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Doctoral Dissertation
博士論文

Process intensification in α- amylase production with *Aspergillus oryzae*: focusing on mixing enhancement

(混合促進に着目した麹菌 *Aspergillus oryzae* を用いたα-アミラーゼ生産のプロセス強化に関する研究)

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Narges Ghobadi

January 2017

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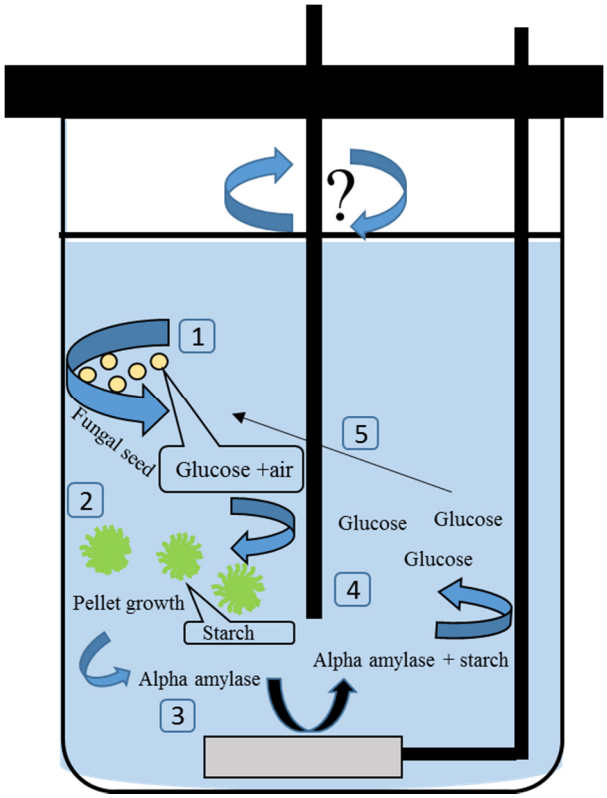
Chapter 1: Introduction



Graphical abstract of chapter 1: [This picture was prepared by Narges Ghobadi, 2016]

Chapter 1 in brief; Based on the requirement of submerged fermentation of *A.oryzae*, obstacles of enzyme production in stirred tank fermenter were mentioned in this chapter. Afterwards, solutions for overcoming the problems (thesis aim and scope) were introduced.

1-1 Introduction into Aspergillus oryzae and its potential for alpha amylase production

The genome of *Aspergillus oryzae* was sequenced in 2005. It is eukaryotes, having considerably more genes than bacteria (**Fig. 1-1**). One of the important advantages of *A.oryzae* was that it could be genetically manipulated. Finally, there were no patents blocking the use of *A.oryzae* [1]. Therefore, it is suitable fungi for production of large amount of heterologous proteins. Large fungal surface area of *A.oryzae* acts like an adsorption layer of extra-polysaccharide matrix, protecting them from inhibitory compounds. *A.oryzae* has high thermal stability and heat sensitivity and is a good fungi for extracellular enzyme production in batch fermentation. The filamentous fungus *A. oryzae* has been used in the production of traditional fermented foods for more than 1,000 years also it is listed as GRAS status by the U.S. food and drug administration [2].

Fig. 1-1 Electro-microscopic image of *Aspergillus oryzae* [1]. (Reproduced with the kind permission of Dr. Kazuya Hayashi, Kikkoman Corp.)

1-2 Mechanism of secretion of alpha-amylase during the fermentation of fungi

The alpha amylases are extensively used in many industries including paper, textile, pharmaceuticals, starch liquefaction, brewing, and food industries [3]. The alpha amylases

produced by fungi are more stable than from bacteria due to high thermo-stability and heat sensitivity [4]. Cultivation of filamentous fungi can be complex in order to produce optimal product yield. Major factors are the type of cultivation, modes of operation, broth rheology, fungal morphology, biomass concentration, and type of bioreactor.

The pathway of secretion in filamentous fungi is composed of following steps [5] (**Fig. 1-2**); translocation across the ER membrane, N-glycosylation and folding in the ER lumen, exit from the ER, and modifications in the Golgi apparatus and finally release from secretory vesicles to the extracellular apace [6]. According to the bulk-flow hypothesis, after folding the proteins are excreted into the extracellular medium at the tip of the hyphae [7].

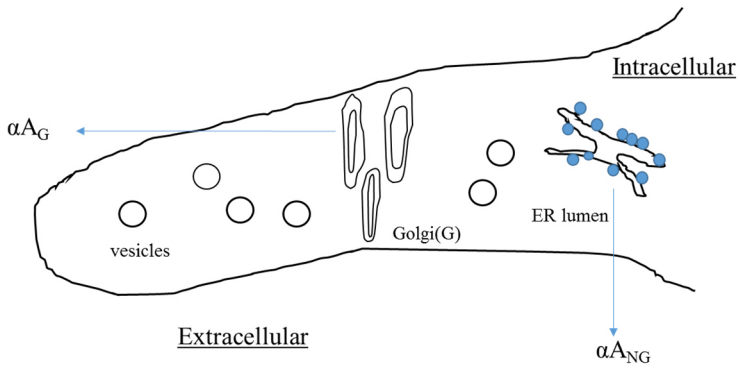


Fig. 1-2 Secretion pathway in filamentous fungi: Synthesized alpha amylase is trans-located and folded in the ER membrane, then glycosylated and folded in the ER lumen [5].

Fungi duplicate their length and nuclei through integration of the processes involved in tip growth, nuclear division, septation, and branching [5]. Fungal cells grow by apical hyphal extension in a highly-polarized manner with respect to their growth, morphology, organelle positioning, and cytoskeletal distributions [5]. Hyphal extension is facilitated through deposition and insertion of new membrane and cell wall material at localized sites on the cell surface [5]. The enzymes and precursors required at the advancing tips for the synthesis of the new material are delivered in vesicles transported to these sites along a polarized cytoskeletal network [5].

1-3 Importance of stirred fungal fermenters development

Industrial bioprocess with filamentous fungi includes the production of a main products in the sense of quality as well as the diversity of metabolites. The development of bioreactor (fermenter) is an essential factor to provide optimum mixing conditions for formation of fungal products by desired rate [8, 9]. Moreover, a dynamic relationship exists between the fermentation condition and growth pattern of fungal. Distinct cultivation condition resulted in morphologic and physic-chemical characteristics of fungal elements and this will be affected on production level of proteins.

1-3-1 Definition and characteristics of stirred fermented systems

The complete mixed fermented system is defined as a uniform distribution of the nutrients and oxygen will be leaded to the efficient mass transfer and achieving in high quality and quantity of production [10]. To reach the complete mixed fermented systems, most of the researcher's study were on the optimum condition of agitation [11-14]. Because mycelial damage at high stirrer speeds or power inputs can limit the capability and volumetric productivity of fermenter [15]. It has been reported that for each submerged culture an optimum condition of agitation partly depends on the resistance of hyphae to mechanical forces and also physiological state [16].

1-3-2 A review on the results of study on the effect of mixing on characterizations of submerged fermentation

According to the last literatures, studying the effect of mixing on the fermentation culture is a complicated issue because it effects on mass transfer, morphology, rheology and hydrodynamic of microorganism both directly and indirectly. The brief review on the effects of mixing (increasing the power of mixing) on the efficiency of submerged fermentation were seen in **Table 1-1**.

Table 1-1 Effect of mixing condition during the submerged fermentation on the morphology of microorganism and fermentation characteristics.

Microorganism	Power input	Agitation Intensity	Bioreactor	Effect on mycelia/hyphae	Comments about fermentation properties	Reference
Penicillium Chryogenium	Increase	High	Stirred tank	Mycelial Length decreased- Short/thick/highly branched hyphae	-	[17]
A. niger/acid citric production	Increase	High	Stirred tank	Densely branched Filamentous- thick hyphae	-	[18]
A.niger /acid citric production	Increase	High	Stirred tank/tubular tube	Increase in mycelial length- Decrease size of clumps	Increase in protein production yield and growth rate	[19]
Aspergillus awomori/ Xylanase production	Increase	High	Stirred tank/shake flask/airlift	Small and compact pellet- large loose hairy hyphae with large number of tips	-	[20]
Trichoderma reesaei/Cellulase production	Increase	High	Draft tube airlift bioreactor	Short mycelia	-	[21]
A.niger	Increase	High	6 L Stirred bioreactor - two Rushton turbines	Entangled mycelia and larger branches- thick hyphae	Protein production independent of agitation, (500-1000 rpm).	[22-24]
A.clavayus/*A.fumugatus* *A.niger*/*A.terreus*/*P.chryognium*, *A.oryaze*	Increase	High	Stirred Tank	Increase in hyphal diameter and hyphal branch frequency.	Decrease in metabolite, increase in viscosity and fragmentation of *A.oryzae*.	[12, 18, 25-28]
Aspergillus Carbonarius/amylase production	Increase	High	Tubular-loop bioreactor	Increase in hyphae length, decrease size of clumps.	-	[29]
A.niger/ glucoamylase production	Increase	-	Ruston Turbine, (RT) and Wide-blade Hydrofoil Upward-pumping, (WHu) were used.	Mixing by WHu was facilitated the formation of pellets.	At similar glucose and oxygen uptake rates, the WHu formed uniform viscosity and mass transfer rates. Using RT was resulted in heterogeneities. larger glucoamylase production rate by WHu impeller	[30]
A. oryzae	-	-	550 L pilot plant stirred tank reactors, Hayward Tyler B_2 and DRT were used.	-	Limiting factor for the productivity was oxygen supply. The energy dissipation function have weak impact on apparent viscosity than that of the biomass.	[31]

					No difference in k_La between B_2 and RDT.	
A.oryzae	increased	-	Stirred tank (80 m^3) with four baffles and 3RT.	Slower growth, altered morphology, or increased hyphal fragmentation.	-	[32]
A.oryzae	-	-	Stirred tank, propeller and turbine impellers were used.	Carbon source feeding and impeller type were controlled the morphology Cells agitated by propeller was in the form of pellet but cells agitated by disk turbine was in the clumped	Propeller agitation reduced the shear stress.	[33]
A.niger	-	-	Stirred tank 22-L 3RT impeller were used.	Pellet size and the overall pellet concentration are affected by hydrodynamic conditions induced by energy dissipation.	Formation of glucoamylase depends on the agitation and was maximum at low energy dissipations.	[34]

1-3-3 Fermentation using stirred mixers

According to the last studies, one of the typical reactors that was used in three phase bio-cultures is stirred tanks. One of the important factors in the stirred bioreactors is mixing time. Scientists and engineers tried to reduce this factor but sometimes, it is impossible to reduce mixing times by raising the power input (P_{in}) into the stirrer. While increasing the stirrer, speed is an obvious way of improving fluid circulation, other techniques may be required.

Mixing can be improved by changing the configuration of the system. The impeller should be mounted below the geometric center of the vessel. Mixing is facilitated when circulation currents below the impeller are smaller than those above. In this condition, the fluid particles leaving the impeller at the same time then take different periods of time to return and exchange material. Rate of distribution in the vessel is increased when upper and lower circulation loops are asynchronous. Another device for improving mixing is multiple impellers, although this requires an increase in power input. Typical bioreactors used for aerobic culture are tall cylindrical vessels with liquid depths significantly greater than the tank diameter. This design

produces a higher hydrostatic pressure at the bottom of the vessel, and gives rising air bubbles a longer contact time with liquid. Effective mixing in tall fermenters requires more than one impeller. Therefore, a device for improving mixing is multiple impellers or some new integrated mixers which intensified the fermentation process. Important goal of mixing intensification in fermenters is remove the limitation of bioreactor by improving the transport phenomena and interphase momentum transfer [35].

1-3-4 Advantages and disadvantages of stirred tank bioreactor for fermentation of fungi

Investigation on the application of stirred tank bioreactor (STBR) for fermentation of fungi was shown that using STBR would be useful for this process for the reason as follows [36]:

- Commonly used
- Ease of scale up (There are a huge number of rules of thumb in the area of stirred aerated fermenters)
- Useful for high viscosity cell culture
- High oxygen mass transfer ability
- Good fluid mixing
- Alternative impellers

Besides of advantages of STBR, some of the obstacles exist when using this kind of bioreactor as a fermenter. It was listed as below [36]:

- High shear stress and shear rate around the impeller tip
- High capital and operational costs
- Heat generation due to the mechanic agitation

- Contamination risk with mechanical seal

1-4 Problem definition

Finding an efficient fermenter with low maintenance and production costs is one of the major challenge that the industries are seeking to find it. The purpose of the thesis is intensification of fermentation of *A.oryzae* using improved mixing flow pattern in STBR. To reach this goal, we focused on the application of different kinds of agitators to overcome the obstacles of submerged fermentation in batch condition. Besides, the idea of changing the common shaft impeller with flexible-shaft was examined. The detail of technical and biological problems existed in this way was explained as below;

1-4-1 Inability to find knowledge on the viscosity and rheological behavior of fermentation culture

By growing the cells, the rheological behavior of cell culture changes from Newtonian to non-Newtonian. There are difficulties involved in measurements with conventional rheological measurement devices. Undesired phenomena such as settling of biomass, phase separation at the rotating cylinder surface, inhomogeneity of the broth in the form of pellets and friction in a narrow gap cylinder, as well as slip velocity near the wall of the pipeline viscometer, are often the cause of inadequate results [37]. It was noticed that overcoming this problem by producing cell suspension with controlled morphology is important because rheology knowledge is the first requirement of modeling the complex hydrodynamics of fermentation fluid [37-39].

1-4-2 Mechanical stress prediction and calculation during fermentation to control the shear damage when using conventional impellers

The exerted mechanical stress exposed to the fungal cells can be either measured or calculated. However, it is not possible to measure the component of mechanical stress via calculation. The required torque for achieving the perfect mixing becomes very high for highly

viscous non-Newtonian fluid. It may lead to destruction of mixing system [40]. Hence, the mixing of highly viscous non-Newtonian fluid is usually carried out at low *Re* in laminar or the early transition regime. Even for the low viscous non-Newtonian fluids, the study of mixing is restricted to the laminar and transition regimes. The coexistence of several phases in the system elevates the difficulty of the simulations also there is various phenomena in terms of phase interaction that can be difficult to consider in the simulations. It is some more challenging to simulate the exact flows, momentum and mass transfer in STBRs in a high viscous submerge culture [40].

1-4-3 Oxygen mass transfer

Oxygen transfer is a limiting factor in an aerobic fermentation, because of poor mixing of the reactor. Highly extended and branched fungi inhibit the nutrient transfer to cell and was resulted in low cell growth. Most of the common agitators disperse the oxygen locally and it leads to local cell growth [41]. Using some other agitators [40] the aeration stress was increased during breaking the bubbles.

1-4-4 Control the morphology of fungi to reach optimal productivity

A. oryzae are morphologically complex and differ structure in different time of life cycle. Therefore, controlling the morphology during the fermentation is difficult. Uncontrolled growing the fungi is leaded to the fluctuation in changing the viscosity during the fermentation and finally resulted in complex rheological behavior.

1-5 Aim of the thesis

It was known that enhancing the mixing in a bioreactor is more required than constructing a new bioreactor. Because, construction of a new fermenter will cost more and is not as fast as the modification in a stirred fermenter. Modifying the hydrodynamic and mixing condition of a stirred fermenter might be an economically main point to reach an improved mixing [36].

The aim in this thesis is to investigate, the effect of mixing (environmental change in hydrodynamic of fungal cells) on the fermentation of *A.oryzae* to overcome to the some of the mentioned obstacles during using the STBR as a fermenter. Moreover, finding an appropriate flow pattern of mixing which to intensify the mixing fermenters and produce greater turbulence with reduced shear stress or damage on the cells. The purpose of the thesis is the development and characterization of new agitators for application to fermentation systems. The work was undertaken in 8 chapters. In chapter 1 a brief review was done on the *A.oryzae* and application of stirred bioreactors in submerged fermentation. Chapter 2 was contained all materials, techniques and analytical methods were used for fermentation of *A.oryzae* and investigating the mixing characterization of different kind of agitators. Chapter 3 was focused on the effect of different hydrodynamic conditions in stirred fermenter on shear rate and shear stress formation in biological fluid. In chapter 4, effect of mixing flow pattern on mass transfer during fungal fermentation has been investigated. Based on the results of shear rate, shear stress and mass transfer macro and micro morphology of *A.oryzae* was studied in chapter 5. Regarding to the strong interaction between morphology and rheology in stirred submerged fermentation, effect of mixing condition and morphology on the rheology of fermentation fluid was investigated in chapter 6. From the combination results of the last six chapters, in chapter 7, there are some suggestions for improving the mixing during the large scale fermentation of *A.oryzae* in stirred fermenter. Finally, in chapter 8, important conclusions of all researches in

this thesis have been shown briefly, also several suggestions were recommended for process intensification of fungal fermentation using batch stirred fermenter.

1-6 Scope of the thesis

The main idea behind this work is to study the feasibility of first; using optimal mixing state in stirred tank bioreactor for process intensification of stirred - batch fermentation of *A.oryzae* at laminar regime to increase the activity of alpha amylase. Finally, it could be concluded that which agitator would be efficient for mixing the non-Newtonian complex bio-fluid and show better adoption with the biological culture containing fungal cells. As was shown in **Fig. 1-3**, the main aim of using large cross-section impellers and flexible-shaft is improving the most important parameters for fermentation intensification such as; mechanism of mass transfer, controlling the environmental stress, controlling the culture viscosity by controlling the macro- and micro-morphology, and also waste biomass reduction. Because, improving most of these factors are considered as a representative of process intensification (PI) during submerged fermentation.

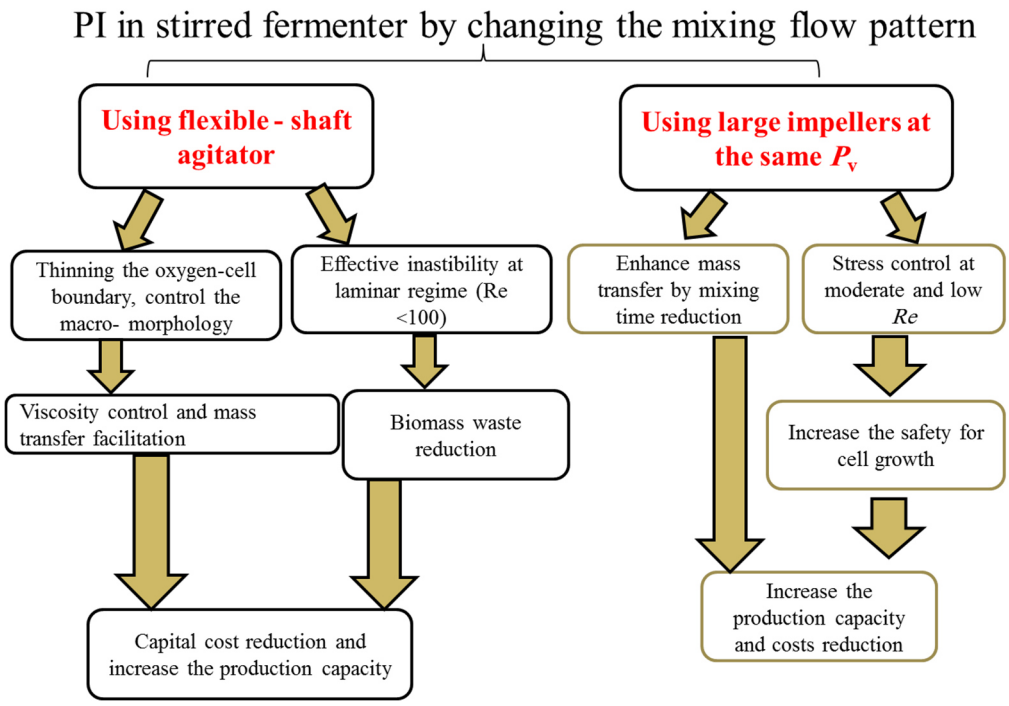


Fig. 1-3 Strategy used in this thesis for PI of stirred-batch fermentation of *A.oryzae*.

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Chapter 2: Materials, experimental and analytical methods

2-1 Materials, experimental set up and methods of fermentation of Aspergillus oryzae in stirred-batch bioreactor

2-1-1 Strain and inoculum preparation

The microorganism used in the present study was wild type *A.oryzae (OSI1013)*. The fungus was maintained in petri dishes of agar. After inoculation, the dishes were incubated at 30 ºC for 5-6 days and subsequently stored at 4 ºC. A suspension of spores was obtained by washing the petri dish cultures using a sterile aqueous solution of Tween-80, 0.05 wt %, (Polyoxyethylene (20) Sorbitan monooleate, Wako Co., Kyoto, Japan). The number of viable spores in the suspension was determined using a hemocytometer (Bürker Türk) (NanoEnTek Inc., Gyeonggi, Korea). The inoculum of *A.oryzae* was prepared in 100 mL Erlenmeyer flasks containing 15.0 mL of nutrient broth with 1.5×10^7 spores mL^{-1}. The flasks were sterilized in an autoclave at 121 °C (10^5 Pa pressure) for 15 min. The medium was aseptically inoculated with suspended spores. The flasks after inoculation were incubated for 3 days on an incubator shaker at 30 °C and 200 rpm.

2-1-2 Fermentation experiments and fermenter configuration

The fermentation experiments for the production of alpha amylase from *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 inner diameter, *H* of 0.114 m, with a flat bottom and a broth height to a vessel diameter ratio of 1.3. It was shown in **Fig. 2-1**.

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 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 up of the following (in g/100mL): Glucose, 3; KCl, 0.2; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.05; peptone, 1.0; yeast extract, 0.5 (all from Wako Pure Chemical Industries, Osaka, Japan); and, soluble starch, 10 (Nacalai Tesque, Co., Kyoto, Japan). The medium was added to a fermenter and sterilized in an autoclave, then it 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-20 μm bottle-top filter (Non-pyrogenic and sterile filter, Corning Inc., California, USA), and the supernatant was assayed for alpha amylase activities.

2-1-3 Geometrical design and size of impellers used in STBR

It is obvious that the structure of the impeller determines the mixing performance. In this study, agitation was provided by four different impellers: (a) the DRT, (b) the Maxblend® (MB), (c) the Fullzone® (FZ), and (d) the Swingstir®. The information about the configuration, geometrical design and location of impellers and other mechanical equipment were explained and illustrated as below.

2-1-3-1 Details of geometrical design and mixing properties of DRT impeller

The DRT had *d/H* ratio of 0.38 and *W/d* ratio of 0.2. 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. The other details of the DRT impeller is shown in **Fig. 2-2(a)** and Appendix 1 and 4. The impeller speeds were varied from 50 to 500 rpm.

