HYDRODYNAMIC DAMAGE OF CULTURED CELLS OF CARTHAMUS TINCTORIUS IN A STIRRED TANK REACTOR

TOSHIYA TAKEDA, MINORU SEKI AND SHINTARO FURUSAKI*
Department of Chemical Engineering, The University of Tokyo,
7-3-1, Hongo, Bunkyo-ku, Tokyo 113

Key Words: Biochemical Engineering, Plant Cell Culture, Safflower, Hydrodynamic stress, Stirred tank, Viability, ATP, Aggregate Size

The effect of hydrodynamic stress on intracellular factors of plant cells was investigated. Safflower (Carthamus tinctorius) cells were cultivated in a stirred tank reactor. The aggregate size distribution was determined with an image analyzer and it was confirmed that the aggregate size became smaller in a stirred tank. The resultant changes in the cells were evaluated on the basis of cell number, dry cell weight, cell viability, ATP amount and cell membrane fluidity. The viability was estimated by growth potential, membrane integrity and respiration activity of the cells. The cells were damaged by hydrodynamic stress in the respiration activity before the occurrence of the cell lysis and the damage of membrane integrity. ATP amount decreased as the agitation rate increased. The extent of ATP loss was larger than that of viability loss. These results indicated that hydrodynamic stress affected the metabolic system and that the loss of viability was probably due to this metabolic change. The degree of ATP reduction showed a power law dependence on the power dissipation rate per unit mass, e, which is calculated from agitation rates.

Introduction

Plant cell culture using dedifferentiated cells in vitro is a useful method for production of valuable components originating from plants. In a reactor operation, cells in suspension are subject to considerable hydrodynamic stress due to aeration and agitation. Although plant cells are surrounded by cell walls and cultured plant cells usually form cell aggregates, they generally have low shear resistance. Hydrodynamic stress causes decreases in cell growth and substrate uptake rate, alteration of the pattern of product formation and change in the productivity of secondary metabolites. The estimation of cell damage induced by hydrodynamic stress including shear stress is important for the design of cell culture system.

The reduction in growth of the animal cells by hydrodynamic stress was attributed to cell death. The cell viability decreased with an increase in the hydrodynamic stress level and exposure time to the stress.

It was also reported that a higher shear stress level was required in laminar flow regime to cause the same degree of damage to animal cells observed in turbulent flow regime. It was suggested that turbulent eddies contributed to this phenomena.

It was indicated that various plant cell lines had significant differences in hydrodynamic sensitivity. Therefore, estimation of hydrodynamic sensitivity of cells is required in designing cell culture systems, but little information is available concerning the mechanism of cell damage caused by hydrodynamic stress.

The damage of plant cells is not necessarily associated with mechanical cell destruction. Changes in intracellular factors caused by hydrodynamic stress should be clarified. Viability assessment is a useful method to investigate the intracellular changes. The viability changes of cultured plant cells due to hydrodynamic stress were detected by growth potential, membrane integrity and respiration activity.

The purpose of this study is to evaluate the effect of hydrodynamic stress on the intracellular factors of plant cells. Correlation between hydrodynamic stress and changes in the cells was investigated using safflower (Carthamus tinctorius) cells. Changes in the cells were evaluated by viability indices, ATP level and cell membrane fluidity. Hydrodynamic stress was evaluated by the power dissipation rate in a stirred tank reactor.

1. Experimental

1.1 Cells and culture conditions

Safflower callus was induced from flower bud by Furuya et al. and subcultured every three weeks. The revised tobacco basal medium supplemented with indolebutyric acid, kinetin and Casamino acid was used for both solid and liquid media. The cells cultured on the solid medium for 3 weeks were suspended in 100 ml of the liquid medium in a 300 ml Erlenmeyer flask shaken at 90 rpm and at 300 K in the dark.

Suspended cells harvested during exponential growth

* Received December 9, 1993. Correspondence concerning this article should be addressed to S. Furusaki.
phase (7-10 day culture) were mixed with one volume of the fresh medium and incubated in a shake flask for one day for conditioning. One hundred ml of the conditioned cell suspension was inoculated into a stirred tank with 450 ml of the fresh medium and cultivation was carried out at 300 K in the dark. The initial cell concentrations were 7-10 g-

cell fresh weight (FCW)/l. The control culture was carried out

in a 100 ml medium in a 300 ml Erlenmeyer flask shaken at 90 rpm using the same conditioned cell suspension that was used in the stirred tank at the same initial cell concentration.

1.2 Reactor and estimation of hydrodynamic stress

The cells were exposed to hydrodynamic stress in a stirred tank with 6-bladed turbine impeller and 3 baffles (Fig. 1). The power dissipation rate for a given agitation rate in this reactor was estimated according to the works by Rushton et al.21) and Nagata et al.17).

