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Some Physical Parameters to Effect the Production of *Heamatococcus pluvialis*

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Abstract: The aim of this study is to optimize the physical parameters affecting the production of *Heamatococcus pluvialis* in photobioreactors and to simulate the process. *Heamatococcus pluvialis* is a green microalgea to have a great interest for production of natural astaxanthin and it can be cultivated in a closed photobioreactor system under controlled conditions. Biomass composition, growth rate and high value product spectra like polyunsaturated fatty acids, pigments, polysaccharides or vitamins depend on strongly the parameters of cultivation process. These are composition of cultivation medium, mixing model and aeration rate, hydrodynamic stress of medium which can be changed by adding some chemicals, cultivation temperature, pH, carbon dioxide and oxygen supply and most important of all: illumination. One of the most important problems during the cultivation is that cells have sensitivity to shear stress very much and the shear stress created by aeration and mixing effects the growth rate of the cell negatively and decreases yield. In this study, physical parameters such as; the rate of the air fed into the reactor, the mixing type, the reduction of the hydrodynamic stress by CMC addition, the effect of the cell size on the cell production and the flocculation speed of the culture, were investigated.

Key words: Photobiorector, microalgea, *Heamatococcus pluvialis*, hydrodynamic stress, CMC, aeration rate

INTRODUCTION

Microalgea has a great importance by producing of high value chemicals and their proven antiviral activities and by ecologically role in climate modeling as the biggest primary producers of oxygen worldwide (Csögör *et al*., 1999). Microalgea can synthesis themselves organic materials to need for cell activities by photosynthesis. Intermediate metabolites by photosynthesis are basically proteins, fatty acids, carotenoides, vitamins, antibiotics and lots of high value chemicals. *Heamatococcus pluvialis* is also a green microalgea grown autotrophically and it has a four cell type following each other along its life cycle. These are phase of microzoides, the phase of macrozoides with big flagelies, the phase of immobile the palmella form and the phase of hematokists having bigger red cells with thick cell membrane. As the culture medium having lower level concentration of nutrients during the cultivation, the number of cells in the palmella phase change their structure into the hematokist phase and astaxhantin accumulation in the cells follows this phase. Astaxhantin is a beta-carotenoid with red color and high value pigments to give pink color to bodies of lots of sea animals like salmon, shrimp or crab (Eriştürk and Akpolat, 2005).

During the cultivation of algae like *Heamatococcus pluvialis* the growth of the culture is determined by quantitative and qualitative methods. Qualitative methods are basically cell numbering, optical density, dry end wet weight, amount of chlorophyll a and directly measuring of carbon context (Boussiba, 2000; Eriştürk and Akpolat, 2005). Biomass composition, that means growth rate and product spectra depend strongly on the cultivation conditions. These are composition of medium, temperature, pH, carbon dioxide and oxygen supply and most important of all: illumination. Microalgeas as photosynthetic organisms need carbon dioxide and light within the Photosynthetically Active Radiation (PAR) to obtain energy. The wave length range of the PAR is between 400 to 700 nm, which is equal to visible light. Additionally it could be remembered that the use of excessive light and oxygen in cultivation has harm full effects. The radiation can be quantified adequately by the photon flux density (quantum of photons per area and time) (Csögör *et al*., 1999; Molina *et al*., 2001).

As to the chemical structure of astaxhantin, it has three esterificated isomers in three types of (3S,3'S), (3R,3'R) and (3R,3'S) although they have the same chemical composition in nature. Astaxhantin included in the cells of *Heamatococcus pluvialis* has the three types

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and the form of (3S,3'S) exists mostly in their mixture. The molecule structure of the astaxhantin being a carotenoid looks like that of beta-carotene very much but small differences between the structures of these molecules create big differences between their specifications chemically and biologically. Astaxhantin has especially more superior and different anti oxidative specifications *in vitro* experiments (Terao, 1989; Miki, 1991; Palozza and Krinsky, 1992; Lawlor and O'Brien, 1995).

