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Case Report
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Study the Relation between Fermentation Characteristics of Submerged Fluid and Improving the Lactic Acid Production by Fungi

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Abstract

In this study, it was shown that how rheology and fluid dynamics effected the behavior of cell growth and L-Lactic acid productivity during cultivation of filamentous fungi (*Aspergillus oryzae*) in a stirred-tank. Submerged culture of *A. oryzae* was a shear-thinning fluid with complex rheology and morphology. It is a shear-sensitive fluid. The scope of this study, was enhancing the gas-liquid flow of fermentation fluid to improve the liquid-gas mass transfer, L-lactic acid production and stagnant zone reduction at low energy consumption by effective shear rate generation using multi-large blade agitator with global axial pattern of mixing. Due to this reason, the advantage of Fullzone® (FZ), impeller was investigated. Experimental results when using multi-large and multi-radial impeller, showed that at the same volumetric power consumption the dissolved oxygen concentration, *Re*, macro and micro-morphology size, biomass and L-lactic acid production when using multi large impeller was higher and more stable than that of the multi radial impeller. Most of above positive properties were due to the reduction in viscosity and apparent consistency index of fermented fluid.

Keywords: Fermentation; Lactic acid; Fungi; Submerged fluid

Introduction

Lactic acid (LA) is a colorless, odorless monocarboxylic acid naturally produced by many organisms. This weak acid has extensively used as an excipient in the food, cosmetics, pharmaceutical and chemical industries. The L-isomer is being preferred for food and pharmaceutical applications [1]. Lactic acid can be produced by several microorganisms classified into bacteria, fungi, yeast, cyanobacteria, and algae. Among these microorganisms, lactic acid production using fungal fermentation showed high efficiency [2]. Widely used method for the production of lactic acid is batch fermentation [1]. Hydrodynamic conditions in the fermenter influence the morphology of the fungus and thus the rheology. High agitation rates, which are required for optimum mass transfer, lead to high shear stress [3]. Consequently, mycelial networks are fragmented, increasing the amount of free filaments and hence the viscosity. Besides, high viscosity at low agitation rate causes insufficient mass transfer and oxygen limitation [4]. Because of an existing dynamic relationship between fermentation conditions and fungal growth patterns, an improved impeller that was sufficiently flexible for submerged cultures would be an advantage in the design of an efficient enzyme production system. One method would be enlargement of multi-stage impellers, and another would be the use of close-clearance agitators. Here, the application of Fullzone® impeller as a large multi-stage agitator for improving mass transfer, biomass/lactic acid production and avoiding the stagnant zone preparation by focusing on the rheology changes have been investigated.

Materials and Methods

Strain and medium

A genetically engineered *Aspergillus oryzae* (NSPlD1/niaD-bLDH/Δ871, *abrr.* bLDH/Δ871) was used in this study. Cultivations of spores of bLDH/Δ871 was conducted in agar plate ($CDNO_3$ medium: 2.0% [w/v] D-glucose, 4.675% [w/v]-NaCl, 2% [w/v]-$NaNO_3$, 1.0% [w/v]-KH_2PO_4, 0.5% [w/v]-KCl, 0.5% [w/v]-$MgSO_4$, 0.002% [w/v]-$CuSO_4.5H_2O$, 0.001% [w/v]-$FeSO_4$.7H2O, 0.0001% [w/v]-$ZnSO_4 7H_2O$, 0.0001% [w/v]-$MnSO_4$.7H2O, 0.0001% [w/v]-$AlCl_3$, 1.5% [w/v]-Agar, with 0.015% [w/v]-L-methionine). This strain was also cultivated in PY-based liquid medium, which contains 1% [w/v]-BactoTM peptone, 0.5% [w/v]-BactoTM yeast Extract, 0.2% [w/v]-KCl, 0.1% [w/v]-KH_2PO_4, 0.05% [w/v]-$MgSO_4.7H_2O$.