The control of dissolved oxygen (DO) concentration was not carried out unless otherwise noted. The DO concentration remained more than 2 mg-O₂/l under the condition of the cultivation without DO control in this study.

In order to control the DO concentration for some incubations, surface aeration was performed by introducing a mixture of air and oxygen into the head space of the reactor at 100 ml/min. The DO concentration was measured with a DO probe and the O₂ partial pressure was regulated to keep the DO concentration at 6.5-7.5 mg-O₂/l.

1.3 Analysis

1) Cell mass Cell mass was determined on a dry cell weight (DCW) or cell number basis. DCW was measured after freeze-drying the cells.

Cell number was measured microscopically using a Burker-Turk hemocytometer. The cells were incubated in a solution with 1 % macerozyme (Yakult Pharmaceutical Ind. Co.) and 12 % mannitol for 1 h to dissociate aggregates prior to the measurement.

2) Assessment of viability Viability of the suspended cells was estimated by growth potential, membrane integrity and respiration activity.

The growth potential of the cells was calculated from changes in cell numbers during 5 days of cultivation in shake flasks after the test cultivations in a stirred tank and in a shake flask as a control. The viable cells cultivated in a stirred tank were assumed to grow at the same specific growth rate as the viable cells cultivated in a shake flask.

3) Membrane integrity The membrane integrity was measured by the Evans blue dye exclusion test. Evans blue is known to stain the non-viable cells28). After enzyme treat-

ment to dissociate aggregate for 1 h as mentioned above, 0.1 ml of the 2.5 % Evans blue solution was added to 2 ml of the cell suspension. Viability was determined by the frac-
tion of cells without staining. Over 1,000 cells were counted in each measurement.

4) Respiration activity The respiration activity was measured using reduction of triphenyl-tetrazolium chloride (TTC). Viability based on TTC reduction was determined using a method developed by Towill and Mazur29).

Phosphate buffer solution containing 1.5 % TTC was added to cell suspension in a proportion of 2:1. The TTC concentration was determined previously as a proper value to be used for safflower cells. This mixture was incubated at 25°C for 18-22 h after introducing nitrogen gas. The red formazan produced in the cells was extracted with ethanol and the absorbancy of the extract was measured at 485 nm.

5) Cell aggregate size and cell diameter The microscopic image of cells was introduced to an image analyzer (Nexus Qube, Shimadzu) and the projected area diameter (PAD) of each aggregate was determined automatically. The volume of an aggregate was calculated from the value of PAD. The projected image was also used for the cell diameter measurement.

6) ATP measurement All procedures were carried out on ice. After taking a sample, 15-20 ml of cell suspension was centrifuged (0°C, 3000 xg, 2 min) and the supernatant was discarded. Then, 7 % perchloric acid solution was added to the obtained suspension in proportion of 1:1. Cells were homogenized with a tissue grinder and extraction was carried out22). The supernatant was collected and neutralized to pH 6.2-6.7 by adding 1 mol/l potassium carbonate solution9). The precipitate was removed with a centrifugal ultrafiltration tube (fractional molecular weight: 10,000) and the filtrate was subjected to assay. The ATP and ADP were separated by HPLC on TSKgel DEAE-2SW column (TOSOH) eluted with 0.2 M phosphate buffer (pH 4.6)-acetonitrile (4:1 by volume) and detected using a UV
spectrometer (260 nm).

7) Cell membrane fluidity Plasma membrane fluidity of intact cells was determined by the steady state fluorescence polarization technique. The cells were washed twice and resuspended with the isotonic solution including 11% mannitol and 0.1% calcium chloride. Diphenyl hexatriene (DPH) dissolved in dimethyl sulfoxide was added to the suspension in order to obtain DPH concentration of 1 μmol/l. The cell suspension was incubated for 5 min and then placed in a spectrofluorometer, FP-777 (Japan Spectroscopic Co.) equipped with rotary Polacooat UV/Vis polarizing lenses. Fluorescence anisotropy which depends on membrane fluidity was obtained according to Ramirez et al. This method was applied for plant protoplast and it was confirmed that nearly the same values were obtained with or without enzyme treatment for protoplast preparation.

2. Results

2.1 Effect of agitation rate on cell viability

Cultivation in the reactor was carried out at 200-500 rpm of agitation rates for 24 h. Relative cell number and DCW after cultivation normalized by the results of the control cultures are shown in Fig. 2. To investigate the influence of oxygen transfer the cultivation with DO control was carried out and the results are shown together.