As regards hydrodynamic stress, there are some references about that it has negative effects on cell growth and cultivation rate and microalgeas are sensitive to shear stress (Gudin and Chaumont, 1983; Merchuk and Wu, 2001; Barbosa *et al*., 2003a). Some other researchers mentions that the sheer stress is effected by mixing model and aeration and adding of CMC in some ratios into the culture medium can protect the cells from the harmful effects of aeration (Chisti, 2000; Bronnenmeier and Markl, 1982; Camacho *et al*., 2001). In some other works, it was suggested that one of the most important problems along the cultivation was cell precipitation and an anionic surface active material like Tween 20 was added into the medium for staying of the cells as suspension in the solution for longer time (Chou *et al*., 2005).

In this study carried out in the light of all these references, it was searched how to effect the physical parameters the cultivation of *Heamatococcus pluvialis* for the production of astaxhantin in panel glass photobiorector. These are the effect of the air fed rate into the reactor, the mixing model and the effect of the reduction of hydrodynamic stress and surface tension addition by CMC and Tween 20 into the medium, respectively (Eriştürk and Akpolat, 2005).

MATERIALS AND METHODS

This study explained as follows was conducted by the facilities of the laboratory in Bioengineering Department of Ege University at 2004.

Experimental set-up and culture medium: In this study, *Heamatococcus pluvialis* Flotow Strain U was used and the strain was isolated by Dr. Giuseppe Torzillo in Italian Institute of Ecosystem National Research Council (CNR). The experiments to be done in a panel glass photobioreactor of 1.5 L volume at 25°C room temperature were carried out under 75 μE m^{-2} sec^{-1} constant light flux by Orsam L brand (18W/10) daylight florescence at the vessel surface The width of the reactor is 5 cm and the air sending to the reactor consists of CO_2 of 1.5% V. The aeration rate was measured as 1.5 L min^{-1} by flow meter and *Heamatococcus pluvialis* strain U was used for inoculation in BG11 medium given in Table 1 (Sukatar, 2002). Strains stored in agar at carrying glasses was firstly inoculated on solid agar with BG11 medium and then incubated along 15 days. Later stock culture was prepared by transferring of inoculated strain into tube of 70 mL, flask of 250 mL and Pyrex glasses of 1 and 5 L, respectively.

Table 1: Composition of the culture medium

Chemical	Concentration (mg L^{-1})	Chemical	Concentration (mg L^{-1})
$NaNO_3$	1500	Na_2CO_3	20.00
K_2HPO_4	40	H_3BO_3	2.86
$MgSO_4.7H_2O$	75	$MnCl_2.4H_2O$	1.81
$CaCl_2.2H_2O$	36	$ZnSO_4.7H_2O$	0.22
Sitrik Asit	6	$Na_2MoO_4.2H_2O$	0.39
Ammonium ferric citrate	6	$CuSO_4.5H_2O$	0.08
EDTA-Na_2	1	$Co(NO_3)_2.6H_2O$	0.05

The sample of the volume to need for inoculation of the reactor medium was taken into sterilized flask firstly. After removing supernatant phase on the precipitated cells, fresh medium was added on the cells. This inoculated culture was grown along 4 days under 75 μE m^{-2} sec^{-1} light flux and 1 L min^{-1} aeration rate conditions. Starting culture is 4×10^4 cell numbers L^{-1} for all experiments to do as three parallels and reactors were sterilized by sodium hypo chloride solution of 1% w/v. Cell accounting, chlorophyll-a, absorbance measurements during the experiments were repeated at the same time interval for all cultivation and the results of measurements were supported by photographing.

During the sterilization all parts of the experimental set-up were washed by distilled water, the contacting of the reactor with the atmosphere was minimized steel covers and airing into the reactor was supplied by steel pipes with air stones at their ends. All the cultivating systems were sterilized under flame.