Fig. 2-1 experimental set up of submerged fermentation of *A.oryzae*; (1): fermentation culture, (2): external jacket, (3): impeller shaft, (4): PH sensor, (5): DO sensor, (6): air filter, (7): anti-foam, (8) alkaline, (9): acid.

2-1-3-2 Details of geometrical design and mixing properties of Maxblend® impeller

The MB impeller had a *d/H* ratio of 0.54 and a *W/d* ratio of 2.35. The other details of MB impellers and fermenter configuration are shown in **Fig. 2-2(b)** and Appendix 4. The impeller speeds were varied from 50 to 500 rpm.

2-1-3-3 Details of geometrical design and mixing properties of FZ impeller

The FZ consisted of two large paddle impellers and the upper paddle was shifted at 45° in the rotating direction. The other details such as size and geometrical condition of the two impellers along the shaft of fermenter were shown in **Fig. 2-2(c)**, Appendixes 1 and 4. The impeller speeds were varied from 50 to 500 rpm.

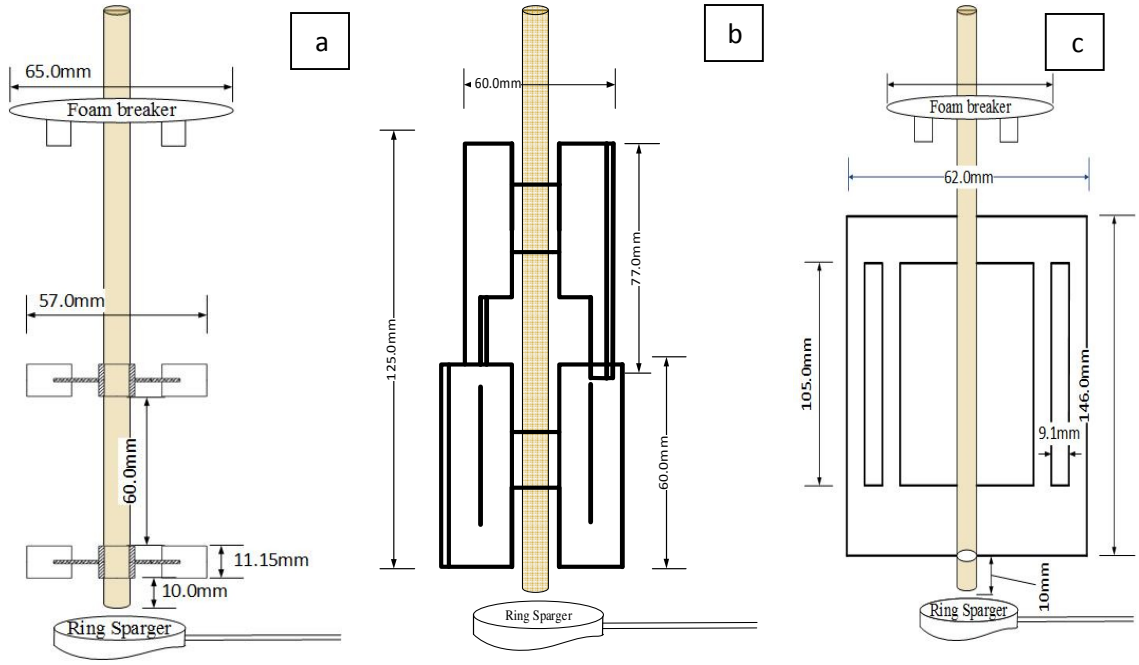

Fig. 2-2 Details of geometrical design of (a) DRT, (b) MB, and (c) FZ impellers used in this study.

2-1-3-4 Details of geometrical design and mixing properties of Swingstir® impeller

Swingstir® (Kobelco Eco-solutions, Ltd. Kobe, Japan) [1], was designed by a flexible shaft for the reason of improvement of accessories and inhabitation of contamination, avoiding the microorganism from exposing to high shear stress, and decreasing the viscosity of biological fluids in a complex bio-process. Flexible sealing of Swingstir® can absorb the negative displacement of agitator and reduce the high-pressure force. Swingstir® is a shaft with eccentric transmission ability attached to the reduction gear located at the output of the shaft. The eccentric transmitted from a bearing to the stirring could convert the cultivation medium from the rotational to the arbitrary movement [1, 2]. Impellers can have important effect on preventing from adherence the cells to the shaft and unwanted growing the microorganism in the fermenter. Cross sectional shape of the flexible seal was shown in **Fig. 2-3**, Appendixes 1 and 4.

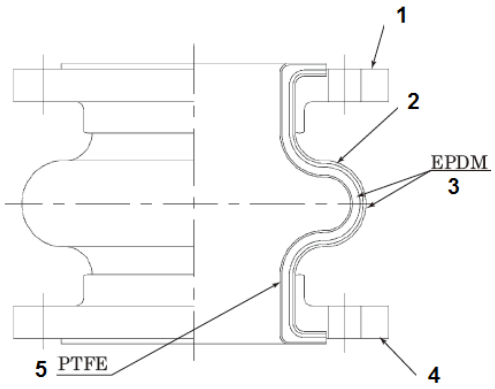

Fig. 2-3 The flexible cross sectional seal of Swingstir®; 1: circling side flange, 2: strength fiber, 3: EPDM (Ethylen-Propylen) rubber, 4: the fixed side flange , 5:inner side PTFE (Poly-tetra Fluoro Ethylene) [recommended to us by Kobelco Eco-solutions, Ltd. Kobe, Japan].

The inner surface of the flexible seal has excellent corrosion resistance. In order to impart the pressure resistance in its outer surface a reinforcing fiber has been attached. The reinforcing fiber was prepared from EPDM (ethylene propylene diene monomer) rubber.

2-1-3-5 Geometrical design of flexible-shaft impeller

The Swingstir® was composed of three wings. Length of each blade was 0.45 m. lower and upper width of the blades were 0.005 and 0.015 m respectively. Length of the flexible-shaft together with the impeller was 0.161m (**Fig. 2-4** and **Table 2-1**).

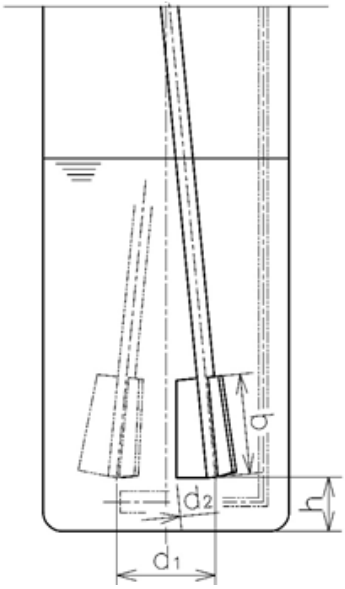

Fig. 2-4 Illustration of geometrical design of Swingstir® impeller used in this study.

Table 2-1 Geometrical properties of impellers used in this study

Parameter	Value [m]
d_1	0.046
d_2	0.036
h	0.025
b	0.046

2-1-4 Statistic efficacy

It was noticed that, all of the obtained data were extracted by doing at least four times independent experimental processes. Each of repeated experimental process and analysis was done at the same condition.

2-1-5 Analytical methods

2-1-5-1 Alpha amylase activity assay, glucose concentration and dry cell weight measurement

Alpha amylase activity was measured for a 1.0 mL fermentation culture containing 0.5 mL of 2.0% (w/v) soluble starch in 0.1 M phosphate buffer (pH 7.0) and the enzyme solution. The reaction was carried out for different intervals at 30 °C, and the reducing sugar produced was determined *via* the dinitrosalicylic acid (DNS) method with glucose as the standard. One unit of the enzyme was defined as the amount of enzyme that would produce reducing sugars corresponding to 1 μmol of glucose from soluble starch in 1 minute under the assay conditions. The culture samples were also analyzed to assay the quality of the glucose. This was determined by 3, 5-dinitrosalicylic acid reaction, spectrophotometrically at 540 nm [3, 4]. Alpha amylase activity and glucose concentration were measured a minimum of 5 times during each sampling. Average of these values was recorded with standard deviation as the data shown in the diagrams.

2-1-5-2 Dry cell weight measurement

Biomass were measured in units of dry cell weight (DCW). The fermentation broth was diluted up to 5 times and filtered. The cell pellet was re-suspended and washed with 20 mL

distilled water and filtered again. The pellet was then transferred to a pre-weighted plate and was dried in an oven at 100 °C until reaching a constant weight.

2-1-5-3 Macro- and micro-morphology analysis (Pellet diameter, hyphae length and hyphae diameter (thickness) measurement

Broth samples were diluted by distilled water then filtered, and this process was performed 3 times followed by storage at 4 °C until analysis. Then, the pellet diameter per sampling was calculated using Hakuran software after taking the photos of pellets using digital camera. In this study, pellet diameter was defined as the total diameter of the hairy portion and the core zone (**Fig. 2-5(b)**). After each sampling, four hundred pellets were taken from the sample. Then diameters of the pellets were measured. The average diameter of pellets, D_p, was calculated as was shown in Eq. (2-1).

$$D_p = \frac{1}{n}\sum_{i=1}^{n} d_i \qquad (2\text{-}1)$$

where n is the number of pellets counted, and d_i is the diameter of each pellet. It was noticed that 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 microscopic morphology parameters. Images of the pellets during each sampling were taken with a digital camera (12.0 Mega Pixels, HDR-XR500, SONY Co., Tokyo, Japan).

The hyphae length and hyphae diameter (**Fig. 2-5(a)**) were measured for more than 300 ~ 400 cells during each sampling. Besides for analyzing the fungal micromorphology at least 200 pictures were taken and on each picture the diameter (thickness) and length of the hyphae was measured 3 times, then average and standard deviation were determined.

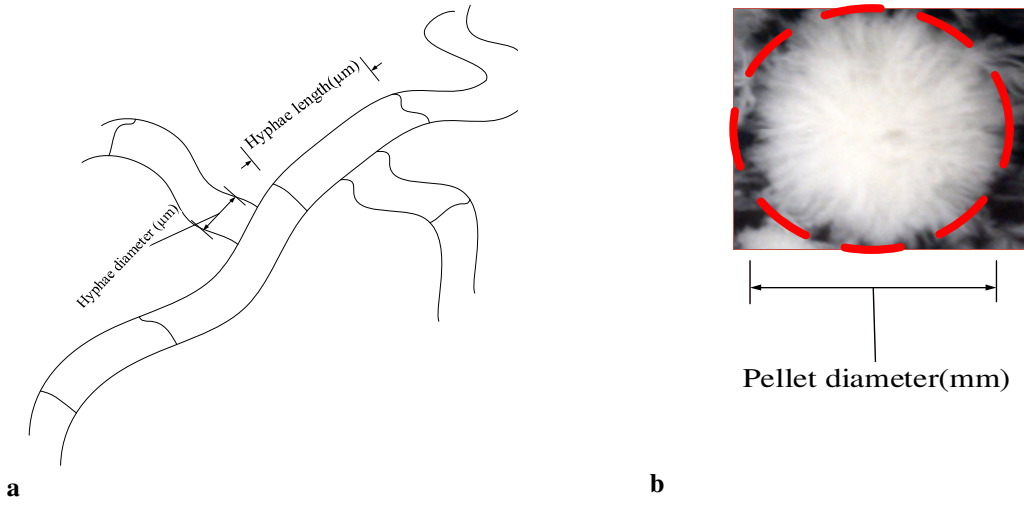

Fig. 2-5 Illustration of (a) micro- and (b) macro-morphology parameters measured in this study.

2-1-5-4 Pellet-cell porosity measurement

To investigate effect of hydrodynamic of fermentation environment on the surface morphology of fungi, pellet porosity (ε_p) of cells was measured. It was measured by recording the settling time of more than 300 cells per sampling in a tube of water with 17 cm length at 25 °C and allowed to settle. The average velocity (u) of the pellets was calculated. Then the apparent density of the wet pellets (ρ_{ap}) was calculated according to Stokes's Law [5] in Eq. (2-2):

$$u = 0.27\sqrt{\frac{D(\rho_{ap} - \rho_w)gRe^{0.6}}{\rho_w}} \qquad \text{for } 1 \leq Re \leq 1000 \qquad (2\text{-}2)$$

where ρ_w (kgm^{-3}) is the water density. It was noticed the water has a density of 997 kgm^{-3} at 25 °C, and the wet hyphae ($\rho_{h,w}$) can be assumed to have a density of 1100 kgm^{-3} [6] thus, from the apparent density of wet pellets calculated above, the porosity of the pellets (ε_p) was estimated from Eq. (2-3):

$$\rho_{ap} = (1 - \varepsilon_p).\rho_{h,w} + \varepsilon_p.\rho_w \qquad (2\text{-}3)$$

The calculated ε_p is representative the comprehensive features of a pellet's structure (dense or loose) and surface conditions (smooth or fluffy, hairs being long or short). Loose and fluffy

pellets would have higher porosity values. It would give relative information of differences in the pellet structure under different fermentation conditions [7].

2-2 Torque measurement of fermentation culture

In this study the torque of fermentation culture was measured by two methods. Because, firstly we need to measure the initial and average torque of fermentation culture to calculate the P_{in} and P_{ave}. The other investigation was measurement the final torque of fermentation culture (after cell growth) calculate the P_v. Based on above explanation the method of torque measurement was done as below;

(a) According to the Eq. (2-4), and N_P ($=P/\rho N^3 d^5$), the power number of mixing system was depended on the torque measurement. During the fermentation by growing the cells, the torque value of culture has been changed. Due to this reason, in each hour the torque of mixing system was recorded by the software joined to the fermenter (the torque recorded at initial hours were used for initial power consumption measurement).

$$P=2\pi NT \tag{2-4}$$

Afterwards the average value of these recorded data was measured as an average torque of process. Using above experimental data the P and N_p of bulk of the fermentation culture were measured.

(b) To measure the torque with high accuracy, the final torque of fermentation culture was measured by attaching the fermentation jar to the SATAKE torque meter (ST-3000 Ⅱ SATAKE Chemical Equipment MFG., LTD., and Japan). This torque meter measured the torque with a high-precision (Appendix 2). With a maximum torque of 0.32 Nm, it supports every level of mixing from very low- to mid- and high-viscosity.

It is important to notice that in the case of using Swingstir®, measurement of torque with high accuracy (using SATAKE torque meter) was not possible. This is because of the limitation of attaching the unique shaft of this impeller to the torque meter.

2-3 Power consumption and power number measurement

The power input, P_{in} [W], was measured via the torque sensor (because the transported energy to the fermentation liquid was only applied by the impeller) and calculated as shown in Eq. (2-4), where N is the impeller rotational speed [s^{-1}] and T is the initial torque [Nm].

2-4 Hydrodynamic and mixing parameter measurements during fermentation in batch-stirred fermenter

2-4-1 Viscosity and rheological model of fermentation culture

Viscosity of non-Newtonian fermentation culture after each sampling was measured using B type viscometer (Model B8L, TOKIMEC INC. Tokyo, Japan, the rotor NO. 1 at 0.6 rpm was used). In the Model B viscometer main case houses a synchronous motor. Motor rotation is transmitted to the rotor through a spring. Viscous frictional torque acts on the rotor immersed in the fluid and the rotor will rotate at a steady speed when equilibrium between the torque and spring force is achieved (see **Fig. 6**, Appendix 2). The size of the torque is indicated as an angular deviation of the pointer fixed to the rotor shaft and is proportional to the reading on the dial directly connected to the motor shaft. Absolute viscosity is determined by a conversion factor based on this reading. The technical properties of BL viscometer were shown in **Table 2-2**.

Table 2-2 Technical properties of BL viscometer.

Model of viscometer	Torque measurement (full-scale torque)	Viscosity measurement range
BL	67.4 μNm	0.001 ~ 100 Pas

It was noticed that viscosity of fermentation culture in Newtonian interval (0 ~ 24h) and the other Newtonian fluids which were used in this study was measured using HAAKE^{TM} viscometer.

In many previous studies on the rheological model extracted for fungi such as submerged fermentation of *A.oryzae* or *A.niger*, the rheological model has been compatible with the power-law model [6, 8-9]. In the present study cell adherence and some problems with dense pellet cells caused unwanted fluctuations while working with the rheometer (HAAKE^{TM} viscometer-550, Thermo scientific, USA) to measure shear stress versus shear rate. Rheological properties of the fluid were measured at 30 ºC in a rheometer. Rheogram were obtained by means of NV and NV-SD rotor. The rheological data were fitted to the Ostwald de Waele model having high regression coefficients. However, these measurements showed that after t = 24h the behavior of culture completely changed from Newtonian to non-Newtonian (shear-thinning), but finding an accurate value for constant parameters of the power-law models was difficult. This is because the fermentation broth was strongly thixotropic fluid. The power-law model of Tang et al. [10] was compatible with the overall and average rheological behavior of culture in this study and was used as an average rheological model of fermentation culture in some of the fermentation conditions. The calculated data follows the Ostwald-de Waele model with n = 0.3, K = 2.5 Pa s^n and (RT case), 1.82 Pa s^n (when using an impeller by the flow pattern similar to that of the MB or FZ). Also, these values were used for average fluid flow simulation. It was noticed that the K_{app} and n_{app} values calculated after each sampling time were considered as the apparent consistency index and flow index of cell-suspension by increasing the biomass and passing the fermentation time. Discussion on the apparent values of power-law model was seen in chapter 6. It was noticed that in the fluid flow, shear stress or viscosity simulation in stirred fermenter at special sampling time, the apparent K_{app} and n_{app} values was used as an input rheological model.

2-4-2 Theoretical method of effective viscosity calculation

For non-Newtonian fluids, the effective viscosity (μ_{eff}) could be defined by the definition used in the Metzner and Otto method [11] shown in Eq. (2-5).

$$\mu_{\text{eff}} = K\dot{\gamma}_{\text{ave}}^{n-1} \tag{2-5}$$

In this equation, μ_{eff} is the effective viscosity (Pa s), and K and n are the consistency and flow index for power-law fluids.

2-4-3 Re measurement

The Re ($= \rho ND^2/\mu$) of fermentation culture was measured using the experimentally measured viscosity. The average Re values during each sampling was shown in Appendix 2.

2-4-4 N_p measurement

The relationship between the impeller rotational speed, N, and the power consumption was shown by the correlation between the Re ($= \rho Nd^2/\mu$) and the power number, N_{P} ($=P/\rho N^3 d^5$) [12]. The glycerol ((99% (mass/mass), Wako Co., Kyoto, Japan) and water were used as a Newtonian fluid. The power numbers of the fermentation culture, as a non-Newtonian fluid, were calculated at different shear rates, and were measured indirectly from the curve of N_{p} against Re.

2-4-5 Velocity and shear stress simulation

Analysis of the flow velocity using different impellers was performed using the fluid dynamics software "R-FLOW" (R-flow Co., Ltd., Saitama, Japan) based on Navier-Stokes equations. In the present study, the Eulerian two-phase model was used to model the gas-liquid flow in a stirred bioreactor. According to the results of viscosity measurement (Chapter 6, **Fig. 6-1**), most of the fermentations were operated in high-viscosity and relatively low Re condition.

Due to a thixotropic behavior of culture (Chapter 6, **Table 6-3**), the maximum and minimum and average viscosity of culture obtained during fermentation was used as input data

to simulate the fluid flow at average *Re* during different mixing condition and this is one of the difficulty of simulation in the present study. In the case of simulation at determined fermentation time the μ_{app} of fermentation culture at defined sampling time was used for simulation and calculating the *Re*.

The cell-fluid interactions were ignored, because these would have been difficult to monitor simultaneously with the gas flow in the tank. We noted that during the simulation, the air-flow rate was the same as that for the fermentation experiment. The gas phase was set as that of 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 a flow-velocity field, uses the mass balance continuity and momentum equation, as follows (Eqs. (2-6) and (2-7)):

$$\nabla.(\rho\vec{v}) = 0 \tag{2-6}$$

$$\rho\vec{v}\cdot\nabla\vec{v} = \mu\nabla^2\vec{v} - \nabla P + \rho g + F \tag{2-7}$$

where ρ , p , v , g , F are the fluid density, pressure, velocity, gravity and external force respectively. F ($F = F_{TD} + F_{D,lg}$) is defined as an interface force between different phases and is defined as assumption of turbulent drag force (F_{TD}), (It was zero in this study because non-turbulent, low-*Re* flow simulation) and $F_{D,lg}$ is the drag force between gas-liquid phases. The $F_{D,lg}$ was calculated according to the Eq. (2-8). Where, α_d, α_c, d_b and C_D are dispersed phase volume fraction, continuous phase volume fraction, bubble diameter and the drag coefficient of one bubble against the fluid, respectively. The drag coefficient defined in Eq. (2-9).

$$F_{D,lg} = \frac{3}{4}\alpha_d\alpha_c\frac{\rho}{d_b}C_D|\vec{v}_c - \vec{v}_d|(\vec{v}_c - \vec{v}_d) \tag{2-8}$$

It is noticed that C_D is estimated with the formula of the resistance near the bubble as can be seen in Eq. (2-9) and (2-10). In Eq. (2-10), ρ_c, v_d, v_c and μ_d are the density of continues phase, velocity of dispersed phase, velocity of continuous phase and viscosity of dispersed

phase respectively. For the two fluid models, the continuous (fermentation culture) and dispersed (bubble) phases are separately expressed.

$$C_{\mathrm{D}} = \max\left\{\frac{24}{Re_{\mathrm{b}}}(1 + 0.15Re^{0.687})/Re_{\mathrm{b}}, \frac{8}{3}\frac{E_{\mathrm{O}}}{E_{\mathrm{O}} + 4}\right\} \quad (2\text{-}9)$$

$$Re_b = \frac{\rho_c|\boldsymbol{v_d} - \boldsymbol{v}_{\mathrm{c}}|d_{\mathrm{b}}}{\mu_{\mathrm{d}}} \quad (2\text{-}10)$$

2-4-6 Visualization of flow pattern of mixing

Visualization of flow pattern of steady-state mixing using Newtonian and non-Newtonian fluids using DRT, MB, FZ and Swingstir® impellers were done. The gas-liquid stirred tank at laminar flow was illuminated by laser slit beam, (SUWTECH laser, LCD-1500 (SHANGHAI UNIWAVE Technology, China)). Glycerol ((99% (mass/mass), Wako Co., Kyoto, Japan) and CMC (2% (w/v), Carboxymethylcellulose, Sigma-Aldrich Co. LLC., μ = 0.096 ± 0.005 Pa s, ρ = 1149 kgm^{-3}), were used as a Newtonian and non- Newtonian fluid respectively. The air-flow rate, temperature, and P_{v} of each study was adjust the same as that for the fermentation experiment.