Fermentation experiments and fermenter configuration

Fermentation experiments for the L-lactic acid production with *A. oryzae* were carried out in a laboratory-scale, 2.0 L, stirred-tank batch bioreactor, (STBR) (DPC-3A Jar, ABLE BIOTT Co., Tokyo, Japan) with a working volume of 1.5 L. Fermentations were conducted in a cylindrical bioreactor with a vessel internal diameter, *H* of 0.114 m, with a flat bottom and a broth height to a vessel diameter ratio of 1.3. Agitation was provided by two different impellers, (a) the FZ and (b) the DRT. The FZ consisted of two large paddle impellers with the upper paddle shifted at 45° in the rotating direction. The DRT had a *d*/*H* ratio of 0.38 and a *W*/*d* ratio of 0.2 (*d* and *W* are impeller diameter and width of impeller respectively). The spacing between the impellers was 1.6*d*, and the lower impeller was located at a distance 1.4 *d* above the base of the tank. Details such as the size and geometrical condition of the two impellers along the shaft are shown in Figure 1. The bioreactor was equipped with monitors, which were used to measure and control the foam, temperature, pH, stirring rate, torque, and dissolved oxygen (DO). The vessel of the bioreactor was equipped with a peristaltic pump

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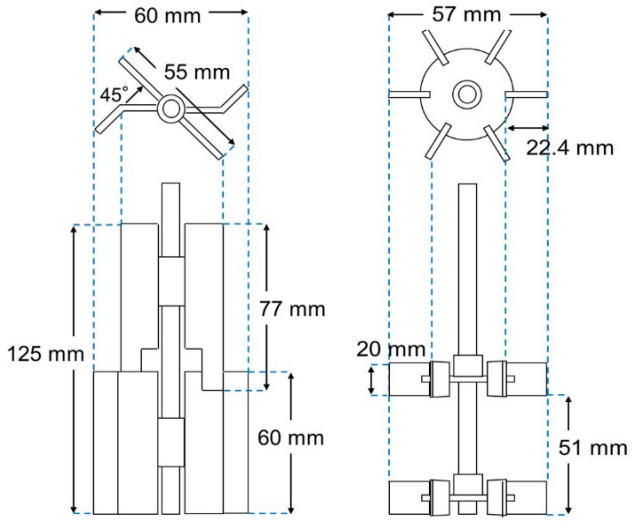

Figure 1: Configurations of FZ and DRT impeller used in this study.

to control the foam and pH *via* the automatic addition of an antifoam agent (KM-70, silicon agent, Shin-Etsu CO., Ltd. Tokyo, Japan) and an acid/base, respectively, also a mechanical foam breaker was used at the top of the culture. The fermentation medium (1.5L) was made from PY-based medium with 3% [w/v]-D-glucose. The medium was added to a fermenter and sterilized in an autoclave. Following cooling, the medium was inoculated with 15 mL of previously prepared seed culture. A ring sparger was used to aerate the culture at 1.00 v.v.m. The DO during fermentation was measured using a commercial sensor (ABLE-DO, SDOC-12FL220, ABLE Co., Tokyo, Japan). An external jacket was used to maintain the broth temperature at 30°C. For a batch operation, the fermenter was run for 72 h. After a fixed interval of incubation, the fermented broth was sampled, then filtered using a 150 ml bottle top vacuum filter, (0.22 µm Pore, 13.6 cm^2 PES Membrane, Fits 45 mm Diameter Necks (Corning Inc., California, USA)) and filtrate was used for glucose and lactate assays. Schematic of impellers used in this study was shown in Figure 1.

Glucose and L-lactic acid assays

The glucose concentration was measured by Wako Glucose C2-Test kit (Wako Pure Chemical Industries Co., Osaka, Japan). The 20 µl-filtrated culture sample was poured into 3 mL-color reagents and warmed at 37°C in 5 min. The absorption at 505 nm of this solution was measured by the spectrophotometer (MPS-2400, Shimadzu Co., Kyoto, Japan).

