EFFECT OF SPARGING IN MICROALGAE (*Dunaliella tertiolecta*) CULTURE USING BUBBLE COLUMN

Hadiyanto*, M. Barbosa and R. Wijffels****

Abstract

Microalgae as a photosynthetic microorganism that contain chlorophyll has high potential to produce novel high value compounds that can be used in food, pharmaceutical and cosmetic industries. With lack of rigid cell wall, microalgae suceptible to have hydrodynamic stress by increasing aeration rate. Increasing gas fow rate will increase the productivity to optimum condition, but after this condition the productivity will decrease due to cell disruption.In this reserach has been focused on effect of sparging on microalgae cell damage. Sparging experiment was carried out by varying gas flow rates between 0.59 to 5.13 L.min^{-1} with a sparger made from needle inserted to a piece of silicon. The needle was used with diameter 0.4 to 1.2 mm and 1-9 needles.With this variables resulted gas entrance velocity between 2.56-104 m.s^{-1}. The cell death rate constant (kd) was calculated by loss of cell viability in time using Coulter counter and Flowcytometer. From the experiments resulted gas entrance velocity was main cause for cell damagae. The small bubble was more detrimental than bigger size of bubble. Bubble rising as like in animal cell culture was not effect to detrimental cell.

Key words : *D.tertiolecta, cell death rate constant, gas entrance velocity.*

Introduction

Microalgae

Microalgae are microscopic and photosynthetic microorganism and grow in aqueous environment ranging from fresh water to extremly salinity, with suitable nutrients compounds such as nitrate, phospate and salt compounds. Microalgae use light as energy source and CO_2 as carbon source. Microalgae are potential sources of novel high value compounds that can be used in food, pharmaceutical and cosmetic industries.

Microalgae can be cultivated in both open systems such as ponds, lakes and raceways, and in closed culture systems where growth and biomass production can be optimised. The open systems, mostly are used in traditional culture that will be cheaper than the closed systems. Closed systems (photobioractors) such as bubble columns and airlift reactors can be used for microalgae cultivation (Merchuck *et al*, 2000). These systems can increase the yield and quality of biomass, but it requires additional energy input for cooling,need particular controllers for temperature, pH and gas suply, resulting in higher costs.

Although many options are available to supply gas, the sparging of the culture broth with a gas mixture is still the most suitable and practicable method. Sparging is the process of bubbling air or another gas through a relatively deep pool of culture broth, usually inside a bioreactor (Chisti, 2000), while sparger is the device through which the gas enters the bioreactor.

In most photobioreactors, sparging has an important role in providing certain gases required for growth like oxygen or CO_2 and for mixing the culture. However, sparging can also lead to cell damage inside the bioreactor and eventually to cell death due to hydrodynamic stress, specially with turbulent flow. The damage arising from gas sparging was considered to be a major barrier in large scale production for microalgae cultures (Suzuki *et al*,1994).

Hydrodynamic Stress

Hydrodynamic stress will depend on: bioreactor geometry, type of pumps, morphology and physiology of microalgae cells and sparging conditions. Hydrodynamic stress can cause cell death of microalgae cultivated in photobioreactors such as bubble columns and airlift reactors at certain condition of sparging (Chisti,2000). Increasing aeration rate up to a certain value will improve the reactor productivity, but after this optimum value cell damage will occur. This normally happens when the turbulence is so intense that the fluid microeddy size approaches cellular dimension. High aeration rates have led to cell inactivation of *Phaeodactylum tricornutum* (Contreras *et al*, 1998) and *Porphyridium sp* (Merchuk *et al*, 2000). The increase of aeration rate also reduced the growth rate of *Dunaliella tertiolecta* (Suzuki,1994; Silva, 1987). Also Camacho *et al*, (2000) realized that the hydrodynamic conditions in a photobioreactor influenced the productivity of *Porphyridium cruentum*. In these studies, they found a positive