2-4-7 *Average and maximum shear rate measurement using Metzner and Otto method*

After growing the cells and producing non-Newtonian cell suspensions, it was useful to show the shear rate in the fermenter via a single parameter such as the fluid dynamic behavior. Many of the terms used in this field were proposed by Metzner and Otto [11]. They proposed Eq. (2-10) to estimate the average shear rate ($\dot{\gamma}_{\mathrm{ave}}$ [s^{-1}]), in a non-Newtonian culture.

$$\dot{\gamma}_{\mathrm{ave}} = kN \quad (2\text{-}10)$$

Where k is the constant used by Metzner and Otto [11]. In this study, k = 11.5 was used for the DRT as in many other studies [13-15], and k = 20 was recommended to us for MB impeller by Sumitomo Heavy Industries, Ltd [16]. The shear rate constant depends only on the impeller

geometry under non-Newtonian conditions. Results of the effective shear rate by MB and DRT impellers were investigated using this method.

2-4-8 Theoretical method for average and maximum shear rate calculation using Brown correlation

During growing the cells and producing non-Newtonian cell suspensions, it was useful to the show the shear rate in the fermenter *via* a single parameter such as the fluid dynamic behavior. In this study, Eqs (2-11) and (2-12) proposed by *Brown* were used [17, 18]. He proposed these two equations to estimate the average and maximum shear rates ($\dot{\gamma}_{\mathrm{ave}}$ and γ_{max}) based on the impeller geometry in a STBR. These equations were selected because they precisely describe the effect of impeller geometry on the $\dot{\gamma}_{\mathrm{ave}}$ and γ_{max}. This is a correlation that suggests a direct dependence between the $\dot{\gamma}_{\mathrm{ave}}$ and γ_{max} values and *N*. According to these equations, the $\dot{\gamma}_{\mathrm{ave}}$ and γ_{max} values were significantly dependent on the geometry of the impeller. Here, d_{T}, *d* and *W* are the diameter of the tank, the diameter of the impeller, and the width of the impeller blade, respectively [17]. In this study this method was used for comparing the $\dot{\gamma}_{\mathrm{ave}}$ and γ_{max} in fermentation culture between DRT and FZ impellers.

$$\dot{\gamma}_{\mathrm{ave}} = 4.2N\left(\frac{d}{d_{\mathrm{T}}}\right)^{0.3}\frac{d}{W} \tag{2-11}$$

$$\gamma_{\mathrm{max}} = 9.7N\left(\frac{d}{d_{\mathrm{T}}}\right)^{0.3}\frac{d}{W} \tag{2-12}$$

2-4-9 Mixing time measurement using decolonization method for qualitative CFD validation and mixing efficiency

A discoloration technique is more suitable for using on a small scale. In this work mixing time measurements was done by decolonization method in stirred tank to investigate the effect of impeller on mixing efficiency. Method of measurement is as follows [19]; a sodium

thiosulfate and an iodine solution react, was used with concentrations 0.1M and 0.2M respectively. In order to color the water, 10mL of iodine solution was put into the stirred fermenter. After the flow had stabilized the sodium thiosulfate solution (12mL) was put into the tank. The decolonization process was video recorded. The mixing time was defined as the time taker for mixing liquid to become completely colorless. Mixing time (T_m) was considered as a non-dimensional quantity given by $N.T_m$. Here, N is the speed of impeller [s^{-1}]. Visualization method was done during mixing by four type of impellers at the same P_v in each fermentation condition.

2-5 Determination of K_La

There are a number of ways in which the value of K_La can be determined. The method is used usually depends on the system being studied. One of the ways is the static gassing out method [20, 21]. The method involves first de-aerating the liquid with an oxygen consuming substance (sodium sulfite). Once oxygen free, after autoclaving the fermenter, oxygen is then re-bowled into the fermentation liquid until reaching to the saturated concentration. The K_La was measured using Eq. (2-13). In this equation, C^* and C_L are the saturated and time-dependent DO concentration in fermentation culture [22].

$$\ln\left(\frac{C^*}{C^*-C_L}\right) = K_L a t \tag{2-13}$$

An important advantage of this method is that it can be used for different medium and does not involved chemical reactions which could affect the measurement precision [23]. Here, the average value of oxygen mass transfer coefficient was used in the next chapters. The maximum experimental error for K_La measurement in this study was up to 25 %, and each K_La values was measured using the experimental data in at least three independent experiments.

Symbols;

Nomenclature

C	Oxygen concentration in liquid medium	[ppm]
C_D	Drag coefficient	-
D	Pellet diameter	[m]
d	Impeller diameter	[m]
d_i	Diameter of each pellet	[m]
d_T	Tank diameter	[m]
F	External force	[N]
g	Gravitational acceleration	[ms^{-2}]
H	Internal vessel diameter of fermenter	[m]
k	*Metzner-Otto* coefficient	**-**
K	Consistency index	[Pa s^n]
K_La	Volumetric mass transfer coefficient	[h^{-1}]
k_s	Shearing constant	**-**
N	Rotational rate	[s^{-1}]
N_P	Non-dimensional power number	**-**
n	Flow index	**-**
P	Pressure	[Pa]
P_v	Power density	[Wm^{-3}]
Re	Reynolds number	**-**
T	Torque	[Nm]
T_m	Mixing time	[s]
u	Average velocity of pellet	[ms^{-1}]
W	Width of impeller	[m]

Greece symbols

μ	culture viscosity	[Pa s]
$\mu_{\mathbf{eff}}$	Effective viscosity	[Pa s]
ρ	Density of culture broth	[kgm^{-3}]
ε_p	Pellet porosity	-
ρ_{ap}	Density of wet pellet	[kgm^{-3}]
$\rho_{h,w}$	Wet hyphae density	[kgm^{-3}]
ρ_w	Water density	[kgm^{-3}]
$\vec{\boldsymbol{v}}$	Flow velocity	[ms^{-1}]
τ	Shear stress	[Pa]
$\dot{\gamma}_{ave}$	Average shear rate	[s^{-1}]
α	Phase volume fraction	-

Abbreviations

DO	Dissolved oxygen	[ppm]
DCW	Dry cell weight	[gL^{-1}]
FZ	Fullzone®	**-**
MB	Maxblend®	**-**
DRT	Double Rushton turbine	**-**
STR	Stirred tank reactor	**-**

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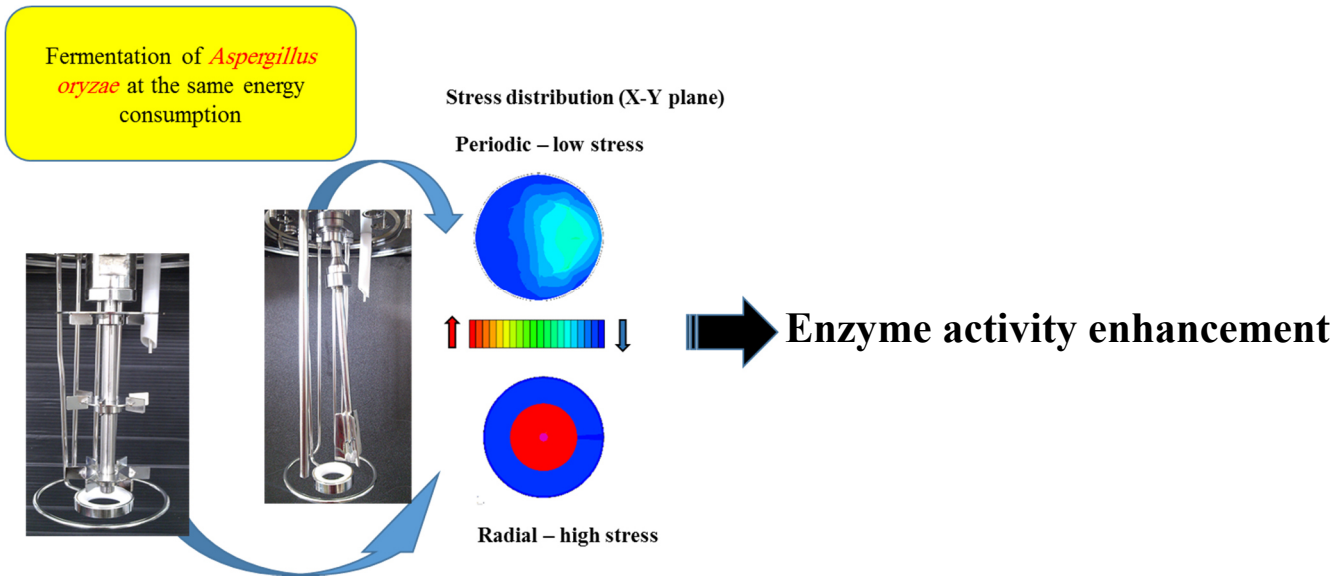
Chapter 3: Study the shear rate and shear stress intensification adopted with the mixing condition in fermentation culture of *A.oryzae*



Graphical abstract of chapter 3: [This picture was prepared by Narges Ghobadi, 2016]

Chapter 3 in brief; In this chapter shear rate and shear stress applied to the fungal cells at different mixing conditions have been studied. Finally the most adoptable agitator that produced low stress with effective shear rate (at low power consumption) has been proposed for submerged fermentation of *A.oryzae*.

3-1 Introduction

Fungal cells are considered as one of the complex microorganism and can be describe as a small factory. Therefore, it is impossible to determine a clear behavior of fungal cells, also explaining the hydrodynamics during fermentation in a bioreactor is difficult. Due to this reason, it is inevitable to do some simplifications [1]. For instance, it could be possible to concentrate on the agitator element and keep the other parameters constant in a stirred fermenter. Such an operation consists of homogenizing a medium in a vessel using a single rotating impeller [2]. Agitation speed of culture broth has a major effect on microorganism such as; damage to cell culture, morphological changes as well as variation in growth rate and product formation [3]. Besides, the shear force will deactivate filamentous mycelia. The magnitude of this deactivation depends on how large and how applied the mechanical force [1].

Intensifying the process of fermentation using mixing enhancement is an attractive approach for commercial interests and groups that work with biochemical products. According to the literature [4-7], achieving complete mixing in fermentation can result in an efficient mass transfer and a high quality and quantity of production. Many researchers have sought optimal conditions for agitation, because mycelial damage at high stirrer speeds or power inputs can limit the capability and volumetric productivity of a fermenter [8]. For the fragmentation of fungal pellets induced by different power inputs, there are 4 main mechanisms: interaction between pellets, turbulent eddies, the impact of the impellers on pellets, and collision among pellets. The interaction between pellets and eddies is expected to be the most important mechanism of fragmentation in turbulent regime. Therefore, study of the flow characteristics in a STR by focusing on power consumption is crucial to the design and scale-up of a fungal fermentation process [9-11]. One suggestion for mixing improvement is the usage of multiple impellers or some new integrated fermenters that will intensify the fermentation process [12]. Studies have shown that the length of a mycelial particle is decreased with increasing power

input per unit mass in a reactor as the increased agitation causes the hyphae to become shorter, thicker and more highly branched [13,14].

The use of standard impellers is often associated with poor bulk motion and an inhomogeneous distribution of the various phases [15]. In addition, Li et al. [5] implied that during the STR-fermentation of *A.oryzae*, agitated by three Rushton-style impellers, oxygen mass transfer occurred mainly in the vicinity of the impeller. Abdullah et al. [16] found that during the production of wild and mutant strains of *A.oryzae* in stirred fermenters, increasing the alpha amylase production had a positive correlation with increasing the mixing intensity by a Rushton turbine (RT) impeller. An improved impeller that was sufficiently flexible for submerged cultures would be an advantage in the design of an efficient enzyme production. One method would be enlargement of multi-stage agitators, and another would be the use of close-clearance designs such as anchors, helical ribbons or Maxblend® (MB). Here the various patterns of mixing have been applied to fungal fermentation. Due to this reason, the agitators as a key element for changing the pattern of mixing were described as follows;

3-2 Introducing MB impeller as a large agitator candidate for fungal fermentation

Hydrodynamic advantage of MB impeller is mixing at lower power consumption and dispersion in a wide range of *Re*. This is a wide impeller that combines a lower paddle and a grid. The paddle at the vessel bottom produces a strong tangential flow and a weak axial flow. It generates a strong recirculation at the vessel bottom that causes flow segregation. The grid part generates an axial pumping, with an upward motion at the vessel wall and a downward flow along the shaft. Common impellers, such as the Intermig, RT and PBT induce mainly radial and axial flows by the moving action of their blades, but in the case of the MB, the nature of the upper part of the impeller reduces the drag and promotes the formation of pressure

gradients. The pumping effect and centrifugal acceleration imposed by MB impeller to the surrounding fluid is minimal [17].

In terms of shearing constant ($k_s = N_p Re$), the MB impeller is more efficient than that of a turbine [2]. The MB is effective when mixing performance is important in non-Newtonian cultures such as with a fermentation broth. Sumi et al. [18] have investigated some mixing characteristics of the MB with highly viscous fluids and compared the performance with that of multistage impellers. They found that the MB created a more uniform solid suspension in comparison with other impellers. Kouda et al. and Hiruta et al. [19, 20] have employed the MB in fermentation processes under aerated conditions and showed the broth culture was kept very well mixed. Yao et al. [21] conducted a numerical investigation on dispersive mixing including the MB and a comparison with double helical ribbon impellers and indicated that the MB exhibited a satisfactory local dispersive mixing performance. Iranshahi et al. [15] performed an experimental study with a wedge-shaped MB impeller and showed that mixing time decreases with the reciprocal of the *Re* with Newtonian and non-Newtonian fluids in the laminar regime. In a study comparing large-scale impellers, Dohi et al. [22] revealed that the MB required the minimum amount of power consumption (*P*) and produced the most uniform solid suspensions**.**

In this study, at first, we have investigated the fermentation process of *A.oryzae* with two impeller configurations generating two distinct flow fields: one with traditional double RT impellers, generating high shear rates and a non-uniform shear rate distribution [11], and the other the straight type of large-scale impeller, Maxblend®, MB (Sumitomo Heavy Industries Co., Ltd., Tokyo, Japan), that does not produce significant radial or axial fluid motion directly [17]. The wide geometrical impeller was used to intensify the incubation parameters compared with the stirring of a 6-blade double Rushton turbine (DRT), as a multi stage small impeller. The DRT is the most frequently used impeller for mixing in aerobic fermentation processes.

At a given power input per unit volume, the DRT can create high shear stress to break air bubbles into small ones, and thus increase the mass transfer rate. However, when the viscosity of the broth is high, the DRT cannot be operated as effectively as in a water-like medium where the viscosity is relatively low. As a result, the overall fermentation efficiency is reduced.

3-3 Introducing FZ impeller as a large multi-stage agitator candidate for fungal fermentation

Optimizing the design and selection of efficient impellers has become a prominent pursuit that is eminently practical [23]. The main factor when selecting an agitator is the nature of the fluid. Biological cultures are usually non-Newtonian, highly-viscos and shear sensitive. To design a mixing system for biological fluids, agitators that are characterized by strong mixing and low power density are necessary to reduce production cost and to avoid high shear stress. Because most biological cultures use different working conditions to grow the cells, finding an appropriate impeller is a major challenge. This is because most of the conventional impellers and agitators are of the low-viscosity type. A method can be used to solve this problem [22], is the application of single-bladed impellers that are large in size. The impellers with larger blades are more competitive for their simple structures, easy dynamic seal and lower cost of operation and maintenance [24]. Large-diameter agitators are operated at low speeds and power densities, and normally provide excellent blending in highly viscos fluids [25]. Among the large cross-section impellers, Fullzone® (FZ) impellers (Kobelco Eco-solutions, Co., Ltd. Kobe, Japan) create a strong liquid recirculation loop. Flow pattern of the FZ impeller is characterized by global axial recirculation. Recent studies [26] showed that the FZ impeller had better homogenization properties in solid-liquid suspension compared with that of a triple small cross-section impeller. FZ impeller was used in studying the effects of a lipase-secreting bacterium on triacylglycerol degradation in a low-shear stress medium during uniform mixing [26]. Viscosity of biological fluid mixed by FZ agitator was lower than that of the radial multi

impellers [27], due to this point, the nutrient mass transfer and biological products has been increased. During agitation, the culture broth containing bacterial cellulose (BC) [28] (mixed by turbine impellers) was characterized by a high degree of shear. However, stress in the other type of large impeller [29], at low energy consumption is low but by increasing the power for mass transfer enhancement the shear stress and also viscosity was not low and controllable. It was seen that culture viscosity was high however the mass transfer was more efficient than that of the radial agitators. Therefore, using adaptable large impeller to control the shear stress and decrease the culture viscosity by increasing the agitation power is desirable.

The transported energy from the impeller to a fluid can have effects on the characterizations of the process, particularly in a power input (P_{in}) sensitive bioprocess. Agitated fermentation at low power is preferred due to the necessity of protecting the cell culture from damage, and minimizing cost. According to previous work [29] done on the submerged fermentation of *A.oryzae*, the main reason for decreases in the growth rate and enzyme activity during agitation by the DRT at low and high P_{in} was a lack of oxygen and mycelial inactivity. Agitation by other large impeller [28], at moderate and high P_{in} however the nutrient mass transfer has been developed but the cells was damaged and finally, enzyme activity was not significantly enhanced. The fermentation of *A. oryzae* is one of the more complex fermentation processes in terms of associated mixing, P_{in}, biomass, mass transfer, enzyme activity, and morphological property variations. In order to obtain an optimal method for agitation in a stirred fermentation process, the fermentation of *A.oryzae* was carried out on a laboratory scale for a STBR using FZ impeller. The primary objective of the present study was to use the FZ impeller to amass experimental data on the relationship between the flow patterns of mixing and fermentation parameters such as mass transfer, biomass production, and alpha amylase activity and cell macro-morphology. In addition, the pattern of shear rate and

shear stress using FZ impeller during fermentation were compared with that of the DRT impeller as a representative example of a typical small, radial multi-impeller.

3-4 Introducing Swingstir® as a flexible-shaft agitator for fungal fermentation

The optimization design and selection of efficient impellers have prominent and practical meanings, particularly in high-viscosity biological fluids [23]. Therefore, design and analysis of impeller system for this kind of bioreactor is crucial [30]. It is clear that different impeller combinations generate various shear environments, to which the fungal morphology of these microorganism can be sensitive, particularly at high shear zone next to the tips of the impellers [31]. Besides, important purpose of enhanced mixing in submerged fermentation industries are high quality product, reduce the risk of inputting the contamination, ease of cleaning, and reduce the negative shear force to the living cells and control the temperature in the bio-culture.

The easiest solution is to use multiple impellers, so that a higher fraction of the biological fluid can be agitated and the power given to the fluid is more evenly distributed. The multi-impellers can be of the same type, such as using three RTs together [7] or a combination of different impellers. Although a helical impeller does not normally create sufficient shear stress to break up the bubbles, it provides a global circulation of the broth with low energy consumption [31-33]. Besides, Elephant ear impeller (EE), has been proposed by many authors as suitable for cultivation of shear-sensitive cells such as animal cell and filamentous fungi [34]. EE is a better impeller than the of the RT for cell sensitive cultures by generating low shear conditions. The pitched-blade turbine (PBT), was produced a simultaneous radial and axial flow. Othman et al., [35] recommend to use the RT impeller combined with axial impeller, PBT, and showed the collision from the circulation loops of blades improves the mixing process.

In addition, a close-clearance impeller, whose diameter is almost as large as the bioreactor diameter, (such as the Maxblend impeller [28]) was used for mixing improvement in fungal

fermentation. Among the family of close-clearance impellers, the axial reciprocating plate impeller (ARPI) produced oscillatory axial movement by a stack of perforated plates. It was used in Karr columns, [36-37, 7] which are found in the pharmaceutical, and environmental industries. It has demonstrated some benefits in bioreactors, such as uniform distribution of the local mixing energy [38]. In addition, it was reported that the stagnant zones were eliminated when compared with agitation using a triple Rushton impeller (TRI). However, its axial movement would make it more difficult to be adapted in an industrial context [39]. Moreover, it was noticed that the dimension of large close-clearance impellers is quite near to that of the reactor and it leads to significant energy cost [40].

Therefore, one of the new approach is investigation on the shaft design of impeller to reduce the power consumption. The effect of the shaft position on the flow field in laminar regime has been experimentally investigated by Alvarez et al. [40] and found important changes in the flow structure and the major improvement in mixing behavior even at low eccentricity. The combinations of two eccentric impellers were studied for single-phase [41] and two-phase gas-liquid systems [42] with the Newtonian and non-Newtonian fluids. The authors showed that eccentricity improves the axial motion which becomes comparable to baffled configuration [43]. Alvarez [40] pointed out the un-baffled stirred vessels were not optimized with large waste of power, because of the presence of large poor mixing regions. Therefore, eccentric mixing is one of the methods of promoting mixing in vessels without baffles [44]. Because, eccentric position of the impeller increases in flow instabilities and axial flow within the laminar range of agitation. Cervantes et al. [45] suggested a disc impeller in eccentric position for the culture of suspended mammalian cell [46]. Additionally, Galleti et al. [43] found that strong circumferential flow and eccentric position of the shaft have generated vortex-shedding phenomena from the flow-shaft interactions. Zhang et al. [39] have intensified the mixing process of viscous Newtonian fluids in a small stirred tank using eccentric mixing.

Due to the past findings, [29], motivation of fungal cells to adhering the surface area in the fermenter is one of the important reason of existing dead and rigid stagnant zone during the fermentation. Therefore, most of the cells were adhered to the typical shaft and produced difficulty in oxygen mass transfer efficiency. Most of the last researches were focused on the advantage of mixing with eccentric shaft impellers but in this investigation, we focused on the centered flexible-shaft impeller (for power consumption, biomass waste and viscosity reduction) in mixing enhancement of the high-viscosity culture during submerged fermentation.

According to the Bagtzoglou et al. [47], parameters such as power input per unit volume, impeller tip blade, mixing time and oxygen mass transfer coefficient or concentration of oxygen strongly influence the fermentation process. Here, a novel flexible-shaft impeller is proposed for studying on the process characteristics and mixing improvement of fermentation of *A.oryzae* in an agitated bioreactor. Also mixing time, volumetric power consumption (P_v), oxygen mass transfer, biomass and enzyme production were studied experimentally. Last findings [29], showed that during mixing at low P_v, by typical shaft impellers by increasing the cell growth in fermentation culture morphology and viscosity was not easily controllable. However, controlling theses parameters can be effective in intensification of mixing and fermentation in homogene medium. Therefore, the main aim of this study is investigation on the possibility of using Swingstir® to control the viscosity, morphology and finally increase the alpha amylase productivity in fermentation culture of *A.oryzae* as a biological culture with complex non-Newtonian behavior.