Measurements and analysis: All measurements and analyses done along the experiments are repeated for three times as parallel and their procedures were summarized in follows shortly.

- Microscopic cell counting was conducted by Olympus BO71 microscope (Model CH40RF200) using Neubauer Lam and nine squares of 1 mm^2 on Neubauer camera were divided in different shapes and the volume of used camera was 9×10^{-4} mL.
- Indication of chlorophyll a concentration was followed daily (Seely *et al*., 1972) and the samples of 5 mL volume taken from the cultures into tubes were firstly centrifuged at 3500 rpm for 10 min. Then, the supernatant phase on the surface of the mixture was removed and the precipitated cells in the tube were extracted by DMSO. After the

samples heating in a bath of 70°C their absorbance were measured at 672 nm by spectrophotometer. The chlorophyll a concentration was calculated by the equation of $E^{1}_{1\%} = 870$.

- The culture viscosities for the samples of 250 mL and at different rpm were measured by a Brookfield viscosimeter.
- The pH values for cultures were determined by a Sartorious pH meter with pH/ATC electrode using 1.5 mL HCl solution.
- The photographs for all visual data were taken by an automated photograph machine with Olympus BO71 microscope and manual Nikon COOLPIX 3100 digital photograph machine. The samples were fixed by the solution of lugol (I-KI) in trace amount.

Determination of growth rate: Growth rate for the cultivation process was calculated by the following equations (Becker, 1994).

$$\mu = \frac{\ln x_2 - \ln x_1}{\Delta t}$$

Where:

μ = Specific growth rate (h^{-1})
x = Cell concentration (cell number L^{-1})
t = Time (h)
Δ = Differences operator ($\Delta = t_2 - t_1$)

Doubling time of the cultivation process was:

$$D.T. = \frac{\ln 2}{\mu}$$

and x_2 is the maximum value of concentration reached for a production period.

Sampling: All samples were taken from the same points of the reactor for homogeneity. Culture was mixed well before sample taking to prevent precipitation of the cells on the bottom corners of the reactor.

RESULTS AND DISCUSSION

Present study examined the growth of *Heamatococcus pluvialis* during the cultivation process, it was investigated the physical parameters in the reactor how to effect the growth specifications of microorganism. That is why, it was studied the growth how to be effected by changing of aeration rate and mixing model, by changing shear stress and viscosity adding carboxy methyl cellulose (CMC) into the cultivation medium and by changing surface tension adding surface active agents like Tween 20 into the cultivation medium. All experiments with the starting culture of $4*10^4$ cell number L^{-1} were done in a panel glass photobioreactor of 1.5 L volume by using BG11 medium at 25°C room temperature under 75 $\mu E\ m^{-2}\ sec^{-1}$ constant light flux, the air sending to the reactor was consisted of CO_2 of 1.5% V and the aeration rate was measured as 1.5 L min^{-1} by flow meter.

The effect of the aeration rate and the sensitivity to the shear stress: The growth rate of the microalgea carried out at different aeration rates was evaluated by cell counting and chlorophyll a concentration measurements of the samples taken from the cultivation medium. Figure 1 and 2 show the effect of different aeration rates on the cell numbers and the chlorophyll a concentration for the cultivation process. As being examined the Fig. 1, it is seen that the cell growth for all culture systems increase from the second day on and they reach to the maximum values at the 7 day for the aeration rates of 0.5 and 3 L min^{-1} and at the 10 day for the aeration rates of 1 and 1.5 L min^{-1}.