L-lactic acid concentration was determined by HPLC, which modified to an organic acid analysis (solvent delivery system, LC20AB; column, Shim-pack SCR-102H; column temperature, 50°C; detector, SPD-20A; Shimadzu Co., Kyoto, Japan). The mobile phase contains 5 mM *p*-toluenesulfonic acid. And the buffer solution was added just before the detector to enhance sensitivity. It contains 5 mM *p*-toluenesulfonic acid, 20 mM bis-Tris and 20 µM EDTA.2Na.

Analyses of dry cell weight of mycelium

Biomass was measured in units of dry cell weight (DCW). Mycelia in the sampled culture were washed with tap water then filtered at least 5 times, next the cell pellet was suspended in 20 ml-distilled water and settled 5 min. After this, the pellet was then transferred to a pre-weighted watch grass and was dried in an oven at 80°C until reaching a constant weight.

Morphology analyses

The investigated morphological parameter was divided into the macro-morphological one and the micro-morphological one. The pellet diameter was measured for the macro-morphological analysis. The mycelia in the sampled culture was washed with tap water then filtered at least for 5 times, then it was suspended in 20 mL-distilled water and settled 5 min. After that, the cell pellets were re-suspended in the glass plate of distilled water whose water level was enough higher than the cell pellet diameter. The suspended cell pellet was taken the photograph with a 5 mm grid. With this picture, the pellet diameter was measured by a ruler. The grid was used for calibrating the length on the picture. The hyphal thickness and length were measured for the micro-morphological analyses. Microscopic images of the fungal pellet were taken using a Digital microscope (VHX-100K, KEYENCE Corporation, Osaka, Japan). The microscope was equipped with image processor software to measure the length between the 2 points. The cell pellets were all transferred on the slide grass, then dried at least 24 h in the room temperature. These dried samples were used for the micro-morphological analyses. The pellet diameter and hyphal thickness and length were measured for more than 50 cells per each sampling time. Those morphological parameters are evaluated with average and standard deviation.

Simulation of velocity distribution during fermentation by R-Flow

To calculate flow velocity in bioreactors using both types of impellers, the fluid dynamics software "R- FLOW" (R-flow Co., Ltd., Saitama, Japan) was utilized to solve the Navier–Stokes equations. In the present work, an Eulerian-Eulerian two-fluid model at low *Re* was used to model the gas-liquid flow with steady- state conditions in a stirred bioreactor. During the simulation, the air-flow rate was the same as that for the fermentation experiment. The gas phase was set for ambient air at 30°C, and was set with a bubble diameter of 2 mm without considering the break-up and coalescence effects. The governing equations in this approach can be derived by ensemble-averaging of the conservation equations for each phase. The governing equation to flow-velocity fields, used the mass continuity and momentum equations, as follows (Eqs. (1) and (2)):

$$\nabla.(\rho\vec{\upsilon}) = 0 \quad (1)$$

Navier- Stokes equation:

$$\rho\vec{\upsilon}.\nabla\vec{\upsilon} = \mu\nabla^2\vec{\upsilon} - \nabla P + \rho g + F \quad (2)$$

where *ρ, P, v, g* and *F* are the fluid density, pressure, velocity, gravity, and drag force respectively. For the two fluid models, the continuous (fermentation culture) and dispersed (bubble) phases are separately expressed using the equations of conversation of momentum and mass continuity. Here, F (F=F_{TD}+$F_{D,\ l\text{-}g}$) is the interface force between gas-liquid phases and is defined as the assumption of the turbulent drag force (F_{TD}), (assumed to be zero in this study) and the drag force ($F_{D,\ lg}$) between the gas-liquid phases. The equation for calculating the $F_{D,\ l\text{-}g}$ is shown in Eq. (3)

$$F_{D,\mathrm{lg}} = \frac{3}{4}\alpha_d\alpha_c\frac{\rho}{d_b}C_D\,|\vec{\upsilon}_c - \vec{\upsilon}_d\,|(\vec{\upsilon}_c - \vec{\upsilon}_d) \quad (3)$$