The cell numbers in the stirred tank at low agitation rate were found to be less than those in control cultures and increased to exceed the values of the control cultures with an increase in the agitation rate. This behavior was observed in the cultures with and without DO control. The DCW also increased slightly with increasing agitation rate. Reduction of cells by hydrodynamic stress was not observed.

The viabilities were determined by growth potential, Evans blue staining and TTC reduction. Relative viabilities after cultivation normalized by the results of the control cultures are shown in Fig. 3. The viability determined by the TTC reduction is the value per unit DCW.

The viabilities determined by Evans blue staining and TTC reduction decreased with an increase in the agitation rate in both cultures with and without DO control. The viabilities determined by the TTC reduction were lower than the values from Evans blue staining. A significant change in the growth potential with agitation rate was not recognized.

These results as shown in Figs. 2 and 3 indicated that the viability decreased without cell lysis and the extent of viability loss was evaluated in the respiration activity larger than in membrane integrity. Therefore, the damage of cells by hydrodynamic stress occurred in the intracellular metabolism. The previous report regarding animal cells indicated that damages in the cells were caused by hydrodynamic stress leading to the loss of membrane integrity and cell lysis.

2.2 Change of viability with time

Cells were cultivated in the stirred tank at 500 rpm of agitation rate for 24 h. During cultivation the viability was measured by Evans blue staining and TTC reduction at 4 h intervals. The results are shown in Fig. 4. Each value was normalized by the initial value. The results from the Evans blue staining in the control culture remained constant within ±3% and the values decreased slowly in the reactor after hour 12. The values determined by the TTC reduction decreased for the first 12 h by 30-40%, then increased during the following 8 h and once again
decreased both in the reactor and in the shake flasks. This behavior was reproducible. These results indicate that the change in activity of TTC reduction depends on the metabolic condition rather than the damage due to hydrodynamic stress. Thus, the TTC reduction is not suitable for estimation of changes in viability with time. The difference between the culture systems by TTC reduction (Fig. 4(b)) appeared at an earlier stage of cultivation than the results by the Evans blue staining (Fig. 4(a)).

2.3 Effect of agitation on aggregate size

Figure 5 shows volume distributions of the aggregates for inoculum and suspensions after 1 day of cultivation in the reactor or the shake flask. The distributions were obtained by combining measured values at four agitation rates. The average aggregate volumes and standard deviations of average volumes at four agitation rates were $0.0357 \, \text{mm}^3 \pm 0.0117 \, \text{mm}^3$ before the cultivation, $0.0540 \pm 0.0115 \, \text{mm}^3$ after the shake flask cultivation, and $0.0272 \pm 0.0104 \, \text{mm}^3$ after the stirred tank cultivation, respectively. The average aggregate volume after cultivation in the stirred tank was smaller than the value after shake flask cultivation. This result indicated that disaggregation occurred in the stirred tank. This result agreed with the previous reports, where the aggregate sizes were determined by sieving. The increase in specific surface area of aggregates by agitation may cause the enhancement of growth shown in Fig. 2.

Figure 6 shows the change in volume distribution of aggregates during 10 h cultivation in the stirred tank. Aggregates larger than 0.1 mm$^3$ of volume decreased by 65% in 2.5 h and the number of aggregates smaller than 0.01 mm$^3$ of volume increased along with time.

Averaged cell diameters were 106 μm in the shake flask culture and 94 μm in the stirred tank culture at 400 rpm of agitation rate. These values were obtained by using more than 20 cells and the significant difference was verified by the variance analysis.

2.4 ATP and cell membrane fluidity

ATP levels in the cells and cell membrane fluidity were measured in order to elucidate the transfer mechanism of hydrodynamic stress into the cell. The cells were cultivated in the stirred tank at 200-500 rpm of agitation rates for 24 h without DO control.

ADP and ATP amounts after cultivation were measured and normalized by the sum of the amounts of
ADP and ATP in the control culture. The results are shown in Fig. 7 as a function of agitation rates. Each value was obtained as an average of 2-3 measured values. Here, the AMP amount was under detection limit. Relative ATP levels in the control cultures were constant at 0.9, while in the reactor it decreased to 0.63 as the agitation rates increased. The ATP content in the cells of control culture was 557-684 nmol/g-FCW. The extent of ATP reduction is larger than the extent of viability loss determined by Evans blue staining and TTC reduction. Therefore, hydrodynamic stress is considered to affect the metabolic system where ATP is synthesized or consumed. Also, the loss of viability seems to be generated from such metabolic change.

The cell membrane fluidity was measured simultaneously. The difference between the values in reactor and flasks was not clear (data not shown). The results show that the composition of cell membrane concerning membrane fluidity did not change significantly in the reactor operation for 24 h.