*) Dept. of Chemical Engineering, Diponegoro University, Semarang 50239
**) Dept. of Bioprocess Engineering, Wageningen University, The Netherland

correlation between cell death and the gas flow rate, the specific gas liquid interfacial area and the height of culture. In addition, an exponential correlation has been found between the cell death rate constant and the power dissipitation due to bubble rupture. In another experiments, Camacho *et al* (2001) used carboxyl methyl cellulose (CMC) to protects *Phaeodactylum tricornutum* culture from shear stress. They found that cell damage could be suppressed by adding a small amount of CMC (<0.02 % wt) to the culture medium, and no physiological effects to cells were found with the presence of CMC. Contreras *et al*, (1998) cultivated the same strains in a concentric tube airlift photobioreactor and the drop in growth rate at high gas flow rate was attributed to micro-eddies in isotropically turbulent field. Silva *et al*, (1987) reported the hydrodynamic effect of gas flow rates on *Dunaliella tertiolecta* grown in two different reactors: Roux bottle and miniloop reactor.

Suzuki *et al* (1994) also studied this strain and suggested the productivity of cells was declined when it was cultivated in an air lift reactor under the same condition with bubble column. It means the hydrodynamic stress by liquid circulation in the air lift reactor was occured.

Shear

The correlation between cell damage and quantities parameters in sparging: shear stress, shear rate and smallest eddy length has been developed by Tramper (1991). Shear rate associated with the laminar flow during sparging may be estimated with equation 1 :

$$\tau = \eta \frac{dv}{dx} = \eta.\gamma \qquad [Nm^{-2}] \qquad (1)$$

where η is the fluid dynamic viscosity ($N.s.m^{-2}$) and dv/dx is the velocity gradients that equal to shear rate (γ). Due to difficulty to estimate length of small coordinate, dx can be represented by cell size (Tramper *et al*, 1988) and v is gas velocity at sparger.

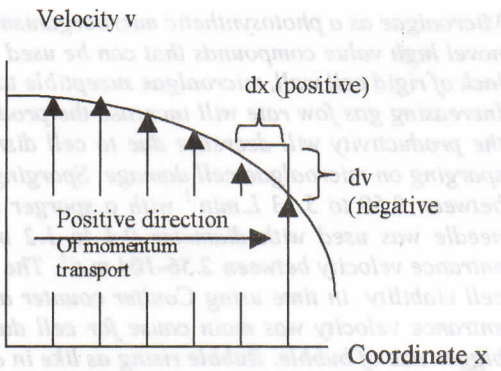

Figure 1. Velocity gradient in shear rate.

Based on figure 1 the shear stress is assumed to be postive in the direction of decreasing fluid velocity. The Cell size measured for several microalgae are presented in table.

Table 1. The cell size for several micro algae.

Species	Cell size (µm)	References
Dunaliella tertiolecta	6-9	Yang, M.M (1988)
	5-8	Osinga, R.(2000)
Porphoridium cruentum	5-8	Yang, M.M (1988)
Prorrocentrum lima	45-48 x 30-33	Yang, M.M (1988)
Chlamydomonas reinhardtii	9-13 x 6-12	http://protist.i.hosei.ac.jp
Dunaliella salina	11 x 6	http://protist.i.hosei.ac.jp
Phaedoactylum tricornutum	12 x 3	Camacho, et al (2001)

The gradient of gas velocity stands for gas entrance velocity (v_l) that will be depend on gas flow rate, number of nozzles and nozzles diameter (Tramper, 1991), as stated in equation 2:

$$v_l = \frac{4.F_g}{n.\pi.\pi_i^2} \qquad [ms^{-1}] \qquad (2)$$

Where n is number of nozzle, d_i is diameter of nozzle (m) and F_g is gas flow rate ($m^3.s^{-1}$)

Cell Death

Cell death in sparged bioreactors follows a first order kinetics and the cell death rate will depend on the changing in viable cell concentration in time (dC_{xv}/dt) (Camacho *et al*, 2000):

$$\frac{dC_{xv}}{dt} = (\mu - kd).C_{xv} \qquad (3)$$

where k_d is the specific death rate constant (hr^{-1}). The specific growth rate (μ) is assumed to be very small when compared to the death rate constant (kd), and so it can be neglected in the determination of cell death.