As to compare the effect of different aeration rates on the cell growth, the highest cell number obtained for the system with the aeration rate of 1.5 L min^{-1} is 35×10^4 cell

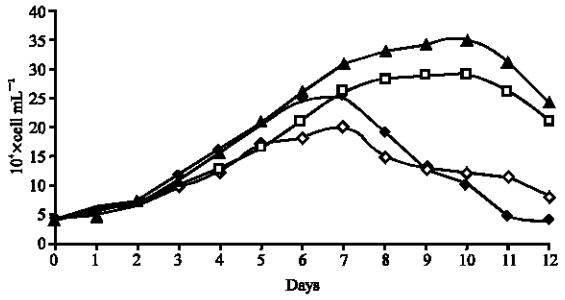

Fig. 1: The effect of different aeration rates on the cell growth (◇) 0.5 L min^{-1} (□) 1 L min^{-1} (▲) 1.5 L min^{-1} (♦) 3 L min^{-1}

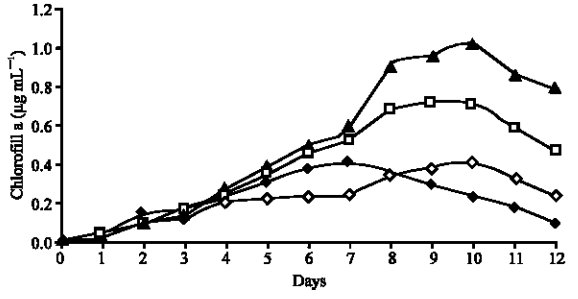

Fig. 2: The effect of different aeration rates on the chlorophyll a concentration (◇) 0.5 L min^{-1} (□) 1 L min^{-1} (▲) 1.5 L min^{-1} (♦) 3 L min^{-1}

number L^{-1} and it is 8.75 times of that of the starting culture value but the growth rates decrease instead of increase the growth decreases instead of increases for higher from the aeration rate 1.5 L min^{-1} because of the cell death formed with increasing of the effect of the shear stress. As being expected chlorophyll a concentrations lined by Fig. 2 have also the parallel results as those of the cell numbers showed by Fig. 1.

At some references it was emphasized that hydrodynamic stress has negative effects on cell growth and cultivation rate (Gudin and Chaumont, 1983; Merchuk and Wu, 2001) and microalgeas are sensitive to shear stress (Barbosa *et al*., 2003b). It also was stated that the growth rate for some microalgea species increases by increasing the turbulence at the beginning because of the light and CO_2 are used more effectively at that time but additionally it was seen that the growth rate decreases by increasing the aeration rate clearly in a optimum turbulence rate (Silva *et al*., 1987; Merchuk and Wu, 2001; Suzuki *et al*., 1995; Barbosa *et al*., 2003b). Silva *et al*. (1987) explained that the higher gas fed rate to the bioreactor has negative effect on the growth of *Dunaliella* and Contreras *et al*. (1998) also declerated that the production of *Phaeodactyylum tricornutum* was effected from hydrodynamic stress at higher flow rate. Chlorophyll a is a basic photosynthetic pigment and the following of the change in the Chlorophyll a concentrations during the cultivation of microalgea is important for their determination of the production (Fox, 1996). For that reason it was investigated four different aeration rates how to affect the cultivation of cell noticing the shear stress created by aeration rate in this study and the results of the study are in parallel those of the references.

The effect of mixing model: As seen the literature there are some references on the shear stress effects on the cell growth. One of them belongs to Merchuk and Wu (2001) and he says that the interaction between the structure of the cell and flow patterns in the culture medium are very complex and it was understood clearly not yet. It is exact that the cells are effected by the shear stress of the solution surrounded them. The most serious effect is being treated roughly or smashing of the cells and it is observed mostly some changings in synthesizes of metabolites and some decreasing in cell growth. According to some other researchers mechanical mixing addition to aeration causes cell damages and decreasing of growth rate especially by crushing of gases bubbles and there is a critical point on hydrodynamic stress related with microalgea growth (Chisti, 2000; Bronnenmeier and Markl, 1982).