3-5 Basic mixing characteristics of DRT, MB, FZ and Swingstir® impellers in stirred fermenter

Before doing the fermentation experiment, the general mixing properties of Newtonian and non-Newtonian fluids have studied to investigate the mixing behavior of different agitators when working fluids with different rheological behavior have been used.

As was seen in **Fig. 3-1**, the slope of N_p versus *Re* when using glycerol for *Re* less than 100 were relatively the same. It means that when using high density Newtonian fluid at laminar regime the N_p of DRT, MB, FZ and Swingstir® impellers at the same *Re* were independent on the pattern upon the mixing and impeller type. The results of correlation shown in **Table 3-1** indicates that at the same *Re*, N_p of DRT was higher than that of the other agitators. However, this difference is not so high. For example, at *Re* = 60 the N_p of four impellers were change from 9.12 by MB to 12.34 by DRT. The lowest N_p was belonged to the condition using MB. Finally, it could be concluded that investigation on the mixing efficiency of four types of agitator at laminar regime in both constant *Re* and constant power consumption conditions could be possible.

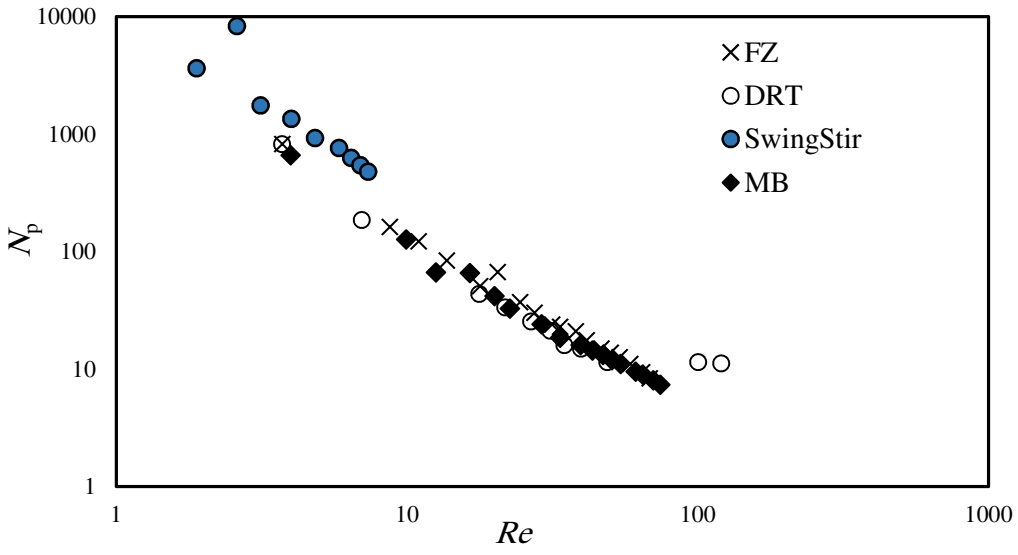


Fig. 3-1 N_p versus *Re* using glycerol at 30 ºC using different kinds of impellers.

Table 3-1 Correlations of N_p - *Re* for different impellers using Newtonian fluid (glycerol).

Impeller	Correlation	(*Re*: ~)	R^2
DRT	$N_p = 1963\ Re^{-1.238}$	3.7~120	0.883
MB	$N_p = 3674\ Re^{-1.465}$	3.97~74	0.987
FZ	$N_p = 4800\ Re^{-1.507}$	3.7~74	0.990
Swingstir®	$N_p = 18657\ Re^{-1.829}$	2.59~7.33	0.870

Investigation on the results of relation between N_p and *Re* for non-Newtonian (Xanthan gum solution 1% wt) fluid shown in **Fig. 3-2** indicates at the same *Re* (*Re* <100) the N_p of mixing system using different types of impellers were the same, and it was not dependent on the impeller type (This investigation was used to approve that N_p-*Re* diagram was unique and independent of kinds of fluid and the experimental values of N_p were reliable).

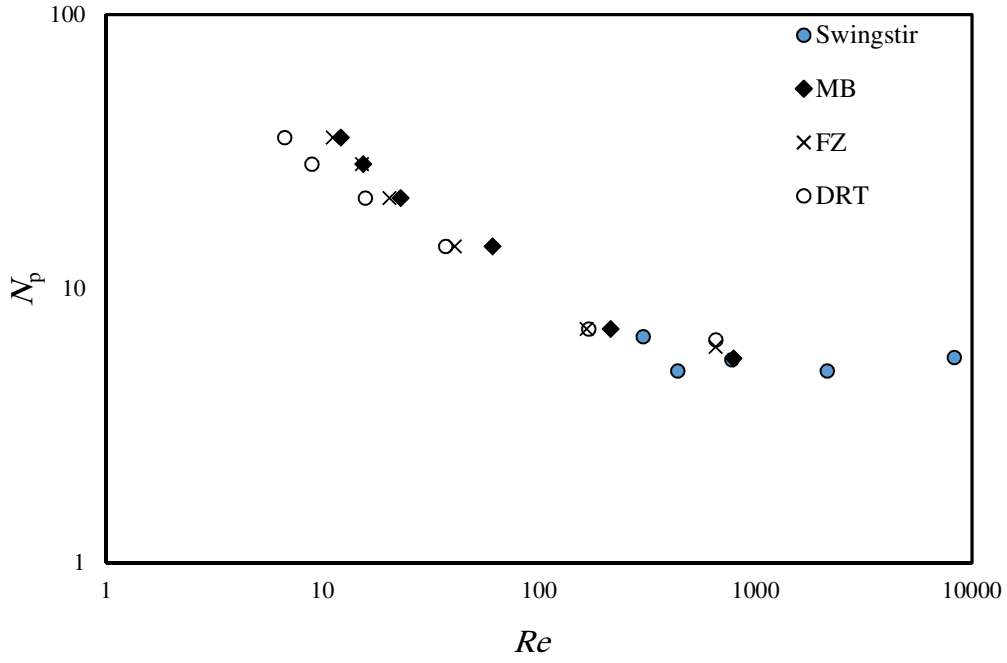


Fig. 3-2 Relation between N_p and *Re* using different impellers using Xanthan gum solution 10 gL^{-1} at 30 ^oC.

3-6 Study the shear rate during mixing the submerge fermentation cultures using STBR

Finding a knowledge about $\dot{\gamma}_{ave}$ and γ_{max} of fermentation culture during mixing with different agitator could be useful for finding the adoptable mixing system. In this study, effect of mechanical forces on cell has been done regarding to the calculation of $\dot{\gamma}_{ave}$ and γ_{max} during the fermentation. In this study for finding the $\dot{\gamma}_{ave}$ and γ_{max} , effect of geometrical design of agitators has been evaluated. Because aim of this thesis was the intensification of the

fermentation focusing on the mixing flow pattern and environmental hydrodynamic of high-viscosity fermentation culture.

3-6-1 Shear stress and shear rate in fermentation culture using large cross-section agitator

3-6-1-1 Properties of $\dot{\gamma}_{ave}$ and velocity distribution at different P_v in submerged culture using the MB and DRT

To investigate the effect of shear rate intensity by MB on fermentation fluid dispersion in fermenter, the flow velocity of fermentation experiment in shear-thinning condition has been simulated. The results of a velocity simulation (**Fig. 3-3(a)**) showed that when the P_v of mixing with the DRT was increased from 152 to 630 Wm^{-3}, the local velocity near the blade tip was increased from 0.31 ms^{-1} to 0.92 ms^{-1} (**Table 3-2**). Also, while the velocity distribution of the MB (**Fig. 3-3(b)**) was uniform at low P_v, the local velocity, or shear rate, for the DRT was changed. However, the P_v of the DRT (152 Wm^{-3}) was lower than that for the MB, but the local velocity at the center of the DRT fermenter, 0.18 ms^{-1}, was higher than it was for the MB (0.1 ms^{-1}). This local difference could result in a local difference in mass transfer and cell growth and could produce a stagnant zone near the tank wall. It is noticed that, besides of flow simulation, the gas-liquid flow pattern of mixing at the same P_v (similar to fermentation conditions) and aeration rate was visualized using laser beam (**Fig. 3-3(a)**). The formation of a stagnant zone at the bottom and wall of a fermenter results in cell fluidization, as shown in **Fig. 3-4**. At a high P_v when using the DRT, a high velocity profile covered the entire cross-section of the tank (0.31 ms^{-1}) and significantly damaged the cells. The comparison of fluid flow velocity (**Table 3-3**) with the recent literatures [17, 48-52] was shown as quantitative validation of flow velocity. Generally, the results of the $\dot{\gamma}_{ave}$, (were measured according to the Metzner and Otto method) agreed with the results from the velocity simulation. This means that by uniform changes in the velocity distribution when using the MB impeller at low P_v. It could be

useful for reducing the K_La gradient in the fermenter. It was shown [49] that the local K_La values in the mixing of Xanthan gum by a 2-RT near the bottom of the impeller were 1.4 times greater with a local K_La in the walls.

Table 3-2 Mixing parameters of submerged fermentation using DRT and MB impellers.

Impeller	$\dot{\gamma}_{ave}$* **(s^{-1})**	P_v **(Wm^{-3})**	**Maximum Flow velocity**** **(ms^{-1})**	**Theatrical impeller tip speed (ms^{-1}) (= πND)**	$Re_{ave,lam}$	N_p	**rps (s^{-1})**
DRT	19.2	152	0.31	0.35	77.43	76.97	1.67
MB	33.4	148	0.30	0.32	106.83	93.23	1.67
DRT	57.5	630	0.92	1.05	31.61	11.85	5.00
MB	100	687	0.90	0.97	162.7	9.6	5.00
DRT	95.5	1487	1.46	1.75	72.13	6.33	8.33
MB	166.0	1524	1.58	1.62	81.53	4.23	8.33

**:$\dot{\gamma}_{ave}$ was calculated using Metzner and Otto method

**: Maximum flow velocity (impeller tip) was used from simulation results by R-Flow software.

Table 3-3 Comparison between the results of flow velocity simulation in this study and literatures.

Impeller type	**Rheological Model of fluid**	**Flow regime- Medium**	**Velocity around the blade (ms^{-1})**	**Velocity Near the tank wall (ms^{-1})**	**Reference**
MB	Shear-thinning (n = 0.18, K = 33.1)	Laminar (Re = 30, 65, 120); Xanthan gum solution 3.5%	(0.33,0.38, 0.66)	(0.27, 0.41, 0.58)	[19]
DPP* 3-6ABDT** 3-6ABDT***	Shear-thinning (n = 0.71, K = 0.032)	Turbulent, (Re = 1156); fermentation culture of *S. avermitilis*	1.50 1.20 1.13	0.60 0.50 0.41	[48]
DRT	Newtonian	Turbulent	2.0	0.48	[48]
MB	Newtonian	Laminar - (Re = 32.4)	0.6 - 0.8	0.45	[51]
MB	Newtonian	Turbulent- (Re = 18000) (Solution of polyethylene glycol 25 wt%)	0.6	0.2	[17]
MB	Shear-thinning (n = 0.3, K = 1.8)	Laminar- fermentation culture of *A.oryzae* ($Re_{ave,lam}$ = 162)	0.85	0.36	This study
DRT	Shear-thinning (n = 0.3, K= 2.5)	Laminar- fermentation culture of *A.oryzae* ($Re_{ave,lam}$ = 31)	0.70	0.30	This study

*; down-pumping propellers

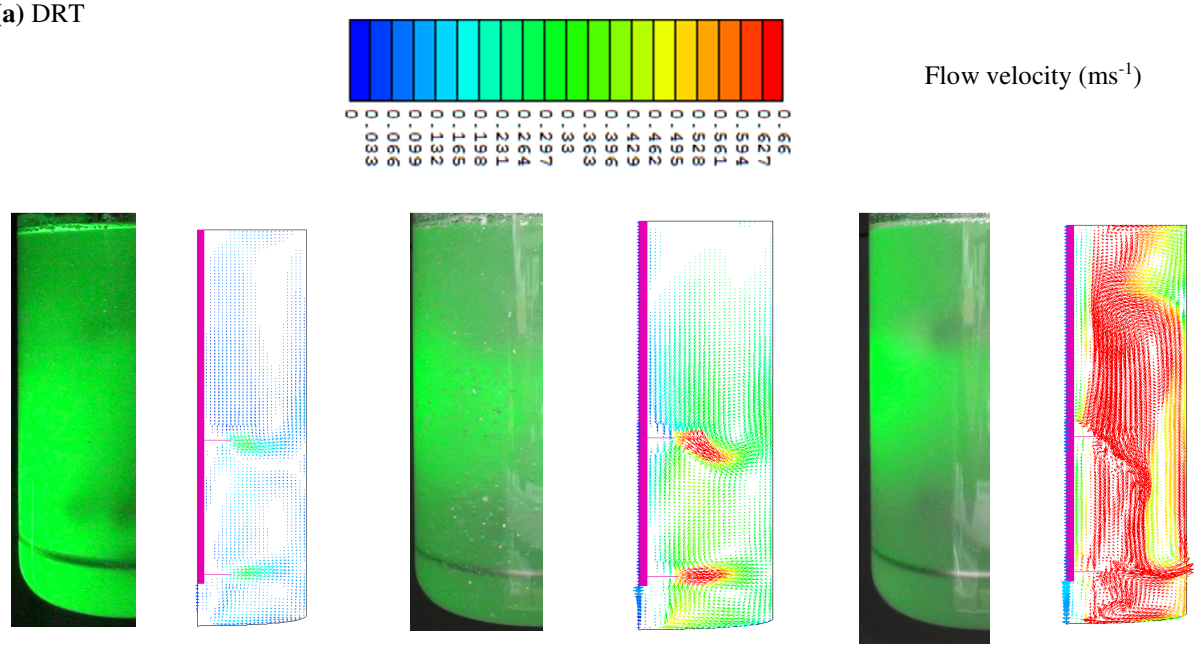
**;6-curved-blade disk turbine

***;6-arrowy-blade disk turbine

The results of the velocity distribution (**Fig. 3-3(a)**), show that mixing with the DRT at 630 Wm^{-3} produced a strong velocity profile near the impellers, and also caused a large degree of loop circulation between the blades, but the region near the shaft and at the inter-zone section of this big loop exhibited high viscosity during the fermentation. The local difference in velocity distribution when using the DRT resulted in a difference between the K_La and the viscosity in the culture. Under these conditions the cells cannot grow sufficiently to produce high enzyme activity. When using the MB impeller (**Fig. 3-3(b)**), however, the velocity distribution was roughly uniform, with the noted exception of a small region near the external grid. The velocity distribution at moderate P_v showed that the velocity of the fermentation culture when using the MB was uniformly distributed. The fluid velocity distribution study, also showed that the velocity of the culture near the wall when using the MB was more uniform than when using the DRT at moderate power consumption.

Besides, according to last studies [52] by increasing the working volume during mixing a non-Newtonian flow by increasing the scale of tanks, the P_v and dimension less mixing time exhibited similar behavior. It indicates that working principles of MB impeller is always the same but exhibits higher or low efficiency depending on the flow regime.

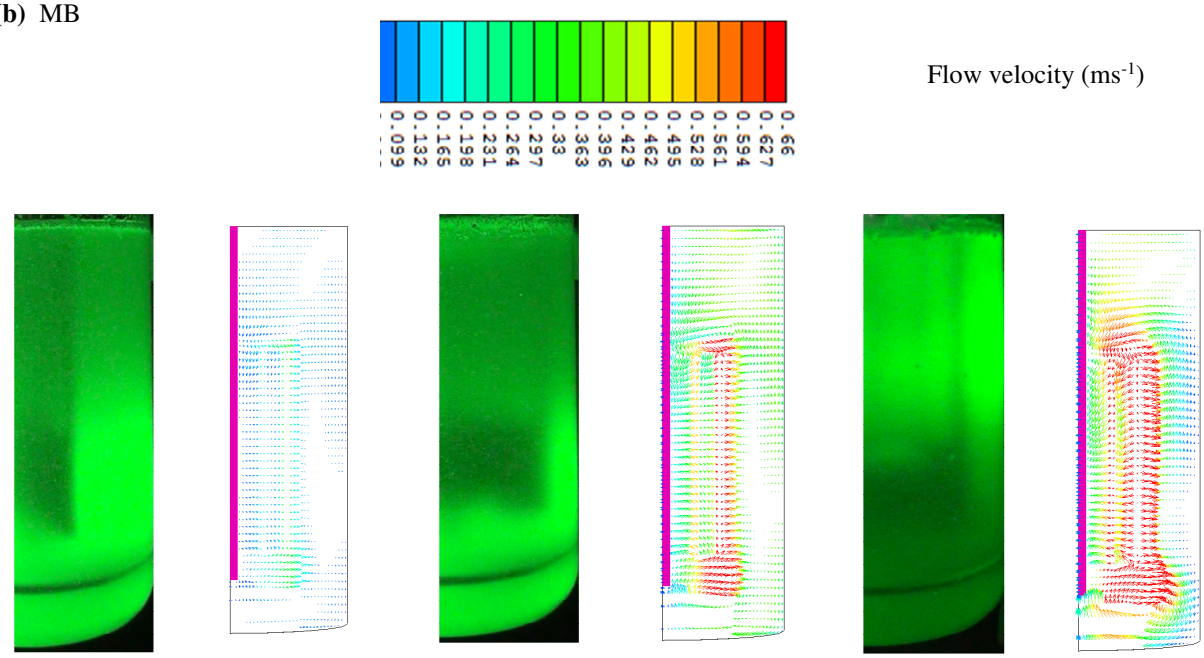
(a) DRT



Low and Same P_v | Moderate and Same P_v | High and Same P_v

N = 1.00 s^{-1} | N =1.67 s^{-1} | N = 3.10 s^{-1} | N = 5.00 s^{-1} | N = 5.17 s^{-1} | N = 8.33 s^{-1}

(b) MB



Low and same P_v | Moderate and same P_v | High and same P_v

N = 57 s^{-1} | N = 100 s^{-1} | N = 170 s^{-1} | N = 300 s^{-1} | N = 267 s^{-1} | N = 500 s^{-1}

Fig. 3-3 Simulation of velocity distribution combined with laser flow pattern visualization (using air-glycerol) in stirred fermentation by **(a)**: DRT **(b)**: MB, in (Y-Z) plane.

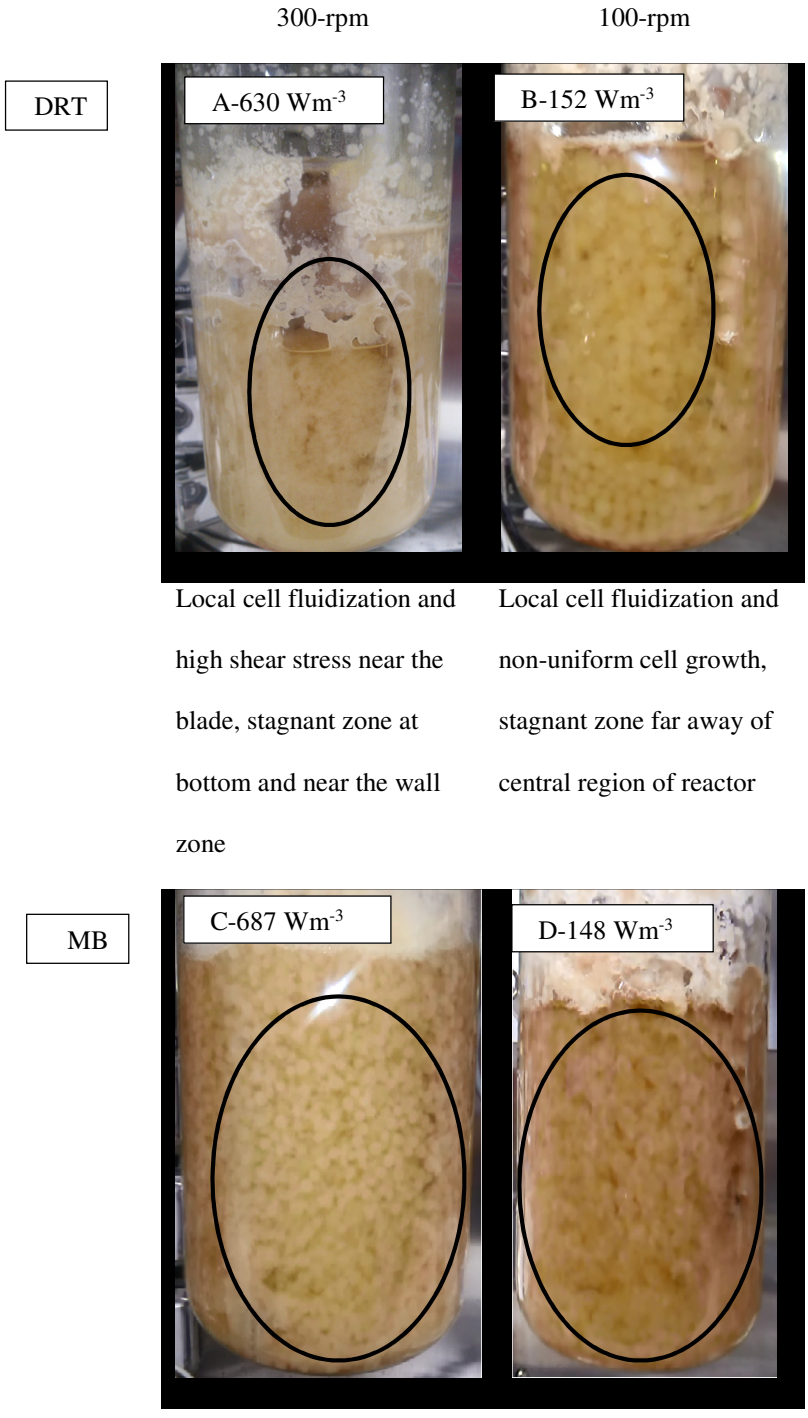


Fig. 3-4 Effect of agitation by the MB and the DRT on cell fluidization in the fermentation of *A.oryzae* in an image study at low and moderate P_v.

