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RESEARCH ARTICLE

Comparison of salinity effects and medium of TMRL, Gillard and Conway cultivations on density and bloom of *Chlorella vulgaris* alga in vitro

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Abstract

In order to compare the effect of cultivation medium and salinity on the growth process of *Chlorella vulgaris* alga, we used a factorial design 3 × 4 (4 is salinity level, 15, 20, 25 and 30 ppt and 3 types of TMRL cultivation medium, Gillard and Conway). In these experiments, 12 treatments were compared. All of treatments had three repeats. The experiments lasted 12 days. According to the results, the most density was for the Gillard at salinity of 25 ppt with average of $125/30\times10^6$ $\pm0/87\times10^6$ cell/ml and the lowest density was for Conway in salinity of 15 ppt with average of $37/18\times10^6$ $\pm1/39\times10^6$ cell/ml, and there was a significant difference between them ($p<0/05$). Also the most growth rate in the first day was to the tenth was for Gillard with average 0/536 ±0/5 at salinity of 20 ppt and the lowest of it was for Conway with average of 0/401 ±1/39 at salinity 15 ppt, and a significant difference was seen ($p<0/05$). This significant difference was also between Gillard, TMRL and Conway ($P<0/05$). So to produce biomass and high density of *Chlorella*, salinity of 15 ppt and 20 ppt was better than the other salinities and Gillard cultivation medium at 200 ppt provides better conditions than others to produce a higher and better *Chlorella* in vitro; but at out cultivation conditions, TMRL medium at salinity of 20 ppt can be better than Conway medium.

INTRODUCTION

Microalgae are the most important organic producers at aquatic environments, and as the first loop of food chain in aquatic ecosystems that due to having chlorophyll are able to do photosynthesis, and via photosynthesis they produce food and energy and change some form of non-eatable energy into eatable one. Some types of microalgae are used for industrial aims and to remove organic materials from sewage (Huguenin, 1974). Since the plants are able to synthesize long- chain polyunsaturated fatty acids, we can say that microalgae supply these vital combination and are the first source of Poly Unsaturated Fatty Acids (PUFA) for all of the creatures of aquatic food chain (Pulz and Gross, 2004).

Microalgae as the food source are necessary for all of commercial breeding stages of various marine fish species including bivalves and mollusks and larval stages of some of the crustaceans and fish. In addition to this, phytoplanktons are used to mass producing Zooplanktons (rotifer, copepoda and Artemia) that are used as food for larvae and the first stages of crustaceans and fish. Also they are used to breed larvae of the marine fish via green water technique that is a common technique (Lavens and Sorgeloos, 1996).

At the aquaculture industry, all stages of shell growth and larval stages of crustaceans and fish directly depend on live food such as microalgae. So generating the microalgae is one of important activities at duplication workshops and subsequently the growth of the species under cultivation (Cho et al., 1999). Physiology of microscopic algae are aggected by the physical and chemical factors such as water temperature, salinity, light intensity, pH and the concentration of nutrients (Thomas, 1975). *Chlorella* is considered a suitable species as a food source; because the species when dried contains high levels of protein and minerals are essential. 45% protein, 20% fat, 20% carbohydrate, 5% fiber, 10% minerals and vitamin have been reported (Belasco, 1997). Alga *Chlorella vulgaris* is widely used at photosynthetic studies, mass cultivation experiments and clearing the waste water of cities. This alga is duplicated very fast and is rich in B vitamins (Belasco, 1997). Now one of the possible solutions to preserve and enhance the nutritional value of microalgae is simple production method and reducing the production time of them (Pulz and Gross, 2004). The purpose of this study was to develop and provide reliable methods as the cultivation medium and good environmental conditions such as salinity of algae species; that specially supplies the food needs of shrimp and fish hatchery and other aquatics. Via recognition of behavior of a species affected by special conditions such as salinity that mass cultivation of duplicable algae at high density can be kept (Affan et al., 2007). Based on the foregoing, the need to identify suitable cultivation medium or suitable salinity has a decisive role in improving live food operations and production in aquaculture industry.