Where α, d_b and C_D are the phase volume fraction, bubble diameter and the fluid-resistance-coefficient respectively, of one bubble against the fluid. C_D is defined in Eqs. (4) and (5), and is estimated with the formula of the resistance near the bubble (Eqs. (6) and (7)).

$$C_D = \max\{16(1+0.15\,\mathrm{Re}^{0.687})/\mathrm{Re}_b, 0.44\} \quad (4)$$

$$\mathrm{Re_b} = \frac{\rho_c \,|\, \vec{\upsilon}_d - \vec{\upsilon}_c \,|\, d_b}{\mu} \quad (5)$$

$$C_D = \max\{16(1+0.15\,\mathrm{Re}^{0.687}) / \mathrm{Re_b}, 0.44\} \quad (6)$$

$$Pv = (2\pi NT) / \mathrm{V} \quad (7)$$

Power consumption, *Re* and viscosity measurement

The volumetric power consumption, *P*v [Wm^{-3}], was measured *via* the torque sensor (Eq. (8)), where *N* is the impeller rotational speed [s^{-1}] and *T* is the average torque [*N*m], from the beginning to the end of the fermentation process. Based on the torque values, the dimensionless power consumption (*N*p (=*P*/ ρN^3D^5)) of fermentation culture was measured.

$$P_v = (2\pi NT)/V \quad (8)$$

The apparent viscosity of the fermentation culture after each sampling was measured using a B-type viscometer (Model B8L, TOKIMEC INC. Tokyo, Japan, the rotor NO. 1 at 0.6 rpm was used). An example of measurement after 2 days from the cell inoculation was shown in Table 1. Then, the *Re* (=$\rho Nd^2/\mu$) of the fermentation culture was measured using the experimentally measured viscosity. The rheological model that was extracted for *A. oryzae* was compatible with the power-law model. Here, the rheological measurements using a HAAKE™ viscometer-550, (Thermo scientific, USA) showed the shear-thinning and non-Newtonian behavior of a fermentation culture after cell growth (Table 1). The data were calculated according to the Ostwald-de Waele model. The calculated values were apparent as the K_{app} and n_{app} values due to the cell adherence and a complex suspension of the fermentation culture.

Effect of large multi-stage agitator (FZ) on rheology properties of submerged fermentation at *t*=48h (same P_v=175 W/m^3) (Table 1).

Statistic efficacy

It was noticed that all of the obtained data were extracted by doing at least four times independent experimental processes. The repeated experimental process was done at the same condition. It was mentioned that all the error bars in the data were standard deviations.

Results and Discussion

Effect of multi-large blade on the hydrodynamic and mixing characteristics of stirred tank at low energy consumption

To investigate the mixing and hydrodynamic behavior of multi-large and multi-radial impellers in an aerated stirred vessel at low *P*v (low flow regime), correlations of N_p - R_e, T_m -P_v, *and* NT_m -*Re* were drawn by measurement of the N_p, T_m, and *Re* at different agitation intensity using Newtonian fluid at relatively low *Re.* Results of these correlations were shown in Tables 2-4.

Correlations of (*N*p-*Re*) for different impellers in using Newtonian fluid (glycerol) (Table 2).

Results of Table 2 showed at the same *Re*, N_p of mixing by DRT was higher than that of the FZ. It means DRT for preparing the same flow regime was needed more energy than that of the FZ. In addition, correlations shown in Table 3 presented at the same P_v mixing time of fluid using FZ was lower than that of the condition using DRT blades. Results of Table 4 also was in agreement with the results of Table 3. Because from the correlation results it could be seen that at the same regime of fluid (same *Re*) by both agitators the non-dimensional circulation time (mixing time) in the vessel by multi-large blades was faster than that of the multi-small impeller. Finally, it could be concluded that at the same and low P_v, using FZ impeller could be resulted in decreasing the NT_m, T_m, and also increasing the effective *Re*. Due to the increasing the Re viscosity of culture have been decreased.

