>0.05$). The results of second experiment showed that 1.59 mg l^{-1} P caused the maximum growth of algae which was not significantly different from that of the control medium (with 1.6 mg l^{-1}; Conway; $P>0.05$). These results showed that due to the lack of significant differences in maximum growth of this alga recorded in 0.3 mg l^{-1} Fe and that recorded in 1.59 mg l^{-1} P in Conway medium, increase or decrease of these doses will have a significant negative effect on algal growth.

Keywords: *Ferrous, Phosphorus, Tetraselmis suecica, Conway medium, Growth.*

INTRODUCTION
Mono-cellular algae are autotrophic organisms that are the foundations of food chains in aquatic ecosystems and have a strong ability for growth and development (Halama, 1990; Phany, 1992). These algae, which contain high amounts of protein, lipid, carbohydrate, minerals and vitamins, are the main food items of many aquatic animal species in their larval and even in their adolescent stages (Brown, 2002).

Tetraselmis suecica is one of the species of Prasinophyceae that has a high growth coefficient and is widely used for feeding Penaeid shrimps, Mollusca larvae, *Artemia* and rotifers (Kawamura et al., 1988; Lavens and Sorgeloos, 1996). Other main consumptions of these algae include the extraction of vitamins, amino acids, antibiotics and refining of industrial wastes as well (Sym and Pienaar, 1993).

The elements that form the tissues of mono-cellular algae quantitatively are two groups including the macro and micro nutrients such as ferrous (Fe) and phosphorus (P) which although are quantitatively very low, are very important for biological activity of the cell and are usually observed as ions in protoplasm structure.

Fe is one of the most important microelements that are necessary for algae growth. Fe is involved in enzymatic activities and is present in the molecular structure of cytochrome (Round, 1996). Fe deficiency interrupts chlorophyll synthesis and photosynthesis rate; however its over-dosage also decreases primary production in aquatic ecosystems (Stanley, 1987).

P is present in aquatic systems in different forms which are directly absorbed by algae as ortho-phosphate (Raymont, 1980). Low levels

of P could limit the growth of many algae species.

The main objective of this research is to determine the effects of different concentrations of Fe and P on growth rate of *Tetraselmis suecica in vitro* and finally to identify the optimum dosage for the maximum growth of this algae as one of the most important used algae in aquaculture, and to compare the optimum dosages with the concentration of these elements in Conway medium.

MATERIALS AND METHODS

Medium preparation and algae culture

Tetraselmis suecica medium was prepared using AQUACOP (1984) method to make the specific medium of Conway. One liter solutions of chemical compounds demonstrated in Table 1 were prepared and then mixed and sterilized. The mixture of chemical compounds was added to filtered sea water filtered by 0.2 µ filter and distributed to 250 ml Erlen Meyer flasks to culture the algae. Pure algae cells cultured and purified in Tarbiat Modares University lab were added to the medium on the basis of 1 mg l^{-1} mass weight (Piri and Ordog, 1997).

Cultured algae were placed on glass tables provided with thirteen, 20 W fluorescent lamps (60 cm in length) at a distance of 15 cm from the glass surface. The light intensity was 2500 ± 100 lux and was automatically regulated for 14L:10D (Robert and James, 1977). In this system, CO_2 was provided by air pumps, and pH and temperature were maintained at 7.6 ± 0.6 and 25 ± 2 ^oC, respectively.

Table 1. Chemical compounds utilized in Conway medium preparation

Chemical compound	Concentration
KNO_3	116 g
NaEDTA	45 g
H_3BO_3	33.6 g
$MnCl_2.4H_2O$	0.36 g
$ZnCl_2$	2.1 g
$CoCl_2.6H_2O$	2 g
$(NH_4)_6MoO_7.4H_2O$	0.9 g
$CuSO_4.H_2O$	2 g
Vitamin B_1	200 mg
Vitamin B_{12}	100 mg
$NaSiO_3$	20 g
$Na_2H_2PO_4.2H_2O$	20 g
$FeCl_3.6H_2O$	1.3 g

Determination of Fe concentration

In each step of the experiment, different concentrations of Fe were measured logarithmically and added to the treatment as $FeCl_3$+$6H_2O$. Finally, 4 treatments and 3 replicates of 0.1, 0.17, 0.3 and 0.5 mg l^{-1} concentrations were analyzed within 96 h and compared with control medium (Conway) containing 0.27 mg l^{-1} Fe. These concentrations were selected according to requirements of algae for culture (Burlew, 1953; Soeder, 1981; Fulks and Main, 1991; Laing and Verdugo, 1991).

Determination of P concentration

Determination of optimum concentrations for each treatment was also obtained logarithmically. Different concentrations (1, 1.26, 1.59 and 2 mg l^{-1}) of P as NaH_2PO_4 were used with 3 replicates for each treatment and 1.6 mg l^{-1} P as NaH_2PO_4 was used as control. Culture conditions were kept similar for all of treatments within 96 h. These concentrations were selected according to requirements of algae for culture (Burlew, 1953; Soeder, 1981; Fulks and Main, 1991; Laing and Verdugo, 1991).