2.Material and methods

-Early preparation of the required tools to cultivate *C. vulgaris*:

First of all accessories required for the cultivation of microalgae, including glass containers are washed with water and detergent and then are dried; all of the containers get full of sea water with the salinity required for experiment and the doors of them are closes and get sterile at the autoclave at 121 C for 20 min at pressure of 1.5 of atmosphere.

- Preparation of water with different salinity:

To prepare water with different salinity, firstly the sea water after filtration is stored at a reservoir and is passed from the filters 1, 5 and 20 micron; thus it is passed from the UV system so that it gets free of any suspended and microbial material. Then the salinity of the sea water is measured and recorded, then depending on the volume of the container and the salinity rate of sea water the required salinity for cultivation medium is calculated using Formula 1 (Grimes, 2002).

Formula 1: $C_1V_1=C_2V_2$

$C_{1:}$ the used salinity for cultivation medium

V1: the desirable water volume

C2: sea water salinity

V2: the required volume for sea water

-Preparing Microalgae and testing:

Microalgae *Chlorella vulgaris* required for this study was prepared from persian gulf and oman sea ecology research institute of Bandar Abbas and the related tests were done at cultivation laboratory of microalgae, section of duplication and aquaculture.

- Preparing and cultivation medium:

In this experiment, three media of Conway (Wallen modified form), and TMRL and Gillard were used. 3 medium was prepared initially in stock form. In this regard, firstly the distilled water at the amount required according t literature recipe was prepared and poured into the container; they were then sterilized in an autoclave. After cooling, the minerals needed to produce each type of medium was added to the water, and then poured into dark sterile containers with lids, and kept in the refrigerator.

- Doing tests:

To study the effect of cultivation medium and salinity on growth of microalgae of *Chlorella vulgaris*, a factorial design 3 × 4 (4 levels of salinity, 15, 20, 25, and 30 ppt and 3 types of medium of TMRL, Gillard and Conway) were used. A total of 12 treatments and 3 replications for each treatment, a total of 36 500-ml flasks were used. The experiments lasted for 12 days. After the salinity of sea water was prepared and sterilized, was transferred to the flask of 500 cc, and the required amount of medium was added to each of the treatments; then based on the size of the container, *Chlorella* with an initial density of one million in cc were added to the flasks. All flasks under identical conditions against light of 3500-5000 lux with gentle aeration, alkalinity 8 and a constant temperature 24± 1 °C and a photoperiod of 12: 12h (light to dark) were exposed for 12 days (Fig. 7). Every other day, 1 ml of each container was taken and fixed with 4% formalin solution, and the cells were counted using a hemocytometer slide.

- Estimating algal cell density and specific growth rate

Chlorella algae cell density was calculated by counting *Chlorella* cells with hemocytometer slide. Cell counts were performed using a magnification of 100 microscopes. To determine the growth rate, from the first day of cultivation, every other day 1 ml sample was taken from each flask and fixed with 4% formalin solution and the cells were counted using a hemocytometer slide. Counting process continued for 12 days, and all information related to the count was recorded. Then, using the formula No. (2) Specific growth rate was calculated:

Formula 2: $SGR = \frac{(\ln W_t - \ln W_0)}{T}$

Wo and wt are the number of phytoplankton at times between two sampling periods and counting (Nan et al., 2004; Schram et al., 2006).

3. Results

3-1- Impact of Conway medium at different salinities on *C.vulgaris* density:

The investigations showed that the most alga density at this medium, with average $69/78\times10^6$ cell/ml is related to salinity of 25 ppt, and the least density is at 15 ppt with average $37/18\times10^6$ cell/ml, and there was a significant difference ($p< 0/05$)(Fig 1).