Mass transfer and shear rate distribution

In this study, the DO concentration in submerged culture with radial and global-axial multi stage agitator was recorded and shown in Figure 2a. However, results of Figure 2a showed no significant difference between recorded DO concentrations of FZ and DRT impellers during the fermentation at very low P_v but simulation results of gas-liquid velocity distribution (Figures 2b and 2c) presented that the air dispersion using global axial mixing by FZ was significantly uniform however, gas flow velocity distribution using radial multi impeller was totally local. Local air dispersion in fermentation liquid was one of the major reasons of low biomass production in comparison with the global axial multi impeller. The other reason of increasing the biomass was related to the rheology of culture at *t*=48 h. As was seen in Table 1 by increasing the K_{app} using FZ agitator the biomass has been increased. It was shown in Figure 3a. In addition, due to the uniform dispersion the mixing time of bioprocess using FZ impeller have been decreased and therefore, the cells can uptake the oxygen and sugar faster than that when using the DRT. Regarding to the results of glucose measurement in Figure 3b, by increasing the rate of oxygen dispersion in stirred tank most of the cells in entire region of stirred tank can receive the oxygen and other nutrient with the same experience. Due to this reason, rate of oxygen and glucose consumption was faster than that of the culture mixed by local flow pattern. One of the positive effects of fermentation at low apparent viscosity and K_{app} was higher biomass production by lower glucose consumption than that of the agitation with DRT.

Effect of double multi large impeller on morphology

The productivity of fermentation broth is influenced by the rheological properties of submerged culture. These properties are determined by biomass and morphology [5]. Investigation on the fungal micro-morphology (Figures 4a and 4b), indicates using global axial mixing, the diameter and elongation of hyphae were more than

Impeller	*N* (s^{-1})	n_{app}	K_{app} (Pasn)	Viscosity(Pas)	Lactate acid [gL-1]
FZ	1.05	0.3	29	0.80 ± 0.05	8.67 ± 2.50
DRT	1.40	0.11	147	1.10 ± 0.08	7.35 ± 3.0

Table 1: Effect of large multi-stage agitator (FZ) on rheology properties of submerged fermentation at *t*=48 h (same P_v=75 W/m^3).

Impeller	Correlation	(Re: ~)	R^2
DRT	N_p=1963 $Re^{-1.238}$	3.7~120	0.883
FZ	N_p=4800 $Re^{-1.507}$	3.7~74	0.990

Table 2: Correlations of (N_p-*Re*) for different impellers in using Newtonian fluid (glycerol).

Impeller	Correlation	R^2
DRT	T_m=2478.0 $P_v^{-1.055}$	0.972
FZ	T_m=2792.9 $P_v^{-0.981}$	0.911

Table 3: Mixing time correlations for different impellers using Newtonian fluid (T_m-P_v).

Impeller	Correlation (*Re*: ~)	R^2
DRT	NT_m=983.3 $Re^{-0.375}$	0.865
FZ	NT_m=157.0 $Re^{-0.247}$	0.582

Table 4: Non-dimensional mixing time correlations when using DRT and FZ in Newtonian fluid (NT_m-*Re*).