The strength of the applied shear and its frequency are important in determining whether disruption occurs [53]. To find how is the applied mechanical force on the culture containing

cells of *A.oryzae*, shear stress formed at t = 48h at P_v = 690 Wm^{-3} was simulated. It was shown in **Fig. 3-5**. As shown in **Fig. 3-5**, τ_{max} was located at the region closed to the outer grid part of impeller blade. The low stress regions were in the region near the tank wall and at the top of the fermenter. The applied stress during agitation with the MB at moderate and high P_v could have high risk of stress damage particularly near the impeller. Existing high stress gradient between the wall (4 Pa) and impeller vicinity (30 Pa) would be resulted in inactivity of fungal cells. Due to this reason, investigation on fermentation by a large blade with less shear stress gradient would be preferable.

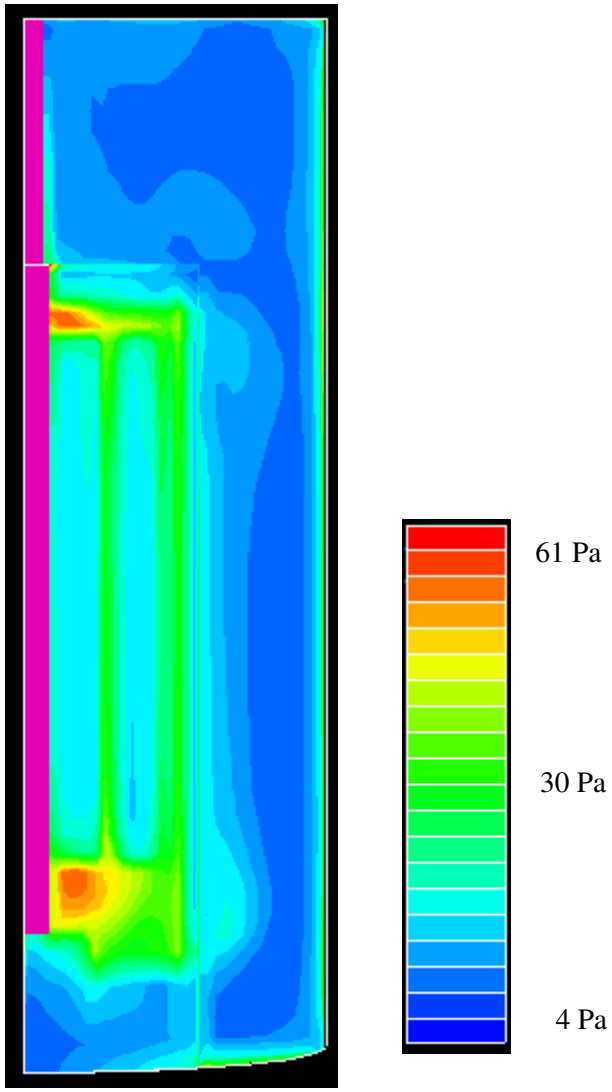


Fig. 3-5 Shear stress simulation during agitation by MB impeller at t = 48 h and P_v = 690 Wm^{-3}

3-6-1-2 Analysis the shear rate and stress formation using FZ as a fermenter agitator

To investigate the effect of mechanical force (when using FZ) moved from the agitator to the fermentation fluid, shear stress has been simulated in two mixing condition (**Table 3-4**). Results of stress simulation were shown in **Table 3-5**. Afterwards, to approve the results of stress simulation the $\dot{\gamma}_{ave}$ and $\gamma_{max}$when using FZ impeller were measured [54-55].

Table 3-4 Properties of samples studied during the fermentation of *A.oryzae.*

Impeller		P_{in} (W)	N_P	$Re_{ave,lam}$	Re^a	N (min^{-1})
Condition 1	FZ	0.42	110.85	35.75	12.20	100
	DRT	0.51	6.40	31.61	46.46	300
Condition 2	FZ	1.14	9.76	50.83	61.28	300
	DRT	1.80	5.50	72.13	76.94	500

P_{in}; Power input of process was calculated by measurement the initial torque produced by impeller at first hour of fermentation

Re^a; Effective *Re* of fermentation culture obtained from diagram of (N_p-*Re*)

$Re_{ave,lam}$; Average *Re* of fermentation flow during agitation in laminar regime (calculated using experimental value of viscosity in each sampling)

When the morphology analysis (**Fig. 5-11(a)** and **(b)** in Chapter 5) and the simulated shear stress contribution (**Figs. 3-6(a), (b), 3-7(a)**, and **(b)**) were combined, it showed that small pellets were formed by the higher τ_{max} (47.50 Pa in **Table 3-5**) in the bioreactor mixed with the DRT impeller. On the contrary, the largest pellets were found in the bioreactor agitated by the FZ impeller, wherein the τ_{max} was lower (9.07 Pa) and uniform. It is interesting to note that using the simulation results of **Table 3-5**, by increasing the P_{in} from 0.42 to 1.80 W when using the FZ impeller, the τ_{ave} remained roughly constant, and also the τ_{max} could be controlled. In addition, during increasing the P_{in} using DRT impeller the τ_{max} was increased from 11.68 to 47.50 Pa, and the enzyme activity and rate of cell growth during mixing by the FZ were higher than cultures agitated by the DRT at nearly the same P_{in}. The distribution of shear stress at low P_{in} when using the FZ impeller was significantly uniform compared with that by the DRT. At high P_{in}, the τ_{max} (11.68 Pa) when using the FZ impeller was located near the tank wall. No

blade tips or sharp edges were near the walls of the fermenter to damage the cells. In addition, the local high shear stress near the walls had the positive effect of removing the stagnant deposits from around the walls. When using the DRT at high P_{in}, the τ_{max} was clearly located at the sharp and 3D edges of the turbine blades (**Fig. 3-7(b)**), and this had a negative effect on the enzyme activity.

Table 3-5 Results of shear stress simulation during mixing in a fermenter using R-Flow software.

Impeller		Maximum flow velocity (m/s)	τ_{ave} (Pa)	τ_{max} (Pa)	Reference
Condition 1	FZ	0.31	2.37	9.07	This study
	DRT	0.89	1.70	9.53	This study
Condition 2	FZ	1.40	3.85	11.68	This study
	DRT	1.49	5.11	47.50	This study
DRT agitation rate = 200 min $^{-1}$ (Air-water)		0.76	τ_{yy} = 2.00 τ_{yz} = 0.40	τ_{yy} = 35.00 τ_{yz} = 10.00	[56]

Finally, it could be concluded that alpha amylase activity directly depends on the shear rate distribution in the tank. **Table 3-6** shows that use of the FZ enhanced the enzyme activity and DCW compared with a typical radial impeller such as the DRT. Using the FZ impeller is a good choice for fermentation intensification of *A.oryzae*, particularly at low P_{in}. For example, it is apparent that in the first 54h after inoculation of *A.oryzae*, the enzyme activity of cells agitated by the DRT was 2507 U/mL lower than that agitated by the FZ.

Table 3-6 Enzyme activity and DCW *via* $P_{in,\,54}$ during using FZ and DRT in the fermentation of *A.oryzae* during the first 54 h after inoculation.

Impeller		$P_{in,\,54}$ (W)	DCW(g/L)	Alpha amylase activity (U/ml)
Condition 1	FZ	0.31	8.20±0.30	5220±400
	DRT	0.89	5.01±0.90	2713±152
Condition 2	FZ	1.40	9.30±0.40	4700±119
	DRT	1.49	4.90±0.90	3305±200

$P_{in,\,54}$; Power input at t = 54h was calculated using the initial torque value recorded at t = 54h after the fermentation.

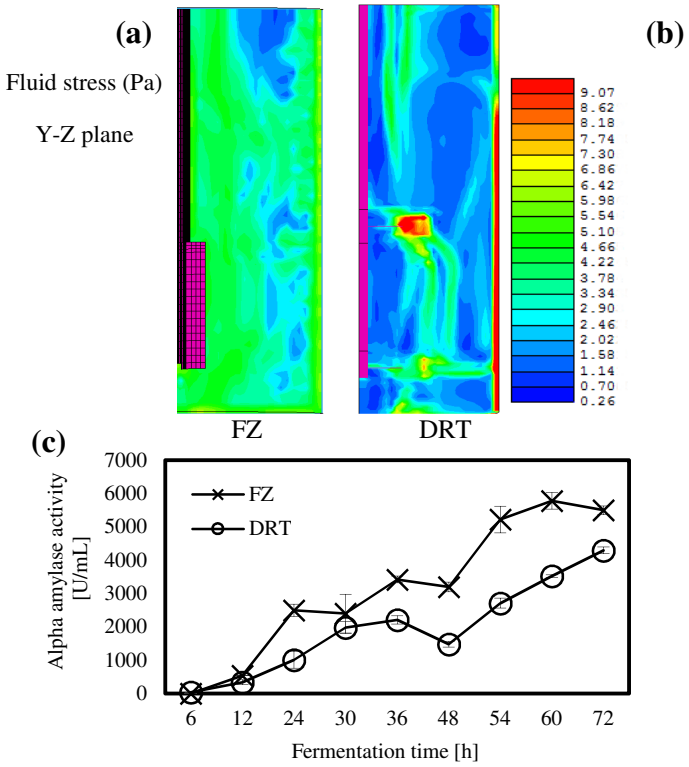


Fig. 3-6 Simulation of shear stress distribution during fermentation using (a): FZ and (b): DRT impellers at low P_{in}. (c): Alpha amylase activity during the fermentation of *A.oryzae* when using DRT and FZ impellers.

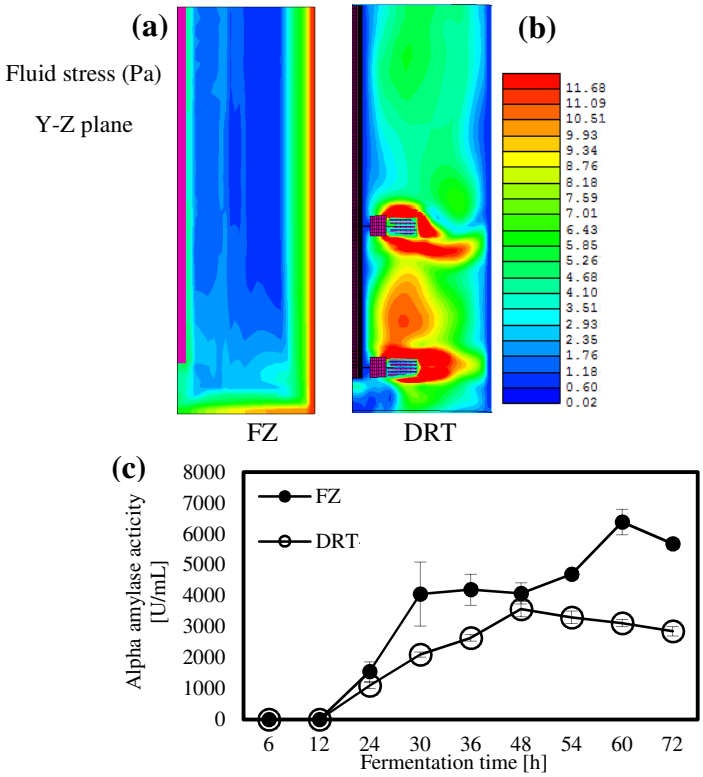


Fig. 3-7 Simulation of shear stress distribution during the fermentation using (a): FZ and (b): DRT impellers at high P_{in}. (c): Alpha amylase activity during fermentation of *A.oryzae* when using DRT and FZ impellers.

Additionally, when growing the cells and producing non-Newtonian cell suspensions, it was useful to the show the shear rate in the fermenter via a single parameter. In this study, equations proposed by Bowen et al. [54] were used. The results of $\dot{\gamma}_{ave}$ and γ_{max} measurements using the Brown equations are shown in **Table 3-7**. Investigation into the results of **Table 3-7** showed that at the same and lower values for P_{in} the $\dot{\gamma}_{ave}$ and γ_{max}values of the DRT impeller were 23 times larger than those when using the FZ. By increasing the P_{in}, the values of the $\dot{\gamma}_{ave}$ and γ_{max} during fermentation with the DRT impeller were 7.7 times larger than that of the FZ. One of the reasons for cell damage when using the DRT impeller is the high values for $\dot{\gamma}_{ave}$ and γ_{max}. The RT impeller previously was used during fermentation [56], and showed large degree of damage due to the fluctuating velocity intensity near the impeller. In the surroundings of the impeller, the shear rates can be 50 to 100 times larger than those in other areas of the vessel. It was noticed that shear forces are unequally distributed within the reactor, so the γ_{max} rather than average parameters should be used to describe the shear rate effect in stirred fermenter. Verification of stress and fluid flow simulation were shown in Appendix 4 (**Fig. 8-11**).

Table 3-7 $\dot{\gamma}_{ave}$ and γ_{max} of DRT and FZ impellers during the fermentation of *A.oryzae* according to the *Brown* equation.

Impeller		$\dot{\gamma}_{ave}$ (s^{-1})	γ_{max} (s^{-1})	N (s^{-1})
Condition 1	FZ	2.98	6.88	1.67
	DRT	68.73	158.74	5.00
Condition 2	FZ	14.80	34.17	5.00
	DRT	114.10	263.52	8.33

3-6-2 Effect of shaft flexibility on shear rate, shear stress formation and intensification of alpha-amylase production in submerged fermentation culture

According to S´anchez et al. [55], a knowledge of shear rate in biological fluid is essential. Because, it influences the average apparent viscosity of non-Newtonian fluids and hence affects power absorption, mixing characteristics and mass transfer phenomena. Microorganisms are

susceptible to damage that is dependent on the prevailing shear rate and associated shear stress. It was noticed that shear forces are unequally distributed within the reactor, so the $\gamma_{\max}$ rather than average parameters should be used to describe the rheology in a bioreactor. Therefore, in this study, $\gamma_{\max}$ has been studied.

Here, CFD simulation of flow velocity and shear stress in fermentation culture during agitating by DRT and Swingstir® have been investigated. According to the simulation results of fluid flow velocity (**Fig. 3-8(a)** and **3-8(b)**), the active mixing regions (AMR) during mixing with the DRT impeller was located around the Rushton blades. Therefore, the center of the tank had good internal-radial mixing but the isolated mixing region (IMR) (near the tank-wall parts) were separated from the center part. Mixing within these regions was significantly weaker than in the AMR regions. However, the flow velocity distribution using Swingstir® showed both vessel center (AMR) and tank -wall region (IMR) could have similar velocity by periodically change of the circular high-velocity region. Therefore, the AMR and IMR sections were not formed. Regarding to the **Fig. 3-8(b)**, the velocity distribution near the shaft and blade of the Swingstir® was axial and uniform. However, high local velocity distribution around the turbine blade of the condition mixing by DRT impeller was appeared. The local-radial high velocity distribution had negative effect on the mass transfer and enzyme activity. Because most of the DO concentration were accumulated around the turbine and this effect was resulted in more cell production near the turbine blade and preparing a rigid biomass-wall around the blades.

One of the positive hydrodynamic behavior of mixing with Swingstir® is due to the formation of unsteady vortices that move slightly, and the impeller velocity profile change with changing the angular position of the shaft. Investigation of Galleti et al. [43] on eccentric mixing also showed that these vortices inducing periodic oscillations in the flow field. As can be seen in the flow velocity simulation in Y-Z plane (**Figs. 3-8(a)** and **3-8(b)**) using Swingstir® there was not an axial symmetry. Lack of axial symmetry was leaded to an inclination of the

vortices. Verification of fluid flow using Swingstir® was shown in Appendix 4 (**Fig.12**). The motion of these vortices extend inducing oscillation superimposed to the main flow field. The axial circulation in the tank destroying the segregated regions and the separation plane between the upper and lower part of the vessel [43]. In addition, shear stress simulation in X-Y plane during the fermentation (**Figs. 3-9(a)** and **3-9(b)**) showed that the τ_{ave}, in the culture agitated by DRT (**Table 3-8**) was 2.2 times larger than that of the Swingstir®. One of the parameter affected on increasing the shear stress when using DRT is high value of consistency index (K_{app}), than that of the Swingstir® (in the condition with relatively the same n_{app}). In addition, for comparing the applied stress by flexible shaft with FZ impeller (as a low stress large blade), based on the K_{app} and n_{app} values in each sampling time, the stress was simulated to show the effect of a flexible agitator on the shear stress distribution in Y-Z plane (**Fig. 3-10**) at P_v (= 690 W/m^3). It was seen that the τ_{ave} and τ_{max} applied by flexible-shaft agitator were lower than that of the FZ. Verification of simulation results were shown in Appendix 4 (**Figs 13-14**)

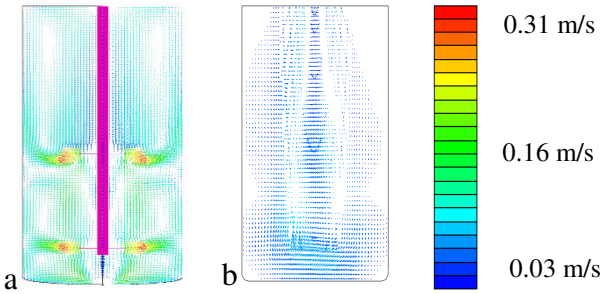


Fig. 3-8 Simulation of fluid flow velocity during the fermentation using (a): DRT and (b): Swingstir® in Y-Z plane, at the same P_v.

Table 3-8 Results of apparent viscosity measurement and shear stress simulation using DRT and Swingstir®.

Impeller	τ_{ave} (Pa)	τ_{max} (Pa)	μ_{ave} (Pa s)	$Re_{ave,\ laminar}$	N (1/s)
DRT	4.17	140.00	0.55±0.05	77.43	1.67
Swingstir®	1.83	30.83	0.21±0.03	127.33	1.42

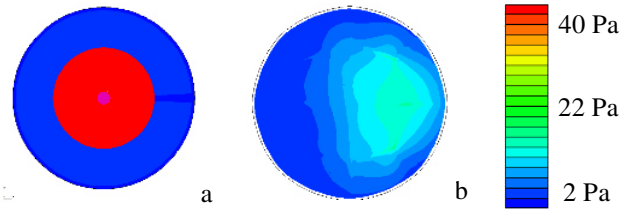


Fig. 3-9 Simulation of shear stress during the fermentation using (a): DRT and (b): Swingstir® in X-Y plane, at the same P_v

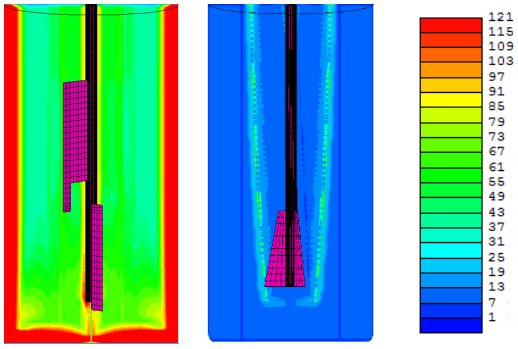


Fig. 3-10 Simulation of shear stress applied to fermentation cultures by (a) the FZ or (b) the Swingstir® at t = 48h at the same P_v.

3-7 Summary

In this chapter, study on the effect of different mixing conditions on the shear rate and shear stress applied to fermentation fluid have been considered. Major results of this section could be summarized as follows;

- The γ_{max} in fermentation using FZ was low in comparison with the condition using DRT and MB (by comparing the results of **Tables 3-2, 3-7**). Flow velocity dispersion when using Swingstir® was totally uniform, and there was no local shear rate even at the end of fermentation. The $\dot{\gamma}_{ave}$ when using MB at the same energy consumption was higher than that of the other three impellers (comparing results of **Table 3-2, 3-7** and **Figs 3-8**). Consequently, it was concluded that Swingstir® and FZ impellers could provide adoptable shear rate in fermented fungal fluid.
- Regarding to the results of stress simulation, at the same energy consumption, the τ_{ave} and τ_{max} during agitation with Swingstir® (**Fig. 3-9, 3-10**) were the lower than that of

the FZ. It means that flexible-shaft could be an agitated system with low risk of cell damage.

- The most uniform fermentation fluid flow velocity distribution was seen during agitation with Swingstir®. However, mentioning to the low and uniform fluid dispersion combining with low stress medium is not enough for selecting the most adoptable condition for growing the fungal cells and intensification of enzyme production but from the shear rate and shear stress aspect of view it could be concluded that using Swingstir® impeller provide a hydrodynamic with uniform culture dispersion and the force transferred from the impeller to the biological fluid was low.

Symbols;

Nomenclature

K	Consistency index	[Pa s^n]
$K_L a$	Volumetric mass transfer coefficient	[h^{-1}]
k_s	Shearing constant	-
N	Rotational rate	[s^{-1}]
N_P	Non-dimensional power number	-
n	Flow index	-
P	Pressure	[Pa]
P_v	Power density	[Wm^{-3}]
Re	Reynolds number	-
V	Liquid volume	[m^3]

Greece symbols

τ	Shear stress	[Pa]
$\dot{\gamma}_{ave}$	Average shear rate	[s^{-1}]

Abbreviations

AMR	Active mixing zone	-
ARPI	Axial reciprocating plate impeller	-
DCW	Dry cell weight	[gL^{-1}]
DO	Dissolved oxygen	[ppm]
DRT	Double Rushton turbine	-
EE	Elephant ear	-
FZ	Fullzone®	-
IMR	Isolated mixing zone	-
MB	Maxblend®	-

PBT	Pitched-blade turbine	-
STR	Stirred tank reactor	-
TRI	Triple Rushton impeller	-

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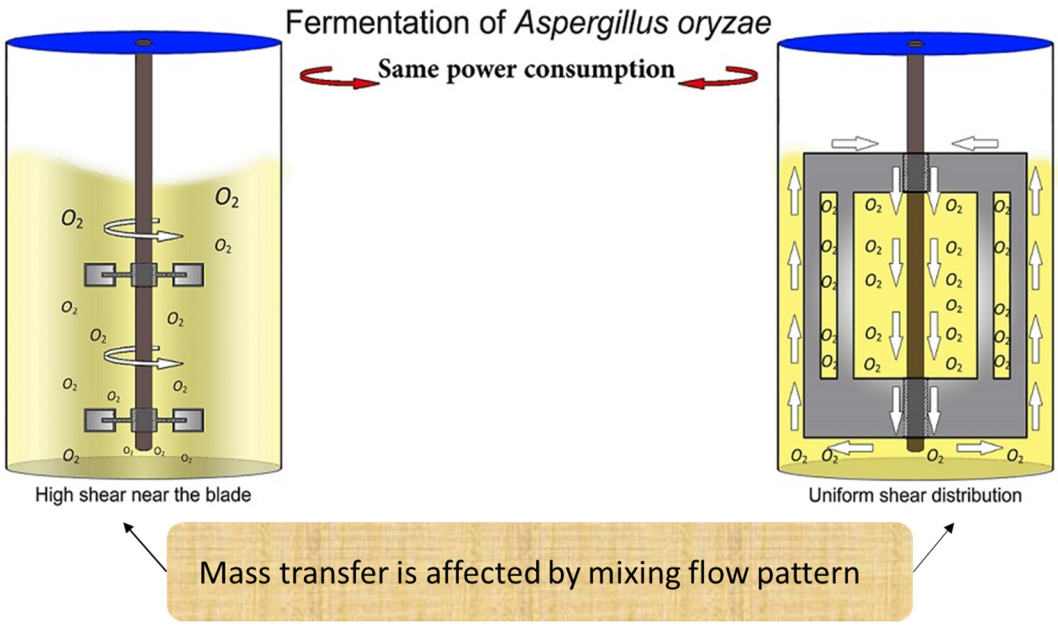
Chapter 4: Oxygen mass transfer intensification in submerged culture of *A.oryzae* using mixing improvement



Graphical abstract of chapter 4: [This picture was prepared by Narges Ghobadi, 2016]

Chapter 4 in brief; In this chapter effect of mixing condition on intensification of oxygen mass-transfer in high-viscosity fermentation culture were shown by fluid flow simulation and experimental analysis

4-1 Introduction

According to the literature, oxygen is one of the most important nutrient for submerged fermentation. Due to this reason, in this chapter intensification of oxygen mass transfer in a complex fermentation fluid has been studied. Process of oxygen transport in a submerged culture consists of the following process [1]; 1) Oxygen transport into the fermenter and dispersion 2) Oxygen transfer from gas to liquid, 3) Oxygen transfer from the liquid to the surface of the fungal particles. 4) Oxygen transport within the fungal particles. 5) Oxygen consumption by the fungi [2].