In this part of the study, after the determination of the negative effect of the higher aeration rate on the cell growth it was studied on the effect of mixing aeration models on the growth. Figure 3 simulates these types of models and fish bait particles having the similar physical

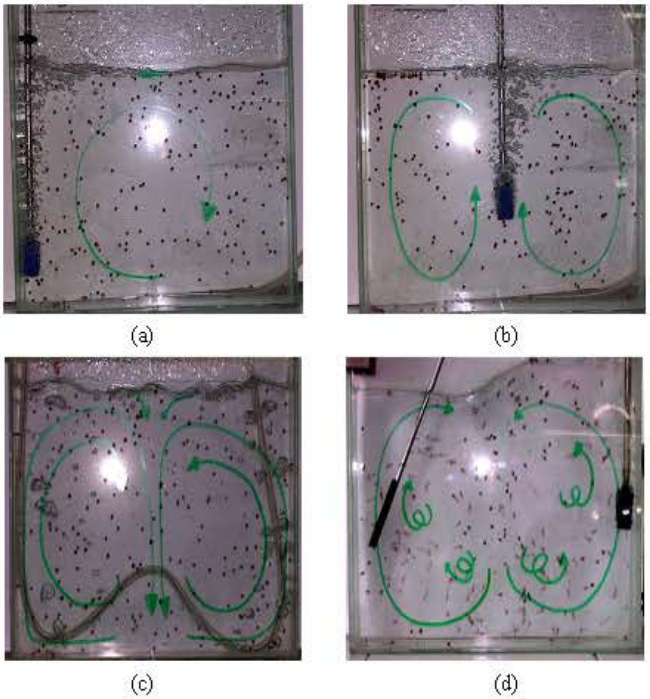

Fig. 3: Mixing model in the reactor (a) aeration from the one corner (b) aeration from the middle of the reactor (c) aeration by the loop pipe and (d) mixing by mechanical pedal

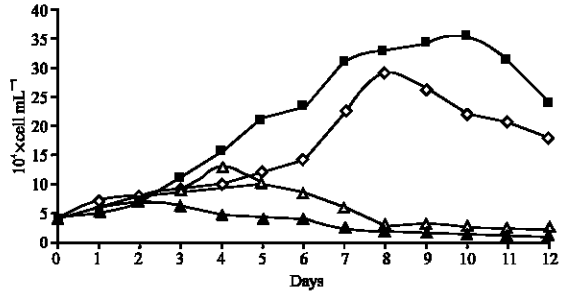

Fig. 4: The effect of different mixing model on the cell growth (◇) mechanic (+) aeration (■) aeration from the one corner (▲) aeration by the loop pipe (Δ) aeration from the middle of the reactor

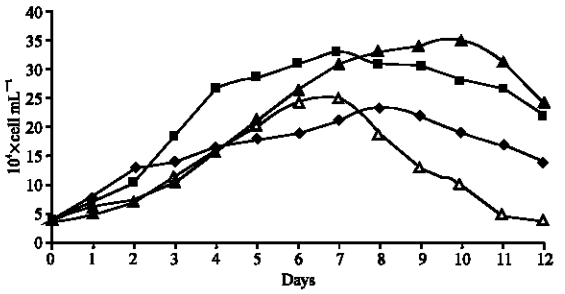

Fig. 5: The effect of adding CMC into culture in different rate on the cell growth for the aeration rate of 3 L min^{-1} (◇) 0.1% CMC (■) 0.05% CMC (Δ) Control (▲) 1.5 L min^{-1}

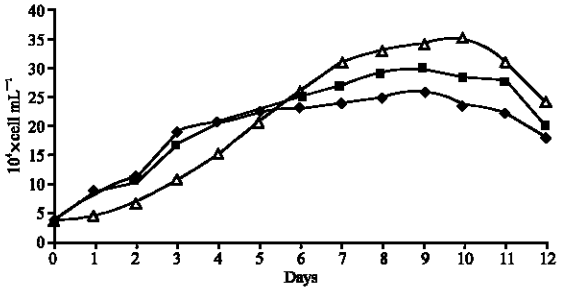

Fig. 6: The effect of adding CMC into culture in different rate on the cell growth for the aeration rate of 1.5 L min^{-1} (♦) 0.1% CMC (■) 0.05% CMC (Δ) Control

specifications with the cells, not precipitating on the bottom of the reactor liquid and not accumulating on the surface of the reactor liquid, was used to follow the motion in the medium created by the aeration in the reactor artificially. Four type mixing models in the reactor were experienced in the study and these are (a) aeration only from the one corner of the medium (b) aeration from the middle of the medium (c) aeration by the loop shape pipe sparger and (d) aeration together with mixing by a mechanical pedal.