Algae counting and determination of light intensity

To determine algae growth rate, two methods were used in each experiment. At first, in two stages of before start and after 96h, the mean cells number of algae per treatment was measured using hemocytometer (×40) in each experiment (Hansen, 2000). Also the light absorbance rate was determined by spectrophotometer (Jenway, 1350 model) at 750 nm wavelength and the differences of cell density in each treatment was obtained.

Statistical analysis

After checking the homogeneity with kolmogorov-Smirnov test, one way analysis of variance (ANOVA) was used to check for differences among treatments. When significant differences were observed, Duncan Multiple Range Test (DMRT) was used utilizing SPSS software (SPSS 12.1, Chicago, IL) to identify the differences at level of $P<0.05$. Data are presented in mean ± standard deviation (SD).

RESULTS

First experiment

The results of average percentage of *Tetraselmis suecica* cell mass increase in different treatments of Fe are presented in Table 2. As shown, the highest and lowest average percentage increase were observed in 0.3 mg l^{-1} and 0.1 mg l^{-1} concentrations, respectively indicating significant differences between treatment groups and control ($P<0.05$). In all three other doses, the average percentage of cell mass increase were higher than control group ($P<0.05$). Besides this, treatment with 0.3 mg l^{-1} of Fe showed higher cell mass increase compared to control, this difference was also significant ($P<0.05$).

Table 2. Different concentrations of Fe and the rate of cell mass growth in *Tetraselmis suecica*

Fe concentration (mg l^{-1})	First numbering (cell ml^{-1})	Second numbering (cell ml^{-1})	Cell mass increasing percentage after 96 h
0.1	15833.3 ± 152.7	162266.7 ± 3370.9^{c}	1024. 9 ± 24.2^{d}
0.17	16066.7 ± 378.6	210333.3 ± 14020.8^{b}	1309.5 ± 91.9^{c}
0.27 (Conway)	15400 ± 818.5	285420 ± 240.2^{a}	1856.9 ± 99.2^{a}
0.3	15333.3 ± 763.8	285766.7 ± 809.8^{a}	1866.7 ± 90.9^{a}
0.5	14900 ± 600	219816.7 ± 6517.3^{b}	1476.6 ± 66.6^{b}

Results of light absorbance rate showed that increasing Fe concentration from 0.1 to 0.3 mg l^{-1} enhanced the light absorbance rate from 0.0103 to 0.0247 (Fig. 1). The variations in cell density on the basis of light absorbance revealed no significant difference between 0.3 mg l^{-1} and control ($P>0.05$), but this difference was significant between the groups with 0.1, 0.17 and 0.5 mg l^{-1} Fe and the treatment group with 0.3 mg l^{-1} and control group ($P<0.05$). Also, the increase of Fe concentration to 0.5 mg l^{-1} decreased light absorption rate which was considerably different from that in 0.17 and 0.27 mg l^{-1} concentration ($P<0.05$).

Second experiment

The percentage increase of cell density in *Tetraselmis suecica* was significantly different ($P<0.05$) with different concentrations of P in the medium (Table 3).

Table 3. Different P concentrations and cell mass growth rate of *Tetraselmis suecica*

P concentration (mg l^{-1})	First cell count (cell ml^{-1})	Second cell count (cell ml^{-1})	Cell mass percentage increase after 96 h
1	21533.3 ± 217.3^{d}	208066.7 ± 2060^{d}	966.4 ± 18.3^{d}
1.26	21900 ± 100^{c}	383266.7 ± 6671.1^{c}	1750.1 ± 33.7^{c}
1.59	24166.7 ± 152.7^{a}	543466.7 ± 2742.9^{a}	2248.9 ± 20^{a}
1.6 (Conway)	24266.7 ± 57.7^{a}	542333.3 ± 2538.4^{a}	2234.9 ± 12.9^{a}
2	23366. 7 ± 321.4^{b}	432466.7 ± 737.1^{b}	1851 ± 23.3^{b}

The maximum percentage increase of cell mass was observed in 1.59 mg l^{-1} P and the lowest value was observed in 1 mg l^{-1}. Therefore, increasing P concentration to 2 mg l^{-1} reduced the percentage increase of cell mass to about 1851 %.

The values related to the mean absorption in different dosages of P showed significant differences among treatments (Fig. 2). So, utilizing 1 and 1.6 mg l^{-1} P will increase the absorption rate, with the highest absorption recorded in the treatment with 1.6 mg l^{-1} P.