In this chapter at first important factors which were important for increasing the mass transfer in a stirred tank fermenter has been investigated using Newtonian and shear-thinning fluids, afterward characteristics of mass transfer during fermentation of *A.oryzae* at various mixing flow patterns was studied.

4-2 Theory of mass transfer in fungal submerged fermentation culture

There are various factors which play an important role in different oxygen mass transfer resistances in stirred bioreactor. Factors were included mixing intensity, bubble size, rheological and composition of the biological fluid [3]. When considering oxygen mass transfer in a mixed fermenter, mostly the following suppositions can be proposed [3]:

1. Transfer of oxygen through the bulk gas phase in the bubble is relatively fast
2. The gas-liquid interface is supposed with negligible resistance
3. The liquid film around the bubbles is considered as the most important source of resistance to the mass transfer
4. In high-viscosity fermentation culture high agitation intensity was not easily resulted in oxygen transfer resistance in the cultivation medium.

5. In fermentation broth the distance between the pellets is small and the intracellular oxygen mass transfer resistance is usually not considered.

In this study, the effect of mixing condition on oxygen mass transfer from the gas to the liquid and gas dispersion in the cultivation media by growing the fungal cells have been investigated.

4-3 Study the effect of impeller type on oxygen mass transfer enhancement

One of the aim of this study is to ensure that the DO concentration satisfies the demand of the fungal cell culture. To reach this goal, some parameters were defined and measured at different mixing flow pattern. These key factors studied in this research are as follows;

- Mixing time study in a batch-stirred fermenter using various agitation condition at the same power consumption
- Two-fluid flow velocity simulation in stirred fermenter
- Comparison between mixing time, power consumption and mixing energy using different agitator in the fermenter
- K_La measurement during fermentation using different flow pattern of mixing
- Investigation on the relation between the DO concentration during the fermentation and pattern of applied shear rate by agitator to the fermentation fluid

Agitation can have an effect on the K_La during the fermentation, also it could help to intensify the aeration and reduce the air bubble sizes in culture media which resulted in mass transfer [4]. Besides, based on the fungal morphology and rheology the mass transfer inside the fermentation media could be quite different. **Fig. 4-1** shows how the mechanism of mass transfer could be affected by fungal morphology and rheology [1].

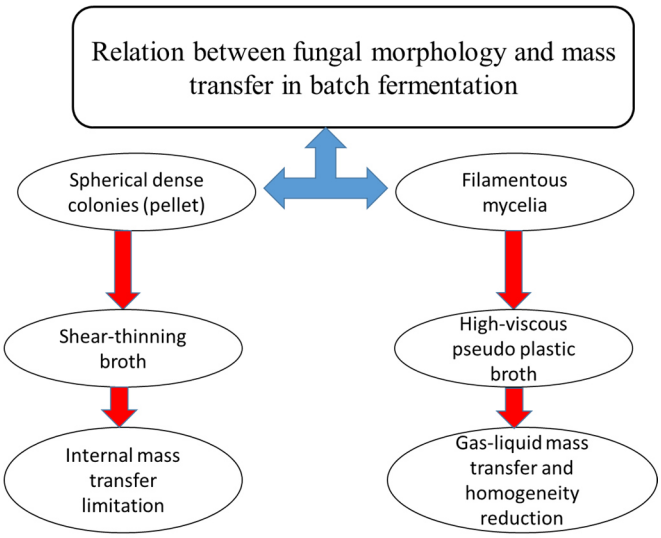


Fig. 4-1 Illustration of the mass transfer challenges come from the fungal morphology in batch fungal fermentation [1].

4-3-1 Correlation between Mixing time, power consumption and mixing energy using Newtonian fluid

Masiuk et al. [5] have proposed empirical correlations for prediction of the power consumption, the mixing time and the energy required to achieve the homogeneity. For Newtonian and non-Newtonian liquids, Masiuk et al. [6] have investigated the interaction between power consumption, mixing time and the geometric parameters of a non-typical helical ribbon agitator. They used the mixing energy as a criterion to select the lowest energy-consuming configuration for mixed process. In this research, mixing efficiency of various agitators with different configuration was investigated using results of mixing time measurement.

4-3-1-1 Relation between mixing time and power consumption

Here, mixing time (T_m) at determined P_v was measured using Newtonian working fluid (**Table 4-1**). Afterwards, correlation between NT_m via *Re* were extracted and shown in **Fig. 4-2**. Results of correlations (exponential function) were shown in **Table 4-2**.

Table 4-1 properties of Newtonian fluid used in this study

Material	Density (kg/m^3)	Viscosity (Pa.s)	Temperature (° C)
Water	1000	0.0012	30
Glycerol	1149	0.0960	30

Results of **Table 4-2** indicates in the condition using DRT, the NT_m was strongly depended on *Re* more than that of the other agitators. Besides, the constant and exponential values of mixing by Swingstir® were the lowest.

Table 4-2 Correlations of NT_m -*Re* for different impellers using water.

Impeller	Correlation (*Re*: ~)	R^2
DRT	$NT_m = 983.3\ Re^{-0.375}$	0.865
MB	$NT_m = 198.0\ Re^{-0.190}$	0.715
FZ	$NT_m = 157.0\ Re^{-0.247}$	0.582
Swingstir®	$NT_m = 33.2\ Re^{-0.174}$	0.660

The experimental data used for finding a correlation between the T_m and P_v was shown in **Fig. 4-3**. These results were in agreement with the results of **Fig. 4-2**, it means that at the same *Re* the value of NT_m when using Swingstir® was the lowest. Finally, it could be concluded that at the same *Re* and P_v the NT_m (or T_m) could be enhanced and decreased using Swingstir®.

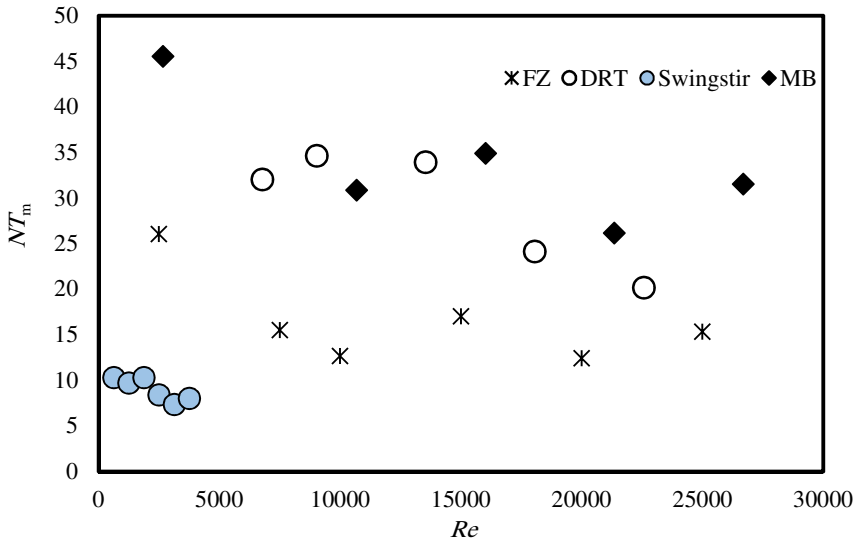


Fig. 4-2 Relation between NT_m and *Re* using different impellers.

Results of **Table 4-3** and **Fig. 4-3** indicates at the same P_v the largest exponent and constant coefficients were extracted during mixing by DRT. Finally, it could be concluded mixing by Swingstir® in a Newtonian fluid would be leaded to a lower mixing time at the same P_v.

Table 4-3 Correlations of T_m -P_v for different impellers using water.

Impeller	Correlation	R^2
DRT	$T_m = 2478.0\ P_v^{-1.055}$	0.972
MB	$T_m = 918.0\ P_v^{-0.747}$	0.855
FZ	$T_m = 2792.9\ P_v^{-0.981}$	0.911
Swingstir®	$T_m = 613.83\ P_v^{-0.933}$	0.996

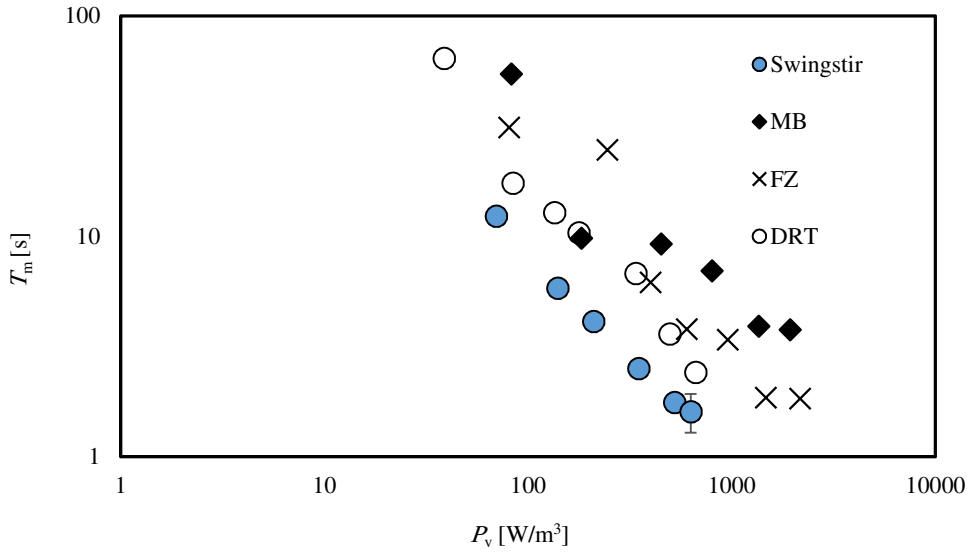


Fig. 4-3 Relation between mixing time and power consumption using different impellers.

4-3-1-2 Relation between mixing energy (π) and mixing time Re number (Re_m)

In this section finding a relation between mixing performance considering the power consumption and mixing time could be useful for scale-up design in stirred tank in future. Due to this reason, the mixing energy (π_3) versus mixing time *Re* (Re_m) [7] for four types of impeller have been evaluated and the results were shown in **Fig. 4-4**. In equations (4-1) and (4-2), *T* was defined as a tank diameter.

$$Re_m = \frac{T^2 \rho}{T_m \mu_a} \tag{4-1}$$

$$\pi_3 = \frac{PT_m^2}{T^3 \mu_a} \tag{4-2}$$

Results of **Fig. 4-4** showed at the same Re_m the mixing energy using Swingstir® was the lowest. The results could be sorted as was shown below;

Mixing energy at the same Re_m: MB > FZ > DRT > Swingstir®

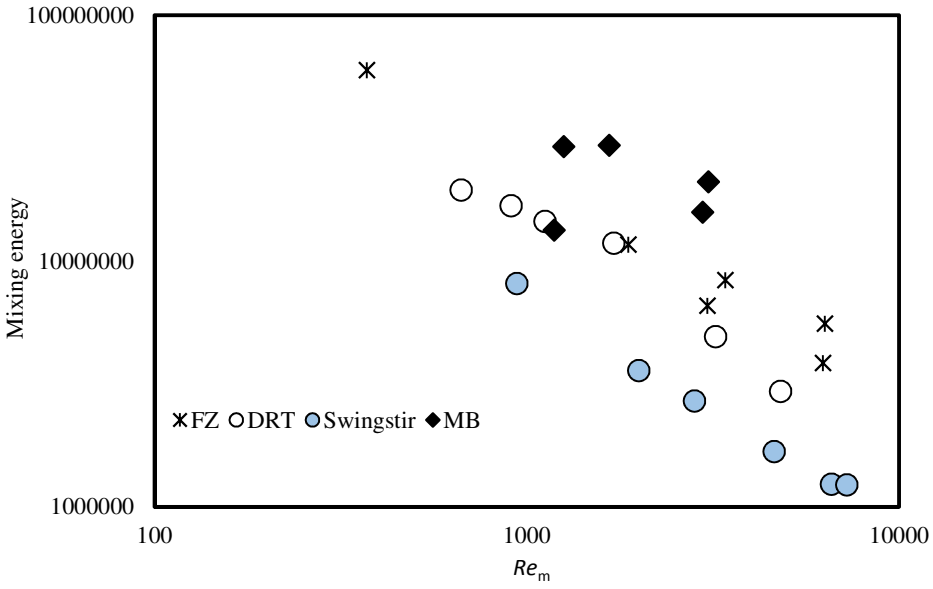


Fig. 4-4 Mixing efficiency of FZ, DRT, Swingstir® and MB impellers using Newtonian fluid.

4-3-2 Correlation between Re and P_v using non-Newtonian fluid

To investigate the mixing time and mixing energy using different kind of agitators when using non-Newtonian fluid with shear-thinning behavior, Xanthan gum solution was used (**Table 4-4**).

Table 4-4 Properties of non-Newtonian fluid used in this study.

Material	Density (kgm^{-3})	Viscosity (Pa.s)	Temperature (^{o} C)	Mass fraction %	K (Pa s^n)	n (-)
Xanthan gum	882	-	30	1	1.32	0.35

Results of **Fig. 4-5** showed when using shear-thinning fluid, at the same Re, the P_v of Swingstir® and MB were higher than that of the DRT and FZ impellers. It could be concluded

that at the same P_v the highest *Re* was belonged to the mixing condition using MB and FZ impellers respectively. Consequently, it was seen that during mixing a non-Newtonian fluid agitation by MB and FZ could prepared a high *Re* in medium.

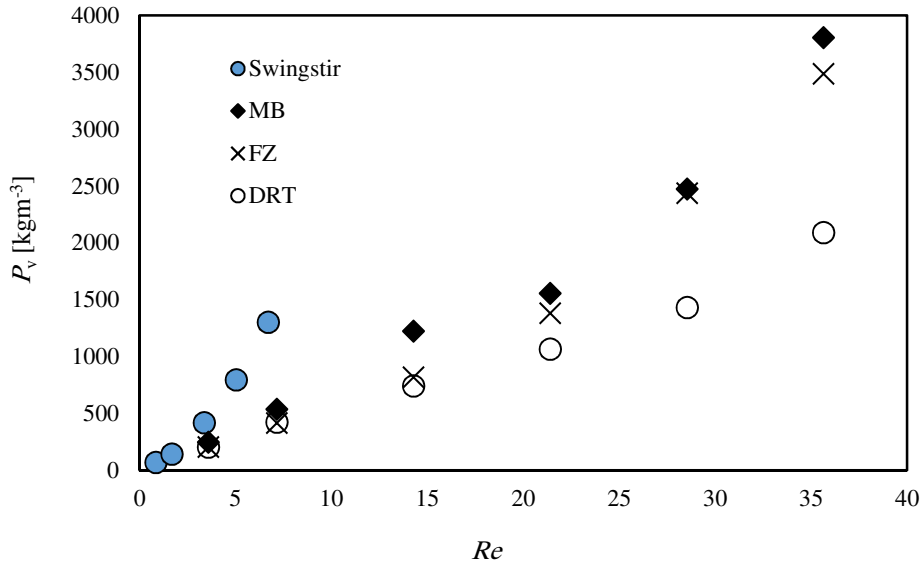


Fig. 4-5 P_v versus *Re* using Xanthan gum solution (10gL^{-1}) at 30 ºC using different kinds of impellers.

4-3-3 Experimental analysis the mixing time using different mixing condition in stirred-batch fermenter

Mixing time is one of the factors when using stirred tank as a fermenter. It can be a representative of the quality of nutrient and oxygen mass transfer. It is usually tried to reduce this factor but it is impossible to reduce mixing time just by increasing the power input into the fermenter. In this study, mixing time of Newtonian fluid using DRT, MB, FZ and Swingstir® by visualization were examined to investigate which kinds of mixing condition could be efficient for fungal fermentation in stirred tank.

4-3-3-1 Comparing the mixing time between large (MB) and multi-large (FZ) impellers

Because of some limitation in agitation rate during using Swingstir®. It is important to compare the mixing time between large impellers because using MB and FZ impellers could

be done in extended agitation or *Re* intervals. Due to this reason, it would be useful to find the optimal large blade for using in wide operational interval during the agitated fermentation. Results of mixing time experiment using two different large blade agitator (MB and FZ as a large multi blade impeller) was shown in **Fig. 4-6**. Results indicates at the same energy consumption, however the mixing energy of condition using MB was higher than that of the FZ but mixing time using FZ was lower than that of the MB. It indicates that FZ could disperse the oxygen and nutrient by higher rate in comparison with the mixing by MB.

Fig. 4- 6 Results of mixing time measurement using FZ and MB impellers by discoloration method at the same power consumption (P_v = 150 W/m^3).

4-3-3-2 Comparing the mixing time between radial mixing (DRT) and agitating by flexible-shaft impeller using visualization

The mixing time results using DRT and Swingstir® were shown in **Fig. 4-7**. High mixing time during the agitation with the DRT indicates it will cost much more time for the substrates, such as glucose, to disperse in bioreactor equipped with DRT impellers than that of the Swingstir®. In addition, experimental results of mixing time *via* P_v, was shown in **Fig. 4-8**. Investigation on the data presented that at the same P_v the mixing time of agitation with Swingstir® was the lowest and the highest mixing time belongs to the reactor agitated with DRT impeller. Finally, it can be concluded that using the Swingstir® would be efficient to decrease the mixing time or fermentation time in comparison with the non-flexible shaft impellers such as DRT impeller.

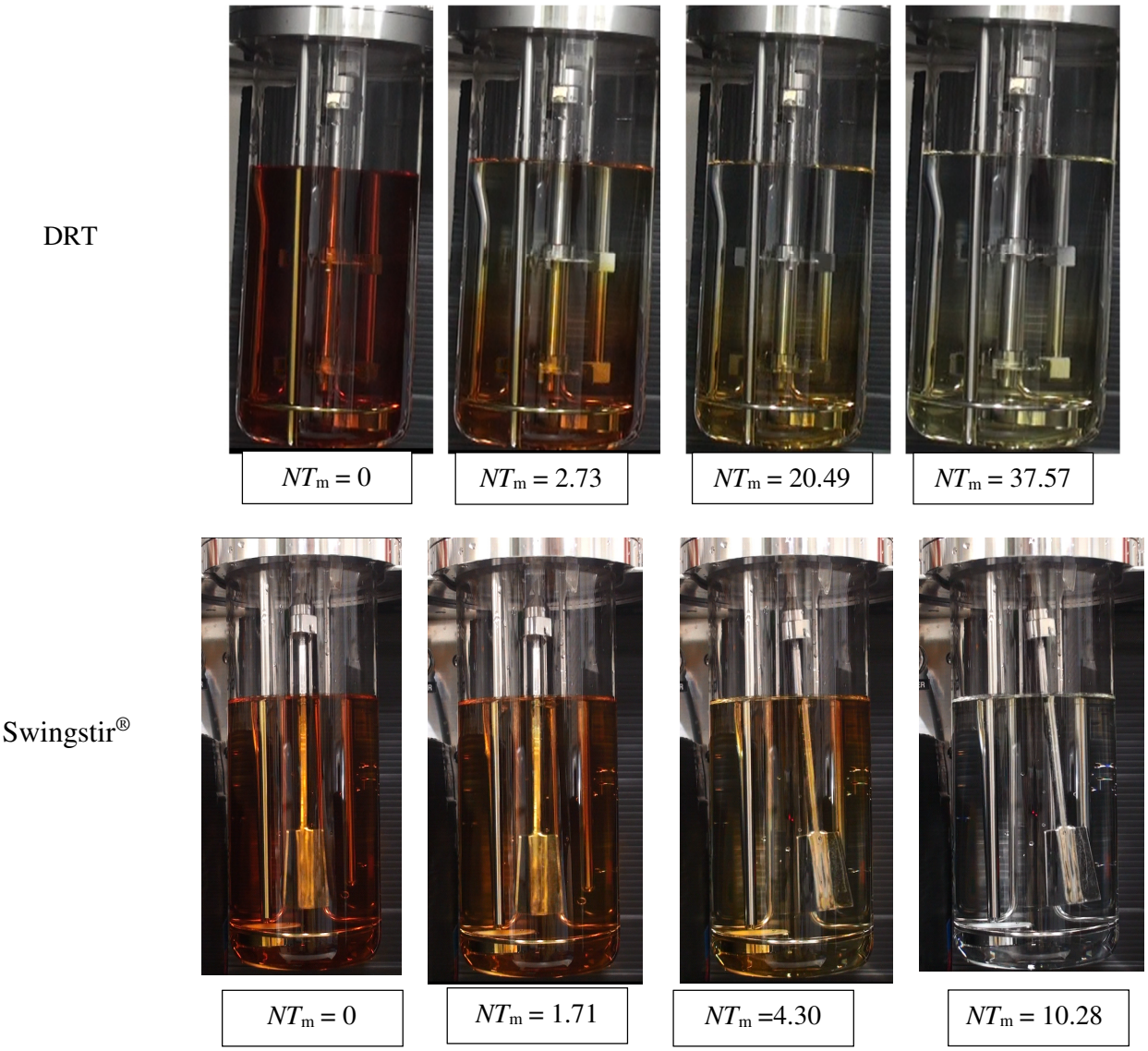


Fig. 4-7 results of mixing time measurement using DRT and Swingstir® by discoloration method at the same power consumption (P_v = 150 W/m^3).