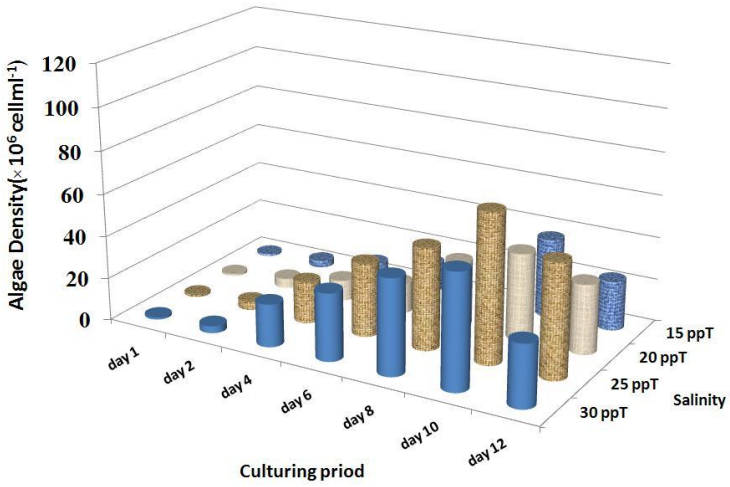

Figure 1: Impact of Conway medium at different salinities on *C.vulgaris* density

3-2- Impact of TMRL medium at different salinities on *C.vulgaris* density:

The investigations showed that the most alga density at this medium, with average $66/63\times10^6$ cell/ml is related to salinity of 20 ppt, and the least density of *Chlorella* is at 25 ppt with average $47/61\times10^6$ cell/ml, and there was a significant difference ($p< 0/05$)(Fig 2).

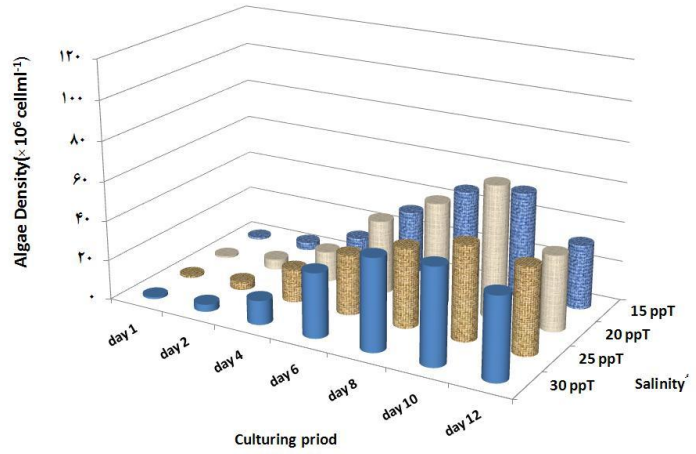

Figure 2: Impact of TMRL medium at different salinities on *C.vulgaris* density

3-3-Impact of Gillard medium at different salinities on *C.vulgaris* density:

The investigations showed that the most and best density at this medium, with average $125/30\times10^6$ cell/ml is related to salinity of 20 ppt, and the least density of *Chlorella* is at 30 ppt with average $10/62\times10^6$ cell/ml, and there was a significant difference between these treatments ($p< 0/05$)(Fig 3).