The sparging with a certain gas velocity lead to collisions between cells and bubbles. Jobses *et al* (1991) proposed two parameters related to collision and interaction between cells and bubbles, i.e : *Chance of collision* and *collision force*. Chance of collision will depend on cell concentration and number of bubbles leaving the sparger per unit time per unit culture volume, and also on bubble size. The collision force has a function in bubble size, and it

will be dependent on operation condition during experiments

Three events or mechanisms during sparging that might lead to cell death have been correlated to cultivation parameters involved in sparging (Jobses *et al*,1991), such as : sparger site, bubble rising and bubble escape.

a. Sparger site

The maximum gas velocity through the nozzle can be calculated with equation 2. If the sparger site is the main cause for cell damage, the decrease in cell viability will be proportional to the chance of collision and collision force (Jobses et al,1991) and of course will also be proportional to square of the gas entrance velocity (v_1).

$$\frac{dC_{xv}}{dt} = -k_1 \frac{v_1^2 . di^2 . n}{d_b^3 Vr} f(d_b) C_{xv} \qquad (4)$$

where k_1 is a proportionality constant, Vr is volume of culture (m^3) and v_1 is the gas entrance velocity ($m.s^{-1}$). This equation was developed assuming constant bubble size, orifice diameter, and culture volume.

b. Bubble rising

During bubble rising, cells will attach to the bubbles until the liquid surface. If the cell death is more likely to happen during bubble rising, the cell death rate constant will not depend on culture height. Jobses et al (1991) concluded that the loss in cell viability would be linearly correlated to gas flow rate. A simple equation has been established with the assumption the cell size, bubble size and rising velocity would be constant.

$$\frac{dC_{xv}}{dt} = -k_2 \frac{F_g}{Dr^2} C_{xv} \qquad (5)$$

where k_2 is a proportionaly constant and Dr is diameter of reactor (m), and this equation shows that cell death rate constant does not depend on culture height, if the reactor dimensions are kept constant.

b. Bubble escape (bursting)

Two regions during bubble break up have been reported i.e bubble cap with retraction process and bubble cavity with its collapse (Chisti, 2000)). When the bubbles carrying cells arrive to the liquid surface, the thin liquid cap is formed (figure 2a). Due to gravity force and pressure differences, the liquid will drain and then a hole will be formed in the thinning film and the retracting toroidal ring rapidly formed (figure 2.b)

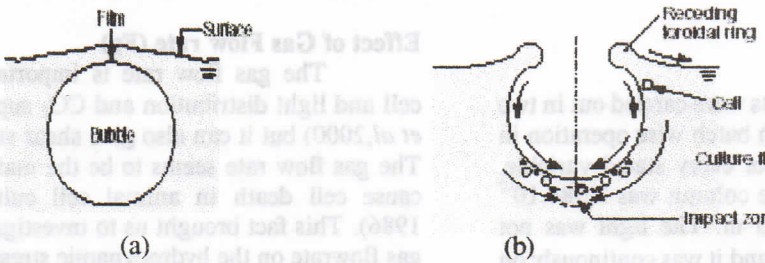

Figure 2. Events during bubble bursting(a) Bubble arrives to the surface and (b) fluid motion in the bubble film cap and bubble cavity.

Jobses *et al* (1991) proposed that collision forces during bubble break up will be dependent on bubble size (db) and bubble rising velocity. Again, Jobses *et al* (1991) expressed the decrease in the number of viable cells with equation 6:

$$\frac{dC_{xv}}{dt} = -k_3 \frac{F_g}{d_b^3 . V} C_{xv} \qquad (6)$$

Where k_3 is a proportional constant, db is the bubble size (m), and V is volume of culture (m^3). This equation is based on the assumption that the forces associated to bubble break up only depend on bubble size.