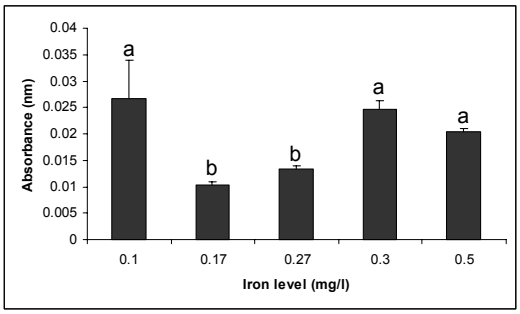

Fig 1. The effect of different Fe concentrations on absorption rate by *Tetraselmis suecica*

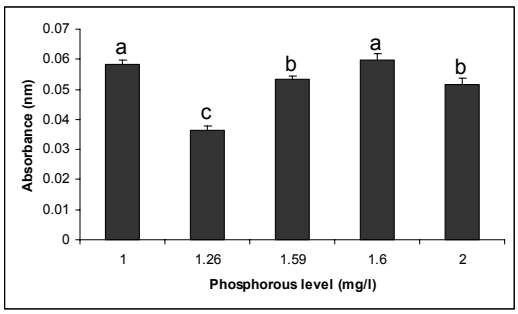

Fig 2. The effects of different concentrations of P on absorption rate by *Tetraselmis suecica*

DISCUSSION

The results of using different concentrations of Fe and P on growth and development of *Tetraselmis suecica* showed that the highest growth rate was observed in treatment with 0.3 mg l^{-1} Fe and 1.59 mg l^{-1} P that did not differ significantly with the concentration of these elements in the Conway medium. Therefore, with regard to concentrations of Fe and P, this medium could specially be used for the culture of this alga.

The commercial culture of microalgae is now over 40 years old with the main microalgal species grown being *Chlorella* and *Spirulina* for health food, *Dunaliella salina* for b-carotene, *Haematococcus pluvialis* for astaxanthin and several species for aquaculture (Borowitzka, 1999). Thus, for better growth in nutrient-rich medium some special media were adjusted for each type of micro alga according to its nutrient requirements. Since Conway medium is a general medium for the growth of marine algae and the amount of its microelements have been determined for the growth of various algae, the improved Conway medium in which the amounts of other elements are determined could be studied for individual species of algae. P is a limiting factor in water (Stryer, 1998) and the results of this study also confirm that its deficiency causes growth reduction and concentrations higher than 1.6 mg l^{-1} could have similar effects on the growth of *Tetraselmis suecica.*

Hang (1996) demonstrated the role of algae size in phosphate absorption rate that there is a relation between algae size with absorption and photosynthesis. Maximum phosphate absorption would be in nanoplanktons like *Tetraselmis suecica.*

The effects of Fe on growth and development of *Tetraselmis suecica*, like the effects of P, has an optimum concentration in that at concentrations more or less than 0.3 mg l^{-1} Fe, growth reduction occurs.

Therefore, with respect to the obtained results, it is suggested that firstly for *in vitro* culture of *Tetraselmis suecica*, Conway medium could be used with improved values of P and Fe. Secondly, since Conway medium is a general medium for other species of algae, the quantitative changes of Fe and P must be studied. Also other microelements have to be investigated for other important groups of algae to obtain the best concentrations for maximum growth which means the decrease of culture costs. The results of this study showed that since Fe and P are essential for algal culture, an increase or decrease from their optimum values could have negative effects on growth and development of algae. Therfore, Conway medium has proved to be more satisfactory than other complex media which have different concentrations and materials for isolation and growth of *Tetraselmis suecica*.

ACKNOWLEDGMENTS

We would like to thank the staff of the Fisheries Department of the School of Natural Resources and Marine Sciences in Tarbiat Modarres University who provided us with the necessary facilities. We also thank Leila Matindoost for preparing the manuscript.

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(Received: Sep. 30-2009, Accepted: Feb. 10-2010)

اثرات مستقل آهن و فسفر بر رشد و نمو جلبک *Tetraselmis suecica* در محیط آزمایشگاهی

ه. ارشاد لنگرودی، م. کمالی و ب. فلاحتکار

چکیده

پنج تیمار مختلف شامل محیط Conway و محیط های کشت حاوی ۰/۱، ۰/۱۷، ۰/۳ و ۰/۵ mg/L آهن در آزمایش اول و محیط های کشت حاوی ۱، ۱/۲۶، ۱/۵۹ و ۲mg/L فسفر در آزمایش دوم به روی نمونه های خالص *Tetraselmis suecica* در محیط آزمایشگاهی آزمایش گردید. نرخ رشد جلبک با استفاده از جذب نوری در طول موج ۷۵۰ nm و شمارش سلول ها با استفاده از لام هماسیتومتر تعیین گردید. نتایج آزمایش اول نشان داد حداکثر رشد این گونه در غلظت آهن ۰/۳ mg/L بوده در حالیکه غلظت آهن در محیط کشت Conway برابر ۰/۲۷ mg/L می‌باشد ($p >$ ۰/۰۵). نتایج آزمایش دوم نیز بیشترین میزان رشد را در مقدار ۱/۵۹ mg/L فسفر نشان داد که اختلاف معنی داری با غلظت این ماده به میزان ۱/۶mg/L در محیط کشت کنترل (Conway) نداشت ($p >$ ۰/۰۵). این مطالعه نشان داد بدلیل عدم وجود اختلاف معنی دار در حداکثر رشد این جلبک در مقادیر ۰/۳ mg/L آهن و ۱/۵۹ mg/L فسفر با محیط کشت Conway، افزایش یا کاهش مقادیر این دو ماده غذایی در محیط های کشت، اثر منفی بر رشد خواهد گذاشت.

کلمات کلیدی: آهن، فسفر، *Tetraselmis suecica*، محیط کشت Conway، رشد