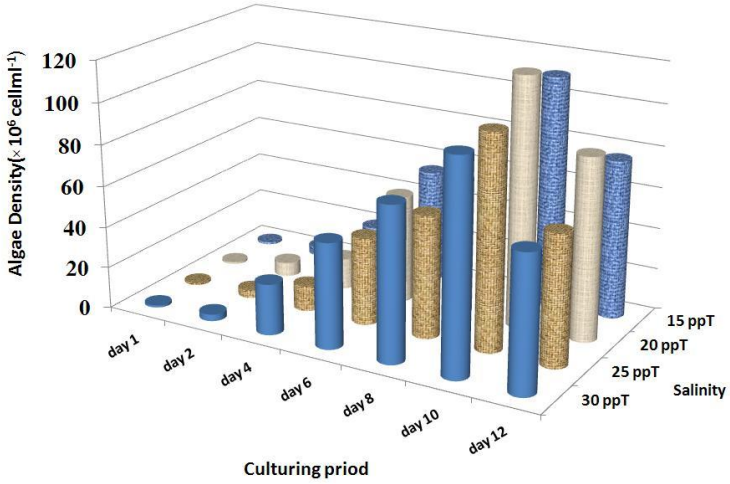

Figure 3: Impact of Gillard medium at different salinities on *C.vulgaris* density

3-4- impact of salinity 15 ppt at three media of Conway, TMRL and Gillard on *C.vulgaris* density :

The investigations showed that the most density of *Chlorella*, with average $111/23\ \times10^6$cell/ml is related to Gillard, and the least density with average $37/18\ \times10^6$ cell/ml is at TMRL medium, that there was a significant difference between these and the two other media ($p< 0/05$)(Fig 4).

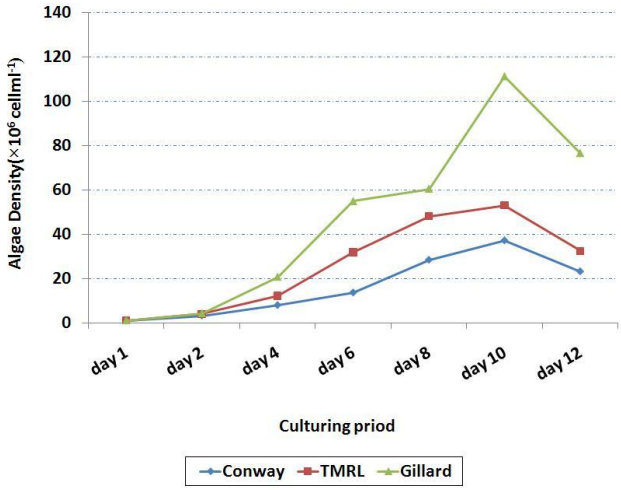

Figure 4: impact of different media at salinity 15 ppt on *C.vulgaris* density

3-5- impact of salinity 20 ppt at three media of Conway, TMRL and Gillard on *C.vulgaris* density:

The most density of *Chlorella* at 20 ppt with average 125/30 $\times 10^6$cell/ml is related to Gillard, and the least density with average 40/96 $\times 10^6$cell/ml is at Conway medium, that there was a significant difference between these and the two other media ($p < 0/05$)(Fig 5).

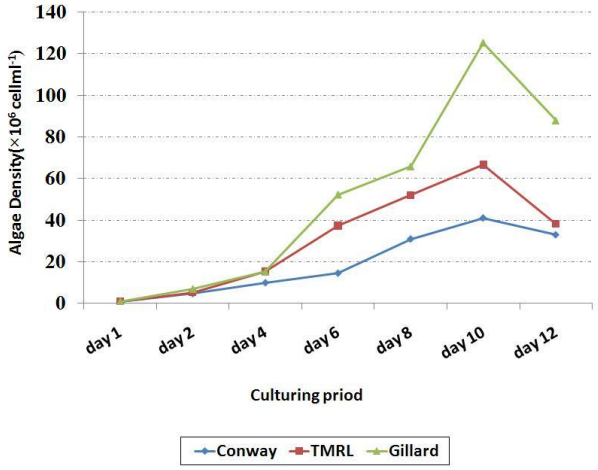

Figure 5: impact of different media at salinity 20 ppt on *C.vulgaris* density

3-6- impact of salinity 25 ppt at three media of Conway, TMRL and Gillard on *C.vulgaris* density:

The most density of *Chlorella* with density average 102/70$\times 10^6$ cell/ml is related to Gillard, and the least density with average 47/61 $\times 10^6$cell/ml is at TMRL medium, that there was a significant difference between these and the two other media ($p < 0/05$). Also, the density at Conway medium is with average 69/78$\times 10^6$(Fig 6).