Jobses et al (1991) stated that if bubble bursting is the main effect of cell damage, the cell death rate constant would be proportional to gas flow rate (Fg). This comes from the equation 6.

This research were subjected to identify and quantify the effect of hydrodynamic stress (lethal effects) by sparging in microalgae cultures. In these experiments, we used bubble columns as closed photobioreactors and the microalgae strains studied were *Dunaliella tertiolecta*. Several variables have been set up in order to study the effect of the following parameters on cell death :Effect of Gas flow rate (Fg), Effect of gas entrance velocity (v_1), Effect of Bubble size (d_B), and Effect of culture height (H_L).

Material and Methods

Cell Culture and Reactor set up

The microalgae *D. Tertiolecta* cells were cultivated in a bubble columns with an inner diameter

of 61.2 mm, a height of 47.5 cm and liquid volume of 1400 ml. The inoculum was 50 ml of pure culture. The cultivation started batch wise until an OD 530 of 0.9 (corresponds to total number of cells of 9.10^6 cells.ml^{-1}) and then it was turned into a chemostat by diluting it with fresh medium at a constant rate by using a small pump (WATSON MARLOW 101 U). The medium consist of artificial sea water (3.3% salinity), Nutrients (KNO_3 16mM, $NaH_2PO4.2H_2O$ 1mM and $NaHCO_3$ 5 mM) and trace elements with Na_2EDTA 61.6μM. The volume of liquid was in constant level by pumping out the overflow with a WATSON MARLOW 505 U pump.

The temperature was 30°C and the pH was maintained constant in the range of 7.7-7.85 by adding pulses of CO_2 into reactor flowing together with air at 1.764 L.min^{-1}. The dilution rate during the chemostate was 0.03 hr^{-1}.

The light intensity for the chemostat culture was fixed constant at 77 μmol.m^{-2}.s^{-1} in order to keep the biomass concentration constant. The air and CO_2 were supplied into the reactor through a sparger with 16 nozzles with 0.8 mm of internal diameter. Two fans were put at the side of the reactor to eliminate the heat produced by the light. In order to keep the temperature constant, the bubble column was coverred with a water jacket that was connected to a water bath (JULABO F25). The pH was kept constant by automatic addition of CO_2 whenever the pH was higher than the set point.

Sparging Experiments

Sparging experiments were carried out in two identical bubble columns with batch wise operation in order to identify the effects of every stated variable. The inner diameter of bubble column was $3.584.10^{-2}$ m and the height was 0.725 m. The light was not changed at 240 μmol.m^{-2}.s^{-1}, and it was continously on during sparging experiments. The light was provided by 10 fluorescent light tubes (LYNX LE, 55 W, sylvania, Germany) placed in front of the reactors and a mirror placed on the opposite side to reflect the light. The light intensity was measured at 12 different spot inside the reactor with a LI-190 SA 2 π PAR Sensor (LI-COR, Lincoiln, NE, USA). The experimental conditions during the sparging experiments, temperature and pH (pH electrode Sen Tix MIC, Germany) were the same as described for with chemostat cultivations.

The experiments were started with OD 680 about 0.4-0.5 and the inoculum was a sample from the chemostat cultivation. This optical density corresponds to the following total cell number: *D.tertiolecta* = $(3.6 \pm 0.4).10^6$ cells/ml. About 2 - 3 ml samples were taken from the reactor and the same volume of medium was added back to the reactor to keep the volume constant. The optical density was measured at 530 and 560 nm using spectrophotometer (SPECTRONIC 20 GENESYS)with medium as reference.

The culture volume was varied between 250-500 mL and gas flow rates of 0.59 to 5.13 L.min^{-1}. The sparger (0.4 –1.2 mm of diamater) was made from needle inserted to a piece of silica. The number of needles were varied between 1-9 needles.