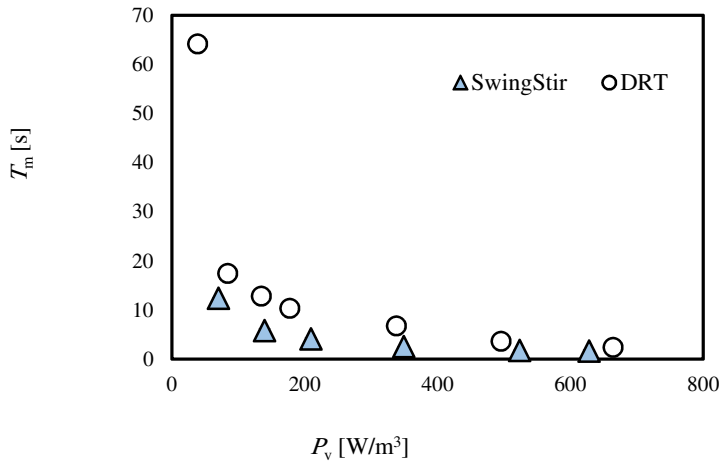


Fig. 4-8 Mixing time [s] versus P_v [W/m^3] using Swingstir® and DRT using water as a working fluid.

4-4 Study on the oxygen mass transfer in stirred bioreactor using large blade agitator during the batch fermentation of A.oryzae

In the present study, a survey on the effect of agitation and impellers on the oxygen mass transfer was accomplished by measuring the DO [ppm] in culture media. The recorded values are shown in **Figs 4-9(a), 4-9(b)** and **4-9(c)**. In this study, it would be important to note that because the cells had adhered to the DO sensor, the real DO concentration could not be easily recorded. Although the culture DO have not reached zero at 48-54 h after fermentation, the DO probe showed that the concentration of the oxygen was near zero. Therefore, qualitative observations show that the MB impeller may provide better oxygen transfer. Also, for approving the impact of impeller design as a secondary gas dispersion object, the monitored DO concentration in fermentation culture using both impeller was compared with the DO values in culture that was not agitated (only used ring sparger for aeration) as shown in **Fig. 4-10**. Results of **Fig. 4-10** indicate that using the MB impeller can control and keep the DO mixing concentration at high value. Besides, the coalescence of bubble in three different mixing condition were agreed with the recorded concentration of DO mixing. These results were compatible with the other findings [8], it was showed when a large cross-section impeller was operated at a high level P_v, the total level of oxygen inside the impeller was increased. Also, it was found that control of the DO concentration during the mixing of BC (bacterial cellulose) cells using turbine impellers was not possible, and controlling the DO level was not effective for maintaining a high degree of BC productivity [9]. The most distinctive difference between the MB and turbine impellers is the configuration around the sparger [9]. To better understand the effect of impeller configuration, a static method [10] was used to measure the average K_La of a fermentation culture. Results of K_La (**Fig. 4-11**) indicates, the MB significantly intensified K_La at low and moderate power input in comparison with high power input. In addition, the decrease in enzyme activity (**Fig. 5-8**, chapter 5) at high P_v during fermentation by the MB was

due to the incomplete growth of the stressed cells. As shown in chapter 5 (**Fig. 5-6**), however, the biomass was increased, but the cells would not grow branches until the end of the fermentation process.

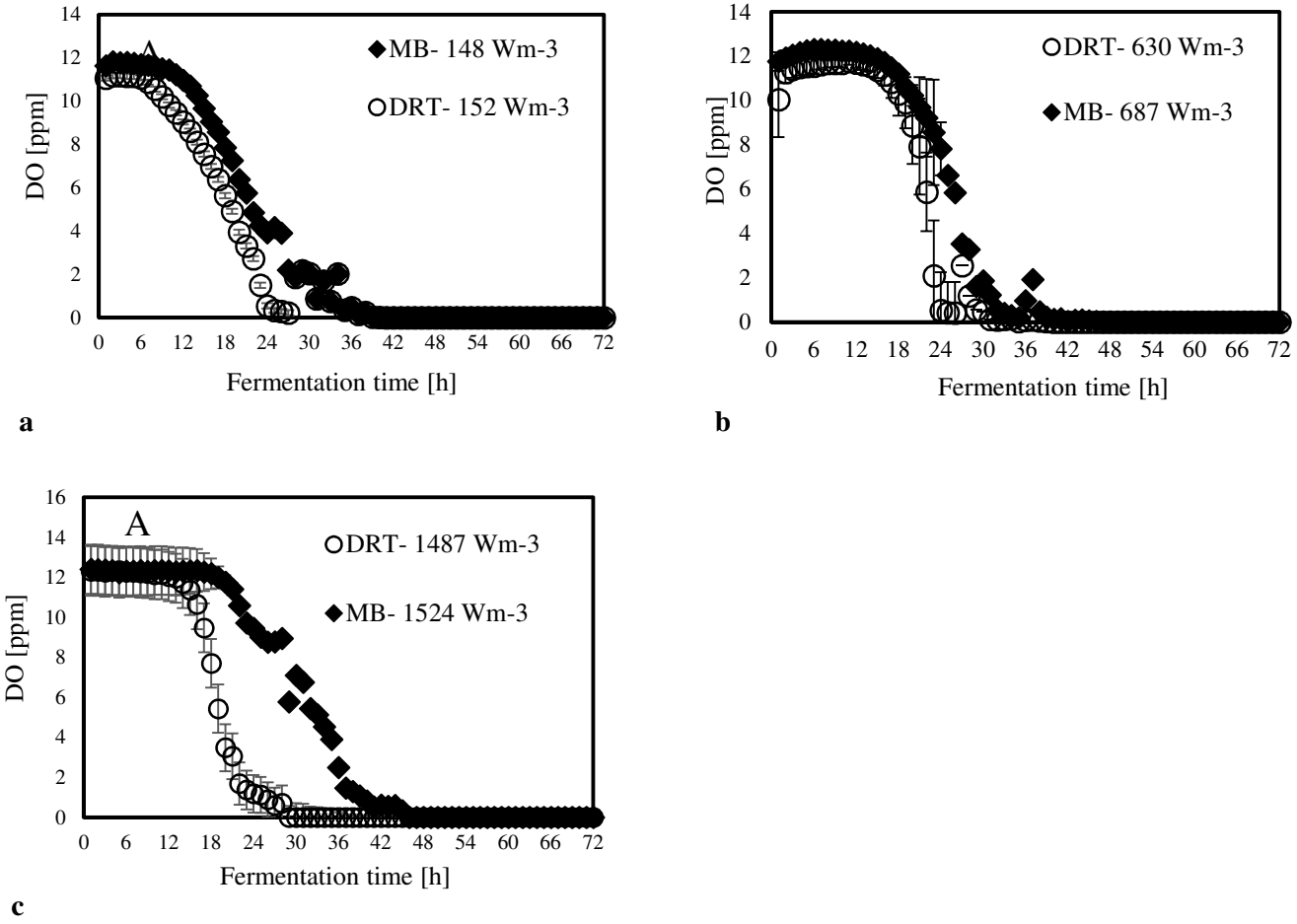


Fig. 4-9 Effect of impeller type and P_v on DO mixing concentration in a fermentation culture by MB and DRT (a): Low P_v , (b): Moderate P_v , (c): High P_v.

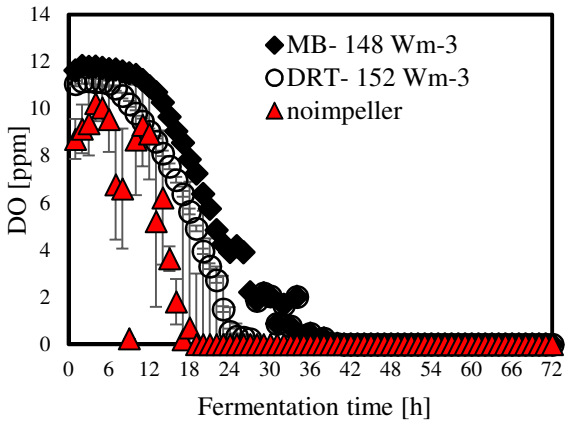


Fig. 4-10 Comparison between the DO mixing values during fermentation with impeller and without impeller.

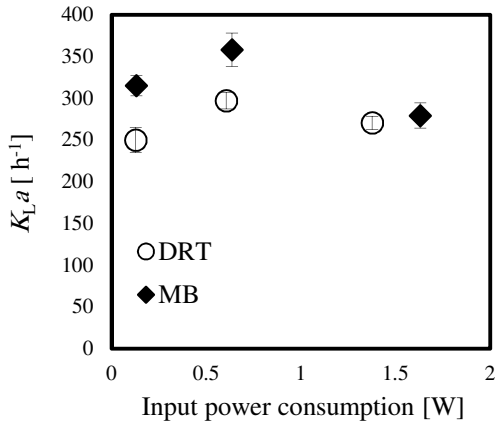
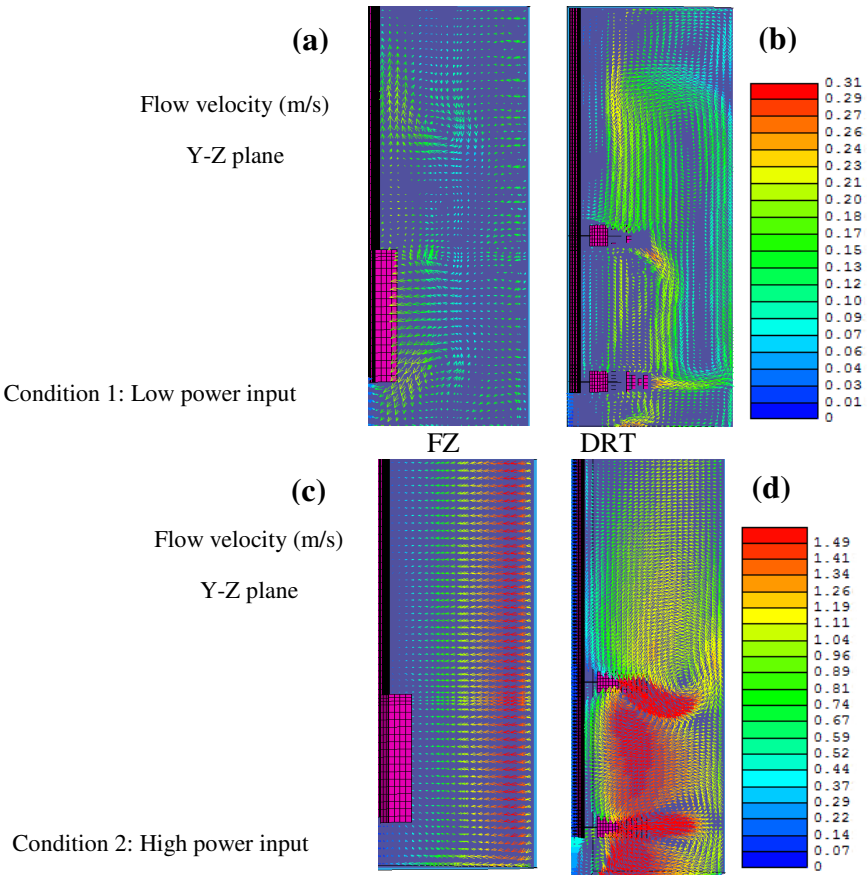


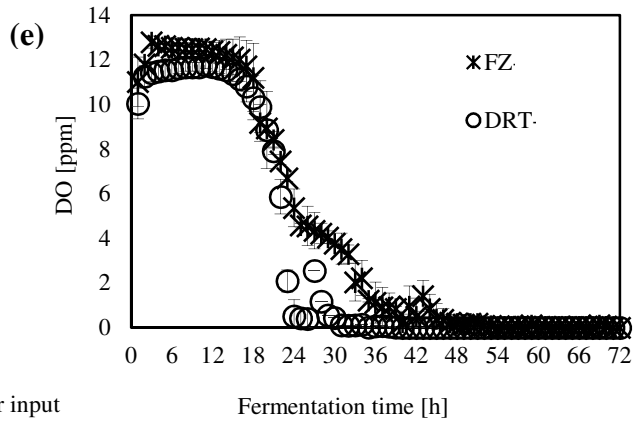
Fig. 4-11 Effect of impeller and input power consumption on K_La intensification.

4-5 Study of oxygen mass transfer and simulation of flow velocity distribution during fermentation using FZ (multi-large) and DRT impellers

Oxygen is a special substrate that is very important for cell growth [11]. In the present study, a survey on the effect that an impeller exerts on oxygen mass transfer was combined with the results of fluid velocity simulation. Velocity simulation of stirred fermentation was done at 100, 300 min^{-1} using FZ and DRT as a different kind of agitator with the same power consumption. Based on the flow velocity simulation (**Figs. 4-12(a), (b), (c) and (d)**), the distribution of the DO concentrations at low and high P_{in} are shown in **Figs. 4-12(e)** and **(f)**. The simulated velocity profiles of the fermenters at the same P_{in} illustrates the differences in flow patterns formed by these two impellers. As a consequence, the different flow patterns caused differences in the mass transfer efficiency in each of the two fermentation cultures [12]. In an aerated tank agitated by the RT impeller, air bubbles accumulated in the trailing vortices, and altered the flow around the impeller region [13] The results show how the uniform culture velocity formed by the FZ impellers at different power consumption created a more appropriate media for oxygen mass transfer during fermentation than that of the DRT impellers at low and high P_{in}. Those results show that simply increasing the flow velocity without regard to the pattern did not increase the mass transfer. At least one study in the literature [14] indicated that for small

diameter impellers, the value of the volumetric oxygen capacity near the wall drops faster than in other areas. The simulation results in **Fig. 4-12(c)** shows a high and uniform velocity profile near the tank wall in a bioreactor equipped with the FZ impeller at 1.14 W. This was the result of avoiding the formation of dead zones and of an increase in the DO concentration during mixing with the DRT at 1.80 W, the velocity near the impeller zone and between the two turbine impellers was higher than that in the surrounding area of the FZ impeller agitating at 1.14 W. The local velocity gradient, using DRT, could result in local differences in DO concentration and ultimately in a reduction in the average DO throughout the bulk of the fermentation culture. Therefore, the average DO concentration recorded by the DO probe was decreased by the high rate (**Fig. 4-12(e) and (f)**). In the case of using DRT at high P_{in}, most of the dead zones were located on the upper side of the fermenter. These dead zones prevented the oxygen bubbles form reaching the top of the fermenter. On the other hand, the combined axial-radial flow impellers, such as the FZ, tend to suspend cells around the periphery of the vessel bottom, so that the flow pattern facilitates cell fluidization in comparison with radial flow impellers such as the DRT.





Low power input

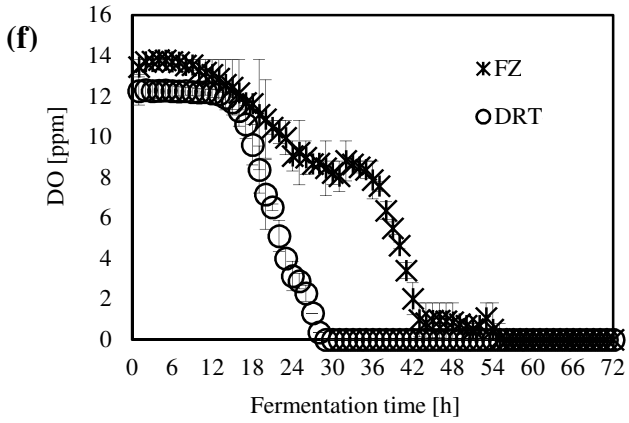


Fig. 4-12 Flow velocity distribution of a fermentation culture using (a): FZ and (b): DRT impellers during the fermentation of *A.oryzae* at low P_{in}, Flow velocity distribution of a fermentation culture using (c): FZ and (d): DRT impellers during the fermentation of *A.oryzae* at high P_{in}, (e): DO concentration in a fermentation culture during the fermentation (FZ and DRT were used at the low and same P_{in}), (f): DO concentration in fermentation culture during the fermentation (FZ and DRT were used at the high and same initial energy transported by impeller to the culture at the beginning of fermentation (P_{in})).

4-6 Effect of flexible - shaft agitator on oxygen mass transfer in fermentation of A.oryzae

According to McFarlane et al. [15] turbine impeller exhibits high unaerated power number, generating high energy consumption and a fall in the power input under aeration particularly in viscous fluids. It was resulted in oxygen mass transfer reduction. Analysis the results of K_La measurement (**Fig. 4-13**) combined with the experimental data of culture viscosity (see **Fig. 6-1**, chapter 6) showed that low culture viscosity was one of the reason of keeping the K_La at desirable value until the end of the fermentation, using Swingstir®. It was due to the ability to control the viscosity at low value. More investigation on the results of **Fig. 4-13** revealed that in the most of the sampling times, K_La of agitation with Swingstir® was more than that of the DRT. Particularly, at t = 48h, the K_La of culture mixed by Swingstir® was 2.5 times larger than of the DRT. In addition, at the end of the fermentation the K_La of the culture mixed by DRT impeller was decreased and reached to $28h^{-1}$ but the K_La of mixed culture using Swingstir® was $76h^{-1}$. Because in non-Newtonian fermentation media, the gas flow tends to be channeled up the center of the bioreactor and leading to inadequate gas dispersion [19]. Finally, the larger K_La when using Swingstir® presented the higher aeration capacity of the fermenter. All above results were in agreement with the results of DO concentration recorded during the fermentation using DRT and Swingstir® (**Fig. 4-14**). It was noted that decreasing the mass transfer between gas-liquid boundary layer was leaded to increasing the K_L part of the overall volumetric mass transfer coefficient [1].

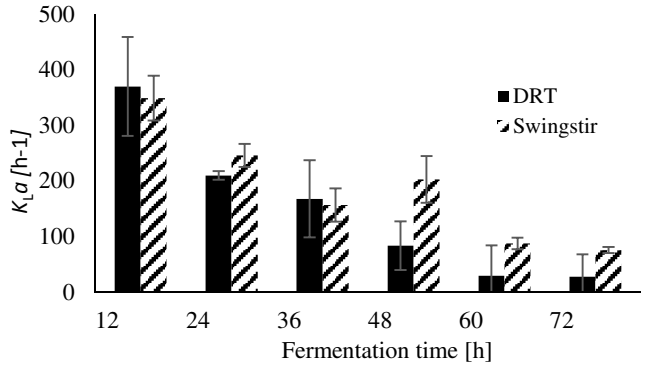


Fig. 4-13 K_La versus fermentation time during the fermentation of *A.oryzae* using DRT and Swingstir®.

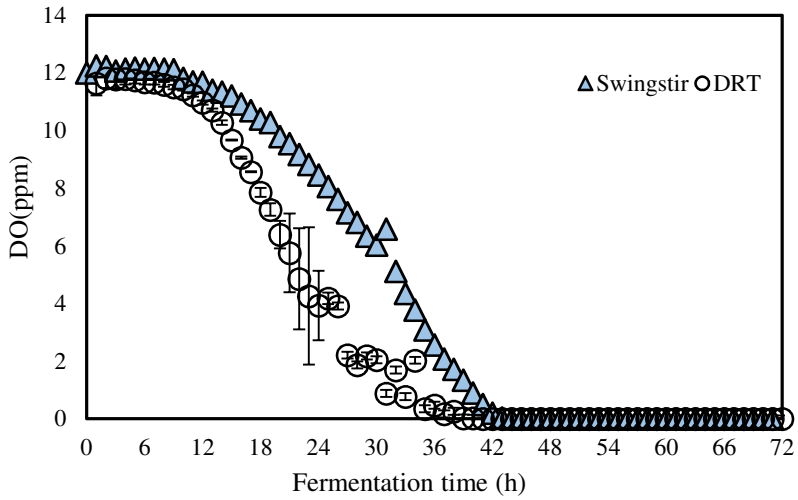


Fig. 4-14 Comparison of DO concentration between Swingstir® and DRT as an agitator of stirred fermentation at the same (low) P_v

4-7 Comparison between mass transfer in stirred fermentation using large blades and Swingstir®

To investigate the effect of mixing by Swingstir® and FZ on the oxygen mass transfer, K_La in each mixing condition were measured and showed in **Table 4-5**. Data in **Table 4-5** showed the values of power-law model parameters and apparent viscosity at t = 48 and 72h when Swingstir® and FZ were used as an agitator at the same P_v. For the reason of showing the effect of rheology on K_La, and alpha amylase activity (as a one of the sign of cell activity for biological production) were calculated and shown in **Table 4-5**.

Table 4-5 Effect of Swingstir® on rheological behavior and enzyme activity of submerged culture in comparison with FZ impeller at the same P_v (= 690 Wm^{-3})

Fermentation time (h)		K_{app} (Pas^n)	n_{app}	K_La (h^{-1})	Enzyme activity ($Um^{-1}L^{-1}$)	Shear rate (S^{-1})	μ_{app}(Pa s)	Average stress (Pa)
48	Swingstir®	8.16	0.20	168	5000±500	4.1	0.50±0.06	10
	FZ	36.60	0.36	101	4000±340	5.0	0.48±0.06	119
72	Swingstir®	49.40	0.20	131	7984±150	4.1	0.55±0.05	65
	FZ	299.00	0.30	60	5687±167	5.0	0.70±0.08	754

Table 5-1 (See in chapter 5), and **Table 4-5** illustrated that when using FZ impeller the dry biomass has been increased and interaction between the cells have intensively increased. Therefore, the oxygen transfer from the liquid to the cells was done in poor condition. Consequently, the final activity of cells has been decreased. It indicated that increasing the dry biomass was not enough for increasing the enzyme activity of cells. Due to the data of **Table 5-2**, at t = 48 h, however the biomass when using Swingstir® is less than the cells agitated by FZ because of high volumetric capacity of oxygen mass transfer, low viscosity and low stress (**Table 4-5**) the biomass was kept at high value until end of the fermentation (t = 72h). Therefore, using the Swingstir® resulted in ample space between the cells for oxygen and nutrient mass transfer. One of the important factor that had positive effect on high K_La when using Swingstir® was low mixing time at the same P_v and Re (**Figs. 4-6 and 4-7**) than that of the FZ. Finally, regarding to **Table 5-1**, at t = 72 h, when dry cell weight (DCW) of both mixing condition were approximately the same, the yield production based on DCW and power consumption when using Swingstir® were higher than that of the FZ.