3. Discussion

The eddy scale effect\(^{17}\), viscous shear stress\(^{3, 7, 11, 18}\), Reynolds stress\(^{3}\) and fluctuating dynamic pressure\(^{11, 18}\) were considered as causes of hydrodynamic stress on the suspended particles in turbulent flow.

Turbulent flow field is governed by three dimensional eddies. Eddies with various scale are generated in a bioreactor. The kinetic energy is transported successively from large eddies with an impeller scale to smaller ones until the energy is finally dissipated by viscous force in the form of heat through eddies of the smallest size.

The smallest turbulent eddies will exist in a state of isotropic, statistical equilibrium. Kolmogorov showed that in isotropic turbulence the specific scale, \(L\), and specific velocity, \(\nu\), of the eddies were functions of kinetic viscosity, \(\nu\), and the power dissipation rate per unit mass, \(\varphi^6\).

That is,

\[
L = \left( \frac{\nu}{\epsilon} \right)^{1/4}
\]

\[
\nu = \left( \frac{\varphi^6}{\epsilon} \right)^{1/4}
\]

The eddy scale effect was estimated using Kolmogorov scales of the smallest eddies\(^{6, 7, 23}\). Eddies larger than the critical value determined by the scale of suspended particles move particles to reduce the net force exerting on the particle surface and the smaller eddies will damage particles\(^{3}\).

The values of viscous shear stress\(^{3, 15, 18}\), Reynolds stress\(^3\), fluctuating pressure\(^{18}\) were reported to be proportional to \(\nu^{1/2}\), \(\epsilon\) and \(\epsilon^{1/2}\), respectively.

Now, the Kolmogorov scales of the turbulent eddies were calculated as 25.8-51.2 \(\mu\)m in the range of agitation rate in our study. These values were 6-14 % of the average aggregate diameters. From the estimation of the eddy scale effect all aggregates will suffer hydrodynamic damage. However, the degree of damage cannot be discussed from the estimation.

The degree of cell damage was estimated by ATP reduction. We speculated that the reduction of ATP occurred only in cells located at the surface of the aggregates where the cells were subjected to hydrodynamic stress directly. The degree of ATP reduction was estimated by the ratio of ATP loss to the intact ATP amount which existed in the cells located at the aggregate surface. The intact ATP amount existing in all cells was obtained as the value of the ATP amount in the control culture.

The ratio of the cells located at the aggregate surface was determined by aggregate size distributions after the stirred tank cultivation shown in Fig. 5 for each agitation rate. The number of cells with diameter, \(r\), in an aggregate with diameter, \(R\), is approximately equal to \((R/r)^2\). The cross section of the cell is \(\pi r^2\) and the surface area of the aggregate is \(4\pi R^2\). Thus the number of cells located at the aggregate surface is approximately equal to \((R/r)^2\). The
ratio of cells located at aggregate surface was, therefore, calculated as $4r/R$ using measured aggregate diameter (PAD) as $R$ and the average cell diameter described above as $r$, under condition $R > 4r$. When the aggregate size is smaller than $4r$, all cells were assumed to locate at aggregate surface. According to these assumptions, the ratio of the cells located at the aggregate surface was estimated to be 67-88% of all cells.

Figure 8 shows log-log plot of the degree of ATP reduction versus the power dissipation rate per unit mass, $\varepsilon$, calculated from agitation rates. Assuming that the ATP reduction is proportional to viscous stress or fluctuating pressure, the logarithm of the ATP reduction is proportional to $\log \varepsilon$ with the slope of 1/2, which agreed approximately with that obtained by the plot in Fig. 8.

Croughan et al. proposed that the death of microbial cells and animal cells on microcarriers occurred with encounter of cells and turbulent eddies and that the specific death rate was proportional to $\varepsilon^{0.4}$. To compare their results with our results, the relationship between the loss of ATP and cell death should be examined quantitatively in a further study.

The consumption of ATP in energy metabolism is caused by hydrolysis of ATP and accompanied with ADP production. In our results changes in ADP levels were not observed. The ATP reduction caused by hydrodynamic stress may be due to the consumption of ATP as substrates or the inhibition of ATP synthesis.

Conclusions

The intracellular change of safflower cells in a stirred tank reactor was investigated. Cell number and DCW increased and the viability of the cells decreased along with increasing agitation rate. The aggregate size distributions were determined using an image analyzer. The deformation of aggregates occurred in the stirred tank.

The extent of the loss of growth potential and respiration activity by hydrodynamic stress was larger than the extent of damage of membrane integrity. Cell lysis was not observed.

ATP reduction in the cells was found to be caused by hydrodynamic stress in the stirred tank reactor. The degree of ATP reduction increased as the agitation rate increased. The result indicated that hydrodynamic stress in the reactor influenced the intracellular metabolism related to ATP synthesis.

Literature cited