To measure the total number of cells, Coulter Counter Multisizer was utilized and Flowcytometer (FCM-Becton Dickinson BV, Etten Leur, The Netherlands) was used to determine percentage of cell alive, cell dead and cell debris. Flowcytometer is a mean to measure certain physical and chemical characteristics such as cell size, shape and cell component that can be detected by a fluorescent compound. The FCM consist of al light source, collection optics, and also a computer to translate the signal into data. Flowcytometer will give information about the relative size (Forward scatter-FSC), relative granularity or internal complexity (Side Scatter—SSC) and relative fluorescene intensity (FL1, FL2, FL3). These parameters were plotted by the computer and gave information about the fluorescence colour and intensity. In these experiments, FL1 and FL3 are important to determine whether microalgae cells were alive or dead, because they detect green and red fluorescence, respectively.

Results and Discussions

Effect of Gas Flow rate (Fg)

The gas flow rate is important for mixing, cell and light distribution and CO_2 supply (Merchuck *et al*,2000) but it can also give shear stress to the cell. The gas flow rate seems to be the main parameter to cause cell death in animal cell culture (Tramper, 1986). This fact brought us to investigate the effect of gas flowrate on the hydrodynamic stress in microalgae culture.

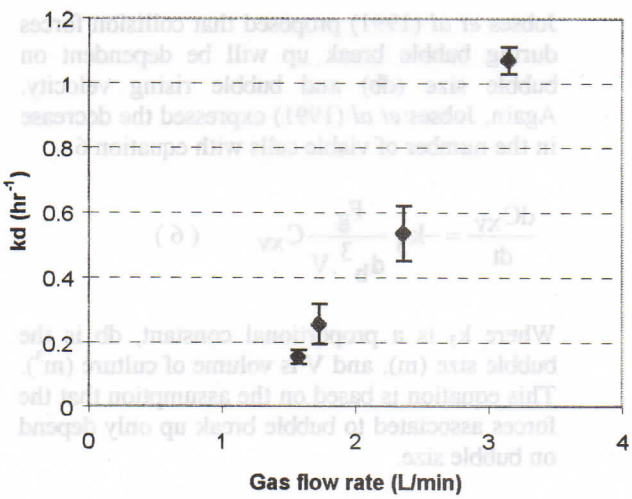

Figure 3. Increasing gas flowrates at fixed volume and number of nozzles lead to an increase cell death rate constant.

As depicted in Figure 3, shows gas flow rate (Fg) higher than 1.57 L.min^{-1} impact to decrease in the cell viability. The decrease of cell viability indicates that hydrodynamic stress occured at these gas flow rates. This figure also shows a positive correlation between cell death rate constant and gas flow rate and according to theory, this phenomenon pronounced bubble bursting could become cause of cell damage. The cell death rate constant (kd) was calculated by determining a slope of a graph between logaritmic of cell loss viability vs time, with came from equation 3. The growth rate constant was assumed very small compared to kd.

However, increasing gas flow rates at constant diameter and number of nozzles (n=1), yield an increase gas entrance velocity (v_L) as well. This means bubble formation at the sparger site also could be considered as cause of cell damage.

Effect of Gas entrance velocity (v_L)

This experiment was executed with varying the number of nozzle between 1 and 5 at a fixed gas flow rate of 3.14 L.min^{-1}. The nozzle diameter was set constant at 0.8 mm and it was assumed that the gas was distributed through every nozzle with the same velocity and there was no interaction between each nozzles.