It was evaluated the effect of different flow ways in the reactor for each model in terms of the cell growth and the sensitivity of the cells to hydrodynamic stress. As compared the models in the Fig. 3 it is understood clearly how and in which way to move the cells. Figure 4 gives the effect of different mixing model on the cell growth and shows that the model with the aeration only from the one corner of the medium supplies the best cell growth of 35×10^4 cell mL^{-1} for 10 day of the production period. For that reason this model with the most homogeneous and regular moves in circular shape was accepted the optimum in terms of its minimal harmful effects on the cells. Calculated specific growth rate for the aeration rate of 1.5 L min^{-1} with optimal result is 0.0094166 h^{-1}.

The protecting effect of CMCNa (carboxy methyl cellulose) on the cells: In the experiments to be done in this part of the study, carboxy methyl cellulose sodium salt (CMCNa) was added into the cultivation medium so that the cells to be damaged by hydrodynamic stress formed by the aeration and mixing would be affected minimal.

Figure 5 and 6 show the effect of adding of the CMC in different concentrations of 0.1 and 0.05% W/W into the medium for the aeration rates of 3 and 1.5 L min^{-1} (the optimum aeration rate). Being examined Fig. 5 and 6 carefully the adding of the CMC in different concentrations into the culture medium increases the cell number by 50 and 100% approximately, as the aeration rate 3 L min^{-1} decreases into that of 1.5 L min^{-1} (the optimum aeration rate) together with increasing of the viscosity. This topic was explained thus by different researchers in the literature.

One of them belongs to Contreras *et al.* (2001) and according to this study broken bubbles by aeration on the surface of the culture medium are main reason of the cells and adding of the CMC (>=0.02% W/W) into the culture medium protects the cells from the harmful effect of the aeration. In another work Camacho *et al.* (2001) shows that the adding of the CMC (>=0.02% W/W) into the culture medium has a protective effect of the cells against the hydrodynamic stress created by the aeration and CMC has a physical effect not a physiological effect. Wu (1995) declerated that some protective material could be used to prevent the cell damages related with the shear stress for animal cell cultures and these type of cellulose based materials effect the physical properties of the culture medium like viscosity and surface tension mostly. In another study, Barbosa *et al.* (2003b) said that cellulose based materials and dextrans were improved liquid viscosity and viscosity to be a property of the momentum

of liquid phase effects mass transfer coefficient (k_L) and interface area for unit volume (a_L). De Swaaf *et al.* (2001) and Elgozali *et al.* (2002) showed that increased viscosity decreases volumetric mass transfer coefficient (k_La) in the fermentor of 1 L volume and bubble column in their work.

It was cleared that increased bubble dimension related with decreased turbulence or in other words decreased specific interface area (a) was the reason of decreased mass transfer coefficient (Barbosa *et al.*, 2003a). As improved liquid viscosity decreases turbulence because of a strong friction resistance to flow the energy of eddies decreases and breaking of bubbles also decreases down. According to the Kolmogrov eddy length theory, eddy length (Λ_K) is proportional to kinematic viscosity (υ) linearly and energy dispersion (ε_T) inversely (Martens, 2005).

$$\Lambda = (\upsilon^3 / \varepsilon_T)^{0.25}$$

This theory says that increased kinematic viscosity increases eddy length and the theory accepts that the cells don't dead over any critical eddy dimension. The theory can explain how to increase the adding surface protective materials viscosity and how to protect the cells. Some surface active materials like pluronik poliols and polietilen glikols protect the cells by decreasing surface tension (Barbosa *et al.*, 2003b).