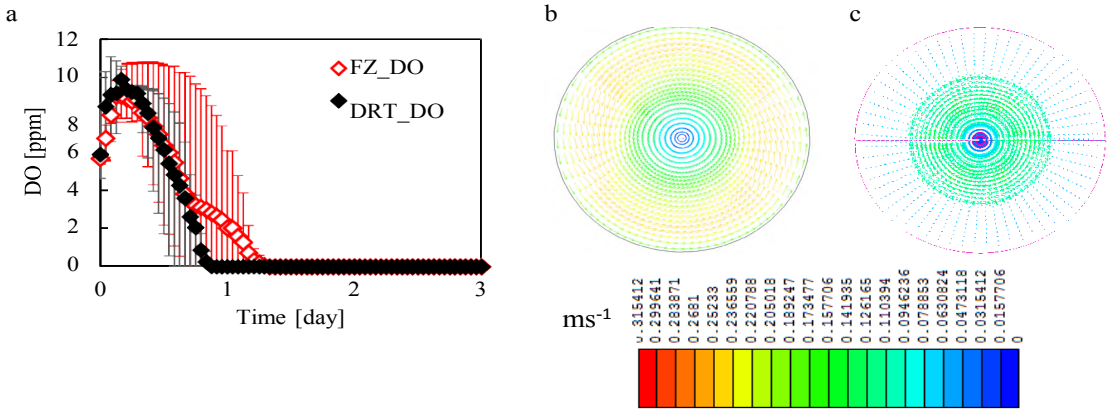

Figure 2: (a): DO concentration in fermentation culture at t=48 h using (1) FZ (2) DRT impeller (b) and (c): Gas velocity profile during fermentation by (1) FZ and (2) DRT impeller in Y-X plane.

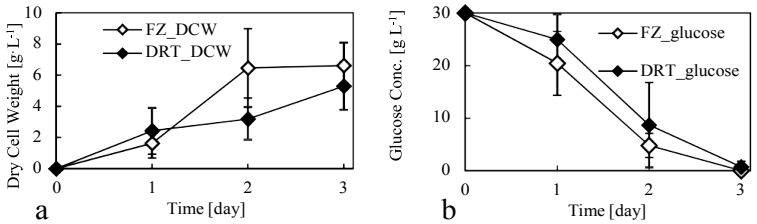

Figure 3: (a): Changing the DCW versus fermentation time using (1) FZ, (2) DRT impeller, (b): Glucose consumption versus fermentation time using (a) FZ and (b) DRT impeller.

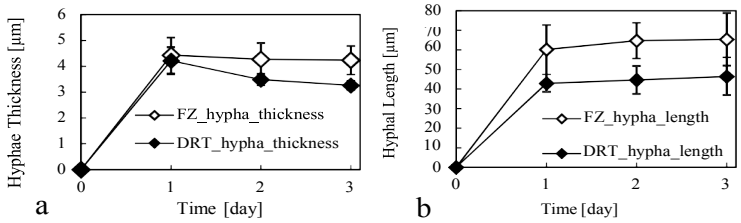

Figure 4: (a): Hyphae thickness versus fermentation time using (1) FZ, (2) DRT impeller, (b): Hyphae length growth versus fermentation time using (1) FZ and (2) DRT impeller.

that of the radial-local agitation at low power consumption. Therefore, axial mixing by FZ at low energy consumption could be an appropriate medium for hyphae growth.

Results of Table 1 and Figure 3a, showed not only using FZ impeller by decreasing the culture viscosity the active biomass and finally the amount of lactic acid at *t*=48 h has been increased when cells mixed in medium with axial and semi-uniform substrate and cells dispersion.

Study the stagnant zone formation during submerged fermentation using FZ and DRT impellers

As already mentioned these experiments were done at very low agitation intensity. In this condition decreasing the stagnant zone by growing the microorganism was an important issue to improve the mass transfer. Illustration of stirred fermenter in each day showed using FZ impeller by decreasing the viscosity of fluid, the volume of stagnant zone at the bottom of the tank have been decreased (Figure 5). Simulation of liquid-gas flow in three directions showed at Z direction the region with low gas-liquid velocity near the tank wall was significantly lower than that of the FZ (Figures 6a and 6b). Additionally, in Y and X directions during mixing by double large impeller the low and high velocity region by changing the blade position during the agitation periodically was changed and this instability formation was leaded to homogene dispersion of fermentation fluid (Figure 6a). However, the flow velocity by the DRT at Y and X direction was symmetrically local and most of the region agitated at low shear rate (Figure 6b). It means that most of the dead zones when using DRT was due to the poor gas-liquid velocity flow at X and Y direction.