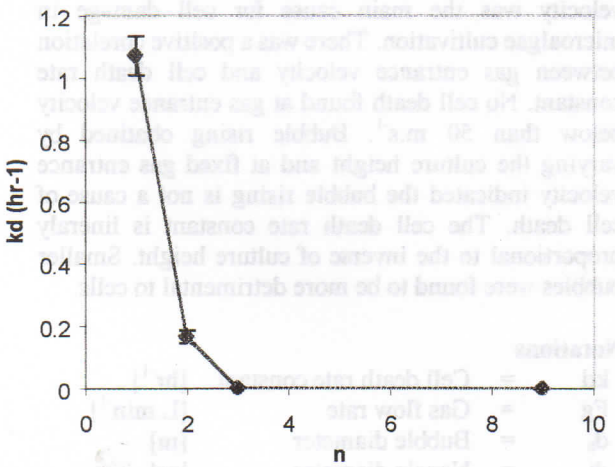

Figure 4. Varying number of nozzles at a fixed gas flowrates and volume of cultures.

As described in Figure 4, increasing the number of nozzle at a constant gas flow rate i.e.: decreasing gas entrance velocity, lead to a decrease of cell death rate constant (kd). The range of gas entrance velocity was from 2.5 to 104.3 m.s^{-1} (Figure 5) and it shows kd has linear correlation to square of gas entrance velocity. High gas entrance velocity will increase the collision frequency between cells and bubbles produced from sparger. High collision frequency give more contact and cell disruption will surely occur.

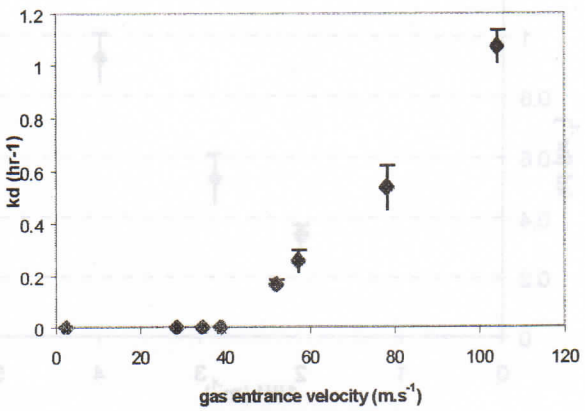

Figure 5. Increase of gas entrance velocity at a constant volume of culture.

In order to further verify the effects of gas entrance velocity on cell death rate, the gas flow rate was increased from 1.57 to 4.72 L.min^{-1}, while keeping gas entrancce velocity constant at 52 m.s^{-1}. It concluded that increasing gas flow rate at this constant gas entrance velocity did not give significantly (with 95% confidence interval) effect to cell death rate constant (kd). Again, this result give more stressing in conclusion that bubble formation at sparger is the main cause for cell death of *D. tertiolecta* culture.

Effect of Culture Height (H_L)

Figure 6 describes the correlation between cell death rate constant (kd) and inverse of culture height. Based on these results, where cell death is clearly dependent on the culture height, bubble rising can not be considered as the main cause for cell death. Jobses et al (1991) reported that bubble rising could be rejected as the main cause of cell death when the cell death rate is dependent on culture height (see eq.5).

This result supported by Tramper *et al* (1988) with animal cell and Suzuki *et al* (1994) with *D. tertiolecta* that obtained the same result. The increase of reactor height led to lower cell death rate constant (kd). Suzuki *et al*,(1994) varied the height of culture between 0.067 and 0.2 m, while keeping superficial gas velocity constant at 0.0167 m.s^{-1}.

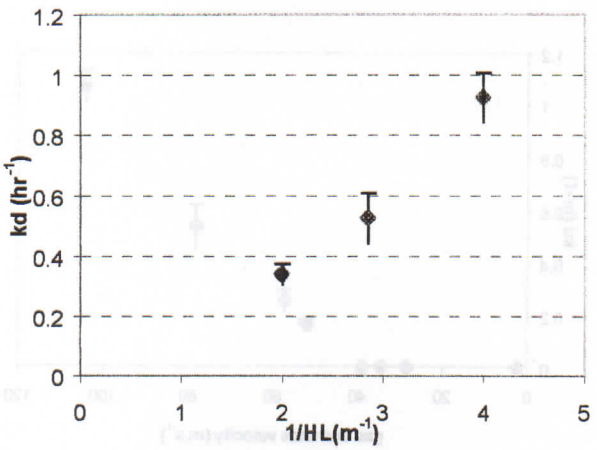

Figure 6. Cell death rate constant (kd) for increasing inverse of culture height

With higher culture height, the power dissipation will decrease as supported by Chisti (2000). Decreasing the power dissipation will give less power contact between cell and bubbles, and then cell still keep growing.