Surface active materials decrease the dynamic interface tension and the bubbles can not carry the cells up because of the changes of the surface, so less amount of the cells are transferred on the surface of the medium where crushing of the cells and so that the cells are damaged less (Camacho *et al.*, 2001).

In another work adding of the CMC into the culture medium increases bubble dimension and crashing of the bigger bubbles on the surface gives less energy from those of the smaller ones thus the cells are damaged less (Miron *et al.*, 2003).

As evaluating of the experiments done by adding of the CMC, it could be understood why adding of the CMC of 0.1% increases the growth rate very little for the aeration rate of 3 L min^{-1}. As the adding of much amount of CMC in the medium increases the viscosity, turbulence decreases and the circulation rate of the cells in the liquid increases, so they can not use the light in the same rate and the mass transfer rate is lower level and photosynthetic productivity also decreases. That is why it is normal that the growth rates decrease for all the experiments from the 6 day on.

Cell precipitation: One of the most important problems along the production of the *Heamatococcus pluvialis*, is

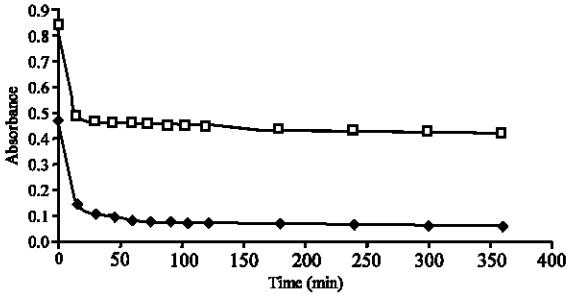

Fig. 7: The effect of adding CMC into the culture in different rate on the cell growth for the aeration rate of 1.5 L min^{-1} for the sample from the top of the reactor (♦) control (□) Tween 20

that the cells gather on the floor and precipitate on the bottom corners of the reactor precipitating and thus an homogeneous culture mixing can not be supplied. In the literature on this topic it was suggested that an anionic surface active material like Tween 20 was added into the medium for the suspension of the cells in the solution for longer time not precipitating. In one literature it was decelerated that Tween 20 was used for protein formed during fermentation, purification, freezing, drying and storing or cell aggregation to increase or to frustrate (Chou *et al.*, 2005).

In this part of the study it was worked by two same cultures in stagnant phase at two panel reactors in parallel and the solution of Tween 20 of 0.02% (V/V) was added into one of them. The systems had the same concentration at the beginning and the spectrophotometric measurements were done for the samples taken from the systems at the same interval of 15 min along a day. The precipitation rates of the samples from the three points of the top, middle and bottom parts of the reactor were determined. It was seen the adding of the Tween 20 solution into the medium how to effect the cell precipitation.

The absorbance measurements of the cultivation solutions for the aeration rate of 1.5 L min^{-1} were shown in Fig. 7-9. Evaluating on the results of the graphics, it was clear that the cell aggregation on the bottom part of the reactor was being more slowly comparing with those of the top and middle parts of the reactor. Adding of the Tween 20 solution makes the culture turbid so it was seen that the spectrophotometric measurements were different although their starting concentrations were the same because Tween 20 is an sorbitan oil acid ester derivative and it creates a suspension as adding into the culture medium. It was seen from the graphics that the precipitation come true quickly at the first 15 min for all and as measured absorbance for top and middle