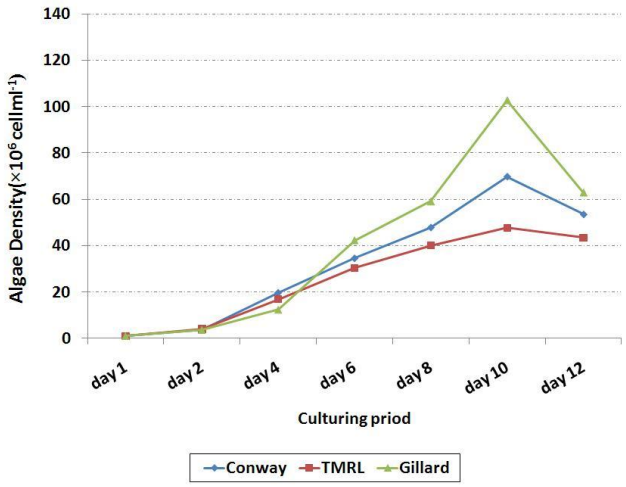

Figure 6: impact of different media at salinity 25 ppt on *C.vulgaris* density

3-7- impact of salinity 30 ppt at three media of Conway, TMRL and Gillard on *C.vulgaris* density:

The most density of *Chlorella* with average $101/62\times10^6$cell/ml is related to Gillard, and the least density with average $48/21\times10^6$ cell/ml is at TMRL medium. The Conway medium had the density of $54/01\times10^6$ but there was a significant difference between different media ($p< 0/05$)(Fig 7).

Figure 7: impact of different media at salinity 30 ppt on *C.vulgaris* density

3-8-special growth rate (SGR):

In this study, the results of unilateral analysis and variance showed that, the most SGR at time interval of first day to tenth day is for the Gillard medium with average 0/536 at salinity 20 ppt and the lowest was for Conway with average 0/401 at salinity 15ppt, that there was a significant difference between treatments ($p<0/05$). This significant difference was laso between Gillard, TMRL and Conway ($p<0/05$).
At salinity 25 ppt, the most SGR was for Gillard mediun with average 0/515 and the lowest was for TMRL, and there was a significant difference between them ($P<0/05$).
Also at salinity 15 ppt, the most SGR was for Gillard with average of 0/523 and the lowes SGR was for Conway with average 0/401, and there was significant difference between the media ($P<0/05$).

4.Discussion and conclusion

The results showed that the best density at salinity 20 ppt was for Gillard, and the lowest density was for Conway at salinity 15 ppt. according to the results, Gillard medium at all salinities was better than other media. However the appropriate density at the lower salinities was seen for *Chlorella* and this difference at low salinities of 15 and 20 ppt at Gillard medium was significant (p<0/05). (Figures 4 to 7)

In this regard, Cho et al. in 2007 studied the salinity effects and temperature on growth of species of *Chlorella ellipsoidea* and *Nannochloris oculata* at 4 levels of temperature)15, 20, 25 and 30 centigrade degree) and 3 levels of salinity (10, 20, and 30 ppt); the most SGR was for *Chlorella ellipsoidea* at 25 centigrade degree and salinity of 10 ppt and the most density at 15 centigrade degree and salinity of 10. For species of *N.oculata* also the most density was at 25 centigrade degree and salinity 10 ppt (Cho et al; 2007).

This study indicates that the species of *Chlorella* at lower salinities generates better density. Also the results showed that the most SGR is for Gillard culture at salinity 20 ppt. So these results are relatively compatible with the results of Cho et al. in 2007 and they suggest that the low salinity is for better growth rate and higher density of this species of *Chlorella*.