Contradictory result has been reported by Camacho et al (2000) using *P. Cruentum*. Increasing height of culture means more cell could be attached to the surface by bubble and consequently, the cell death rate constant (kd) will increase.

Effect of Bubble size (d_B)

Effect of bubble size was done by varying nozzles diameter (0.4-1.2 mm) at the same gas entrance velocity at 78 $m.s^{-1}$.If cell damage occured, it could be only caused by differences in bubble size, due to gas entrance velocity which was found to be main cause for cell death. No exact measurement for bubble size, but varying needles diameter could represent this variables.

Figure 7 summarized that small bubbles are more detrimental to cells and that no significant differences were found between bubble sizes produced from diameter of nozzles 0.8 mm and 1.2 mm (based on 95% confidence interval). This is in good agreement with Handa *et al* (1989) who reported that for animal cell culture, smaller bubble (< 2mm) are more hazardous and no effect was observed for bubble with diameter between 2-6 mm.

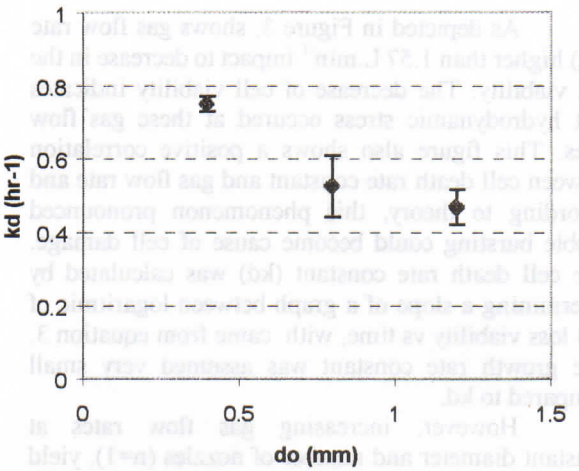

Figure 7. Bubble size is represented by varying nozzle diameter.

Bigger bubbles give less surface contact area between cells and bubbles, consequently less cell attached and lead to lowering cell death rate constant. This phenomenon has been developed by Chisti (2000).

Conclusions

The effect of hydrodynamic stress on microalgae Dunaliella tertiolecta cultivated in bubble column reactor has been studied. The gas entrance velocity was the main cause for cell damage in microalgae cultivation. There was a positive corelation between gas entrance velocity and cell death rate constant. No cell death found at gas entrance velocity below than 50 $m.s^{-1}$. Bubble rising obatined by varying the culture height and at fixed gas entrance velocity indicated the bubble rising is not a cause of cell death. The cell death rate constant is lineraly proportional to the inverse of culture height. Smaller bubbles were found to be more detrimental to cells.

Notations

kd	=	Cell death rate constant	[hr^{-1}]
Fg	=	Gas flow rate	[$L.min^{-1}$]
d_b	=	Bubble diameter	[m]
di	=	Nozzle diameter	[m]
H_L	=	Height of culture	[m]
n	=	Number of nozzle	[-]
v_L	=	Gas entrance velocity	[$m.s^{-1}$]
Cxv	=	Viable cell	[cell/ml]
V	=	Volume of culture	[m^3]
OD	=	Optical density	[-]

Greek symbol

μ	=	Growth rate	[hr^{-1}]
η	=	Dynamik viscosity	[$N.m.s^{-1}$]
γ	=	Shear rate	[s^{-1}]
τ	=	Shear stress	[$N.m^{-2}$]

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