Conclusion

Stirred fermentation of *A. oryzae* using global-axial large multi impeller at very low agitation intensity showed several advantages in comparison with the condition using radial multi impeller as follows;

Air dispersion when mixing by FZ was more uniform than that of the DRT

Biomass and L-lactic acid production have been increased in high-viscosity and complex fermentation time. It was due to the decreasing the apparent viscosity and apparent consistency index of fermented suspension.

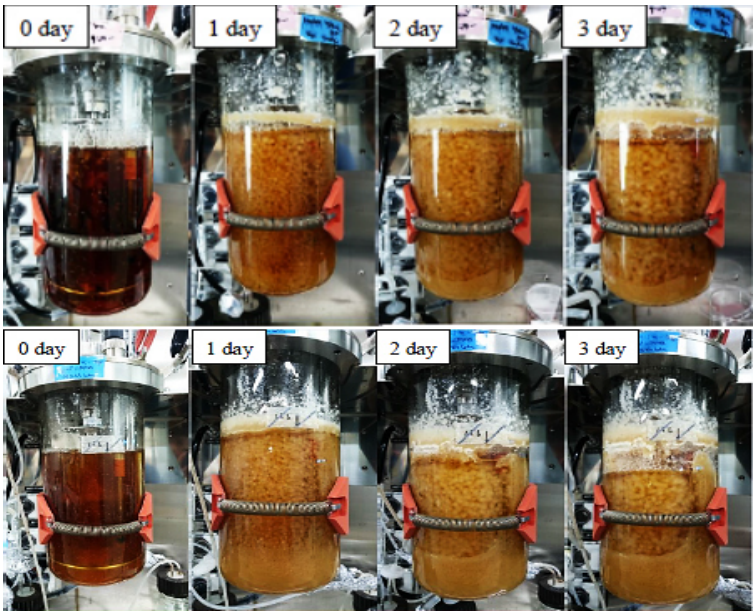

Figure 5: Pellet-cell fluidization during submerged fermentation of *A. oryzae* (a): using FZ, (b): using DRT.

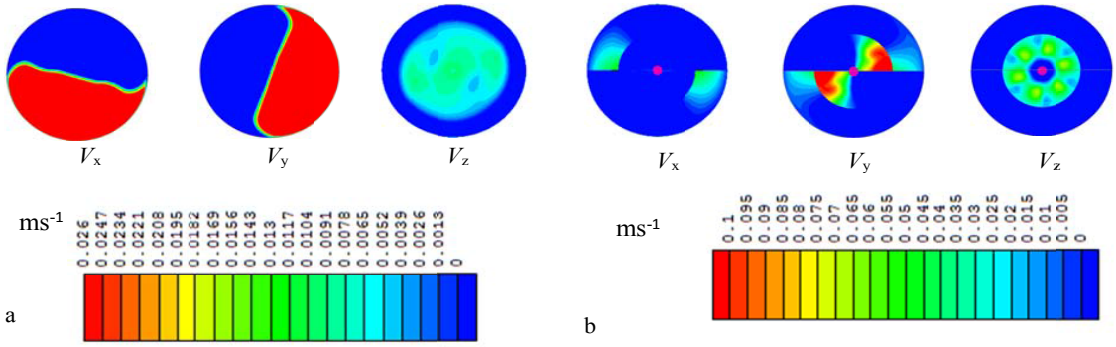

Figure 6: Flow velocity distribution of gas-liquid when mixing by (a): FZ and (b): DRT during fermentation in second day of fermentation at Y-X plan in three direction.

Local gas-liquid dispersion in three directional flow velocity has been decreased during agitation with axial and large multi blades.

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