In 2013, Adenan et al. studied the impact of salinity and temperature on species of *Chlorella* sp. and *Chaetoceros calcitrans*, so that the best conditions for biomass production under the impact of these two factors are assessed. In this study, 3 levels of salinity (20, 25, 30 ppt) and 3 temperature levels (20, 25, and 30) were studied. According to the results of *Chaetoceros calcitrans* and *Chlorella* sp had higher growth respectively at salinities of 30, and 25 ppt (Adenan et al; 2013). This shows that better growth is at lower salinity for *Chlorella*.

At another study in 1986 by Gopinathan, the effect of culture media of TMRL, PM, Conway and F/2 (Gillard) on growth of 3 microscopic alga species of *Isochrysis galbana*, *Tetraselmis chuii* and *Nitzschia dosteritim* was investigated. According to the results, *Isochrysis galbana* alga had the most density at Conway medium. However for *Tetraselmis chuii*, the fast alga growth till the fourth day was seen to be under effect of PM culture medium; but the most density was under effect of TMRL medium and at the 7th day. For *Nitzschia dosteritim*, the best result was under the effect of PM culture medium (Gopinathan, 1986). These results can say that at present study also at chlorella, the Gillard ulture medium was better and then for more production, the TMRL can have less cost with better density than Conway.

At present study the results showed that the *Chlorella* growth process at lowe salinities in a 12-day period has been more appropriate and this process daily at Gillard medium is at most density than other media. Another study was done in 2010 by Mathad Hiremath on *Clorella vulgaris* at different salinities (20, 20, 30 and 40 ppt) at constant temperature 26±2 centigrade degree that showed at high salinities the chlorophyll and photosynthesis would be reduced and also there was the least growth rate. In this regard, the protein rate that was measured at high salinities was reduced with reduced photosynthesis, but the most amount of beta carotene was at salinity of 30 (Hiremath and Mathad, 2010).

At another study in 1991 by Latala, the impact of salinities (0, 2.5, 5, 7, 11.5, 25, 25, and 30 ppt) and different temperatures (و 35 ،32 ،29 ،26 ،23 ،20 39 15،18C) on growth rate and chlorophyll of 9 alga species was investigated. The results showed that use of high salinity and very low salinity would reduce the growth rate and chlorophyll. Also the best domain of temperature is between 20-26 centigrade degrees. Also the results showed the optimal growth of *Chlorella vulgaris* between 7/5-30 PPT (Latala, 1991).

The results of this study indicate the obtained results at the performed study and says that *Chlorella* alga has a good growth at salinity that is relatively low at 15 and 20 ppt and the best culture medium was at Gillard laboratory medium; and after that TMRL culture medium for the cultures out of laboratory that it is because of less cost.

At another study in 2009 by Mathad and Hiremath, the impact of different salinities at 14 levels and at different temperatures was studied on chlorophyll and beta carotene of *Chlorella saccharophila*. The results of this study showed that the good growth was between 15-35 ppt and the optimal was at 25 ppt, and at other salinities was very slow and at zero salinity and 60 ppt the growth was stopped. This is while that the optimal temperature for its growth was at temperature between 20-23 centigrade degree (Hiremath& mathad; 2009). In 1993 he showed in a study that with increase of salinity from 30 above, the growth rate and photosynthesis and also chlorophyll would be reduced (Ria&Abraham 1993).

We can conclude that the direction of biomass production and high density of *Chlorella* at salinity of 15 ppt and 20 ppt is better than other salinity levels and from the three culture media, the Gillard one has better condition for higher and better production of *Chlorella*, although at all of salinity levels, the Gillard culture medium had the best density and had created biomass, and there was a significant difference between the other two media. The Gillard culture medium was very good in vitro, but because of high cost of it at outside the laboratory environment is not affordable. So with respect to this that at low salinities there was a better density of *Chlorella* alga with use of TMRL, so TMRL culture medium can be used as the best culture medium for the cultures out of laboratory (mass production).

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