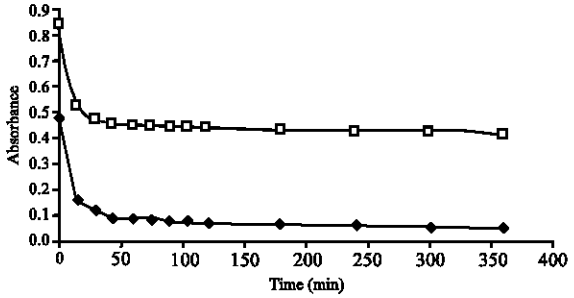

Fig. 8: The effect of adding CMC into the culture in different rate on the cell growth for the aeration rate of 1.5 L min^{-1} for the sample from the middle point of the reactor (♦) control (□) Tween 20

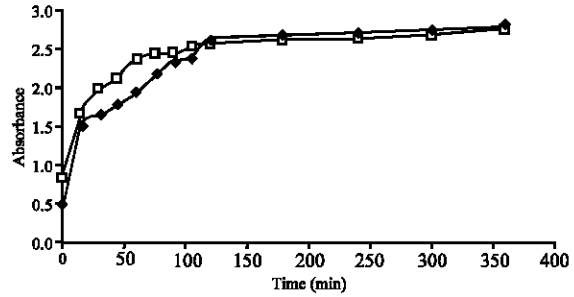

Fig. 9: The effect of adding CMC into the culture in different rate on the cell growth for the aeration rate of 1.5 L min^{-1} for the sample from the bottom of the reactor (♦) control (□) Tween 20

parts of the reactor increase from 0.838 to 0.5 and 0.525, respectively, it decreases to 1.653 for that of the bottom part of the reactor.

In literature there are not much studies on the effect of the adding Tween 20 into the cultures for the production of *Heamatococcus pluvialis* but it is known that it promotes both to hydrolyze of enzymatic cellulose and to foam as a surface active material (Chou *et al*., 2005; Sukan *et al*., 1989). Tween 20, Tween 40 and Tween 80 as buffer decrease surface tension changing the forces of inter molecules but this effect is related with their concentrations inversely (Keane *et al*., 2002; Mardikar and Niranjan, 2000). Chou *et al*. (2005) says that surface active materials like Tween 20 and Tween 80 protect albutropin protein from aggregation by agitating.

Based on all these explanations it may be thought that thudding of Tween 20 into the cultures increases the surface tension between the cells of *Heamatococcus pluvialis* and medium and the inclining towards forming the groups of the cells of *Heamatococcus pluvialis* similarly and the cells can be suspended for longer time in the culture medium.

CONCLUSION

In this study it was investigated the physical parameters how to effect the growth specifications of *Heamatococcus pluvialis*. Comparing the effect of aeration rate on the growth the highest cell number for the system with the aeration rate of 1.5 L min^{-1} is 35×10^4 cell number L^{-1} and it is 8.75 times of that of starting culture value but by increasing of the aeration rate from that of 1.5 L min^{-1} on the growth decreases instead of increases because of the cell death formed with increasing of the effect of the shear stress.

After the determination of the negative effect of the higher aeration rate on the cell growth it was studied on the effect of mixing aeration models simulated with fish bait particles artificially on the growth. Examining the models it is understood that the model with the aeration only from the one corner of the medium supplied the best cell growth of 35×10^4 cell mL^{-1} for 10 days of the production period. Calculated specific growth rate for the aeration rate of 1.5 L min^{-1} with optimal result is 0.0094166 h^{-1}. Being examined the results of the experiments the CMC added into the culture medium it is understood that the adding of the CMC increases the cell number by 50 and 100% approximately as the aeration rate 3 L min^{-1} decreases in that of 1.5 L min^{-1} (the optimum aeration rate) together with increasing of the viscosity. As to adding of the Tween 20 as a surface active material into the medium it was seen from the results that the precipitation comes true quickly at the first 15 min for all and as measured absorbance for top and middle parts of the reactor increase from 0.838 to 0.5 and 0.525, respectively, it decreases to 1.653 for that of the bottom part of the reactor. It may be thought that Tween 20 added into the cultures increases the surface tension between the cells of *Heamatococcus pluvialis* and the medium and the cells can be suspended for longer time in the culture medium.

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