To compare the oxygen mass transfer when using Swingstir® and MB, the recorded DO concentration during the fermentation by each impeller were shown in **Fig. 4-16**. Results of **Fig. 4-16** presented that the DO concentration in culture agitated by Swingstir® was quite higher than that of the MB, however it was not any difference between DO concentration of agitating by Swingstir® and FZ. At the same energy consumption, the DO concentration in culture agitated by MB was reached to zero at t = 30h. However, during mixing by flexible

shaft the recorded DO was more than zero until t = 48h. According to the results of **Fig. 4-16**, at the same energy consumption the value and stability of DO concentration when using Swingstir® was higher than that of the MB.

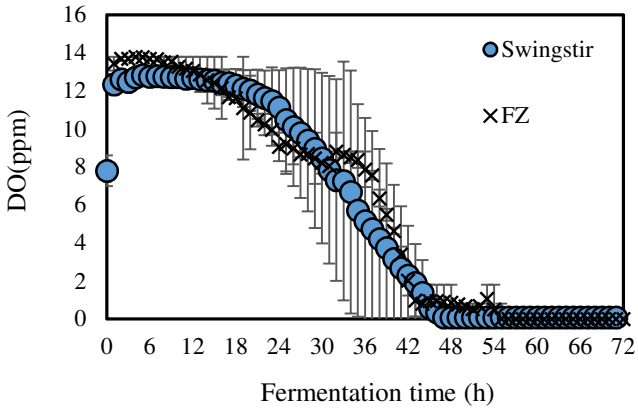


Fig. 4-15 DO concentration in fermentation culture during the fermentation (FZ and Swingstir® were used at the high and same P_v).

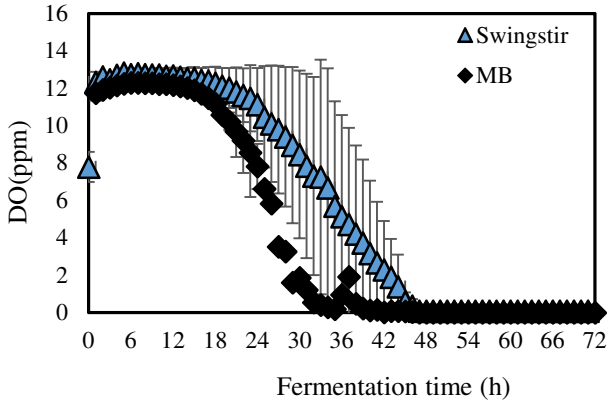


Fig. 4-16 DO concentration in fermentation culture during the fermentation (MB and Swingstir® were used at the high and same P_v).

4-8 Overall investigation on the volumetric capacity of oxygen mass transfer during the fermentation at different hydrodynamic condition

Investigation on the data in **Fig. 4-17** showed the effect of mixing with different impeller on changing the K_La during the fermentation. Due to the results of **Fig. 4-17**, among the four types of agitation only mixing with Swingstir® could provide the potential of controlling the

volumetric capacity of oxygen mass transfer at around $150h^{-1}$ until the end of fermentation. It was seen that using DRT and MB could not keep the initial high K_La values until the end hours of fermentation. Another result is that increasing the P_v using FZ impeller did not have any effect on increasing the K_La. Finally, overall investigation on the results of **Fig. 4-17** showed several applicable and important results as follows;

- Rate of K_La reduction by increasing the fermentation time when mixing by DRT and MB were higher than that of the agitation with FZ or flexible - shaft impeller (however the initial K_La values during using MB and DRT were high)

- K_La of fermentation culture agitated by Swingstir® were kept relatively constant after growing the cells (after t = 24h).

- K_La values of fermentation broth using FZ impeller at different P_v after t = 24h were approximately the same. It means after growing the cells K_La of culture mixed using FZ was independent on P_v.

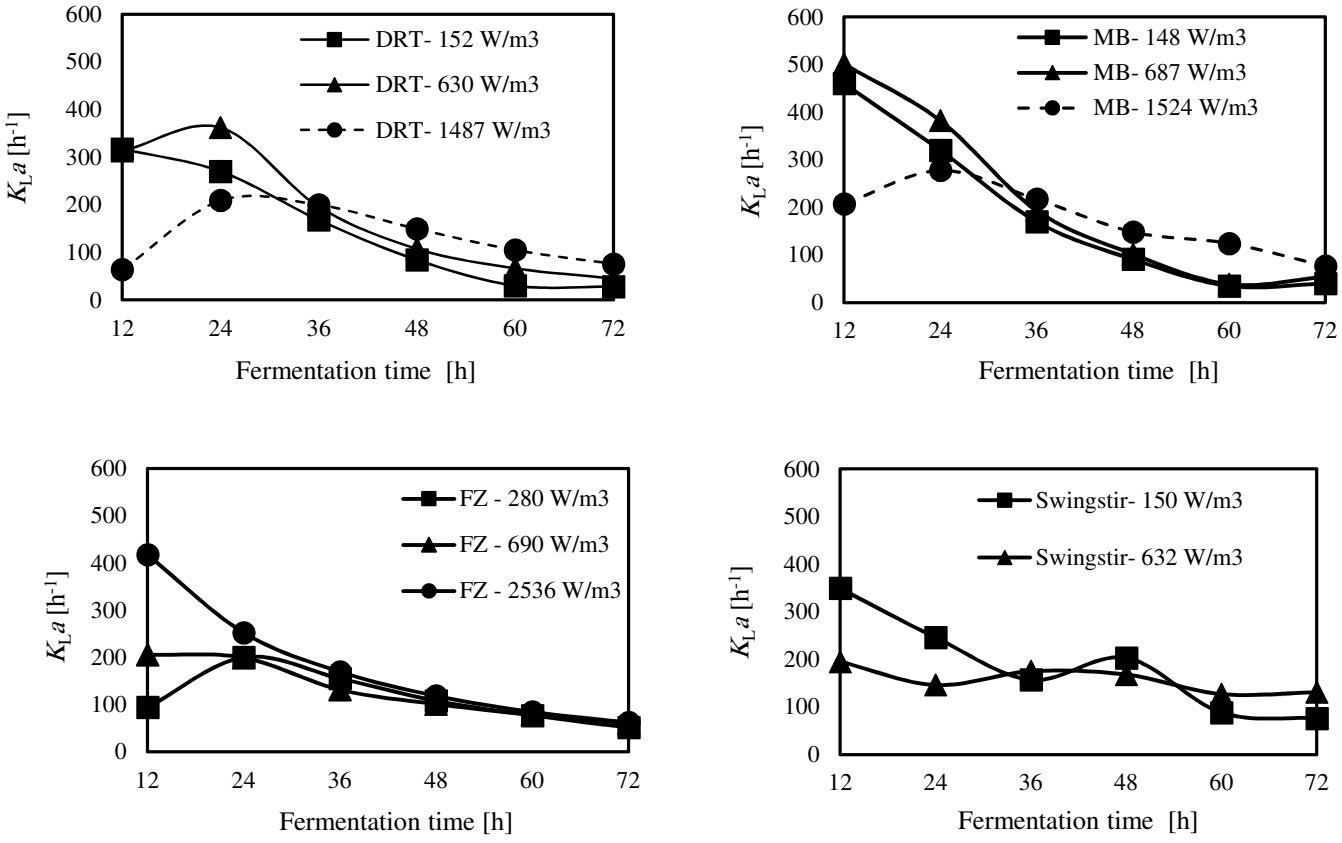


Fig. 4-17 Effect of mixing by different impeller on K_La improvement

4-9 Effect of mixing on glucose consumption in submerged fermentation culture; a comparison between two large impeller and DRT impeller

The results of **Fig. 4-18**, indicate that changing the pattern of mixing from radial (DRT, multi radial impeller) to axial (MB) did not have any effect on sugar consumption. However, in the case of changing radial mixing (the DRT impeller) with global axial pattern of mixing (FZ impeller, **Fig. 4-19**), at the same energy consumption, the rate of glucose consumption when using FZ impeller was higher than that of the DRT. It was due to the decreasing the mixing time when using FZ impeller. Consequently, because of reduction in mixing time the rate of carbon source mass transfer in fermentation culture has been increased. Generally, sugar consumption in biological culture except the mechanical agitation and environmental hydrodynamic was dependent on different parameters such as physiological condition and

kinetic. Therefore, analysis the rate of glucose consumption just by mentioning on the mixing effect could be complex. Consequently, in this study finding a clear result from the effect of flow pattern of mixing on the rate of glucose consumption was not possible.

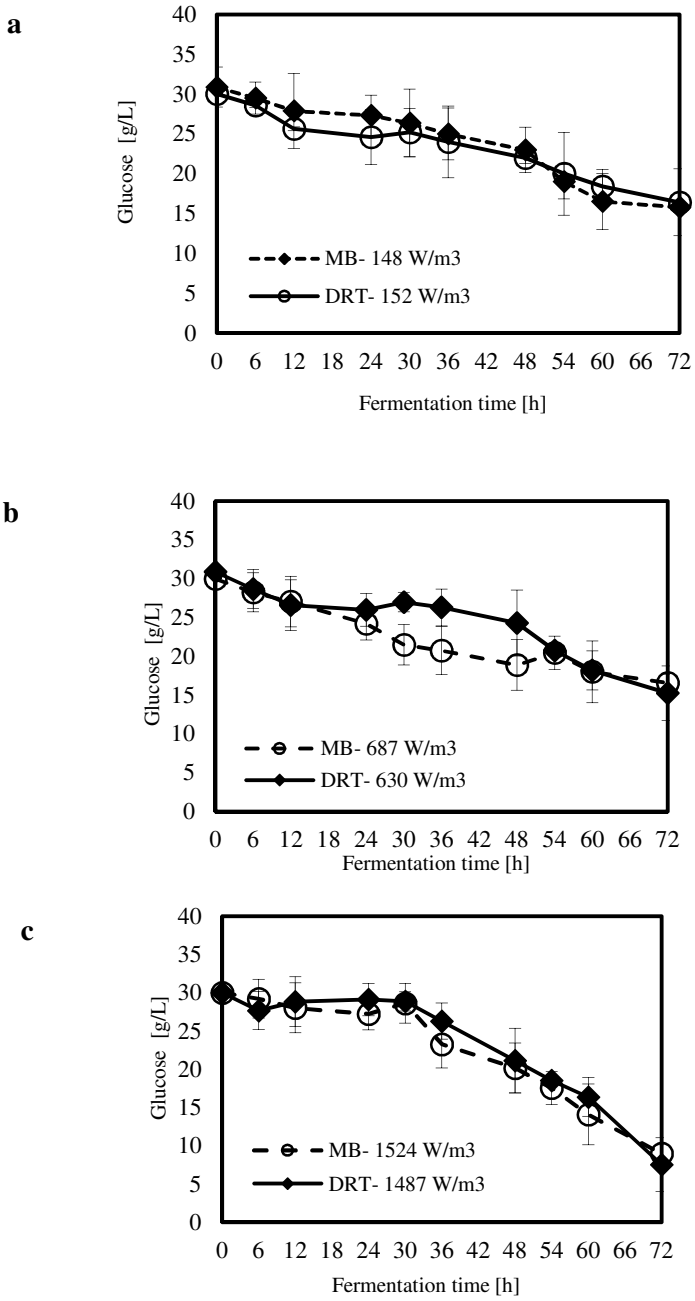
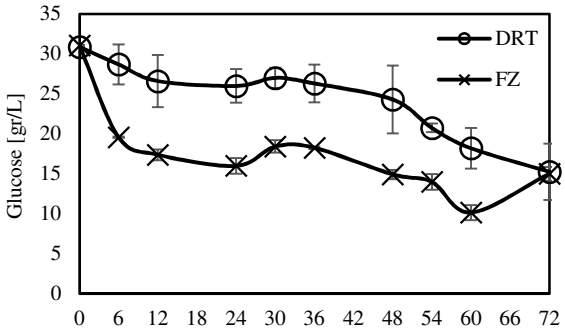
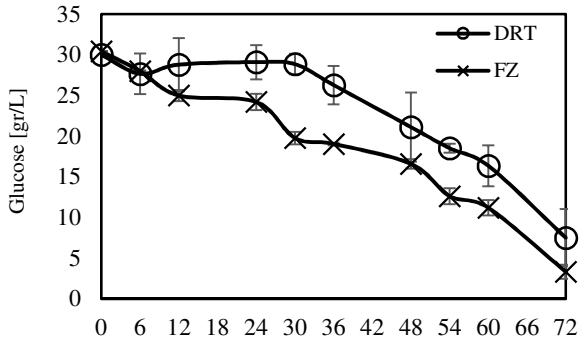


Fig. 4-18 Effect of flow pattern of mixing using MB and DRT impellers on carbon source mass transfer



(a) Low power input



(b) High power input

Fig. 4-19 Effect of flow pattern of mixing using FZ and DRT impellers on carbon source consumption at (a); Low power input, (b); High power input

4-10 Summary

Investigation on the effect of hydrodynamic and mixing condition on mass transfer in submerged fermentation were summarized as follows;

- In the condition using DRT, the NT_m was strongly depended on *Re* more than that of the other agitators. At the same *Re* the value of NT_m when using Swingstir® was the lowest.
- Mixing by Swingstir® in a Newtonian fluid would be leaded to a lower mixing time at the same P_v. At the same Re_m the π_3 using Swingstir® was the lowest. However, the π_3 of condition using MB was higher than that of the FZ but mixing time using FZ was lower than that of the MB.
- The MB significantly intensified K_La at low and moderate power input in comparison with the DRT. In addition, the decrease in enzyme activity at high P_v during

fermentation by the MB was due to the incomplete growth of the stressed cells. At the same energy consumption, the value and stability of DO concentration when using Swingstir® was higher than that of the MB.

- Uniform culture velocity formed by the FZ impellers at different power consumption created a more appropriate media for oxygen mass transfer during fermentation than that of the DRT impellers at low and high P_{in}. K_La values of fermentation broth using FZ impeller at different P_v after t = 24h were approximately the same. It means after growing the cells K_La of culture mixed using FZ was independent on the P_v. When FZ was used for mixing, the DO was higher and more stable than that of the MB and DRT
- Low culture viscosity was one of the reason of keeping the K_La at desirable value until the end of the fermentation, using Swingstir®. Finally, the larger K_La when using Swingstir® presented the higher aeration capacity of the fermenter.
- Rate of K_La reduction by increasing the fermentation time when mixing by DRT and MB were higher than that of the agitation with FZ or flexible-shaft impeller. K_La of fermentation culture agitated by Swingstir® were kept relatively constant after growing the cells (after t = 24h).
- However, at the same P_v, DO concentration of FZ and Swingstir® was relatively the same but because of effective instability preparation by Swingstir® K_La at middle and end times of fermentation was higher

Symbols;

Nomenclature

K	Consistency index	[Pa s^n]
$K_L a$	Volumetric mass transfer coefficient	[h^{-1}]
N	Rotational rate	[s^{-1}]
N_P	Non-dimensional power number	-
n	Flow index	-
P	Power consumption	[W]
P_v	Power density	[Wm^{-3}]
Re	Reynolds number	-
T	Tank diameter	[m]
T_m	Mixing time	[s]

Greece symbols

μ_a	Apparent culture viscosity	[Pa s]
μ_{eff}	Effective viscosity	[Pa s]
ρ	Density of culture broth	[kgm^{-3}]
v_g	Superficial gas velocity	[ms^{-1}]
(π_3)	Mixing energy	-

Abbreviations

DO	Dissolved oxygen	[ppm]
DCW	Dry cell weight	[gL^{-1}]
FZ	Fullzone®	
MB	Maxblend®	-
DRT	Double Rushton turbine	-

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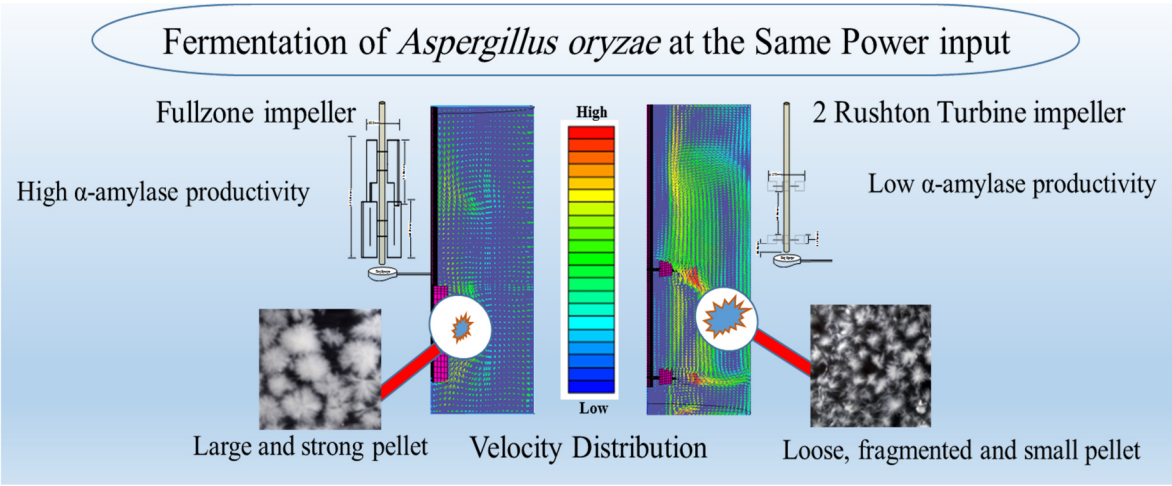
Chapter 5: Morphology control for intensification of fermentation of *A.oryzae* using adoptable mixing condition



Graphical abstract of chapter 5: [This picture was prepared by Narges Ghobadi, 2016]

Chapter 5 in brief: Results of this chapter presented the dependency of fungal macro- and micro-morphology on the quality and pattern of mixing force applied to the pellets of *A.oryzae*. Besides, showed the convenient morphology condition for intensifying the activity of alpha-amylase

5-1 Introduction into fungal morphology in submerged fermentation

Changes in environmental conditions during the fungal fermentation was leaded to desirable morphological characteristics for improved productivity. Morphology has a significant impact on protein secretion either directly (tip number) or indirectly (by affecting mixing and mass transfer). Generally, morphological forms of fungi are described on two levels : macroscopic and microscopic [1, 2]. Using macro-morphology, the morphology parameters such as pellet size, clump or freely dispersed mycelia are described. The micro-morphology is used to show properties like branch frequency, hyphal diameter and length [3, 4]. The micro-environment of hyphae is determined by the process conditions and the mixing of the culture. The resulting micro-morphology can have a direct effect on metabolic pathway activity and can influence productivity due to the segregation of hyphae. Fungal morphology is one of the important factors that could increase or decrease the biological products. **Fig. 5-1** provides a schematic of the interactions between process conditions, morphology, and productivity in submerged fermentation.

Special attention should be done to fungal morphology because it influences the rheology of the fermentation culture, and has a major effect on biological fluid homogenization, and mass transfer [5]. According to the finding of Casas L´opeza et al. [6] during the fermentation of *Aspergillus terreus* the agitation speed significantly affected the pellet diameter, morphology and production. It was reported the agitation speeds of more than 600 rpm have damaged the fungal pellets with 1200 micrometer in diameter. More investigations presented that during the fermentation of *A. terreus* broths of small and dense pellets had a shear thickening rheology however, the large fluffy pellets were strongly shear thinning.

Macroscopic morphology also determines the micro-environment of hyphae through effects on mixing, mass transfer, and bio-fluid rheology. Pellets may have dense and inactive cores due to the poor diffusion of nutrients [7], which may lead to cell lysis and loss of the interior

pellet structure [7]. Furthermore, the products of autolysis, which may be growth inhibitors, could diffuse through the pellets into the medium and prevent from the growth of the culture. Thus, development of macro-morphologies indirectly affects the productivity of a culture.

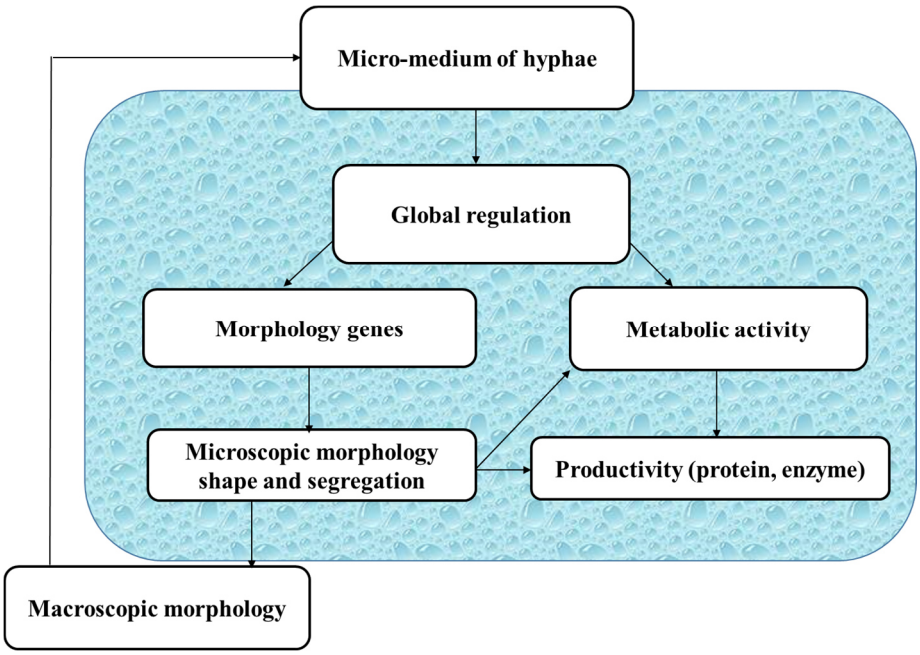


Fig. 5-1 Representation of interaction between process conditions, morphology and productivity [1, 2]

5-2 Morphology obstacle during the fermentation of Aspergillus oryzae

Fungal bioprocesses are inoculated from spores [8]. After germination: branching, extending, and entanglement of single hyphae introduce disperse hyphal networks (**Fig. 5-2**). Sometimes pellets develop by growing from single spores or by agglomeration. High shear forces and nutrient limitation cause pellet disintegration. One of the disadvantages of fungal growth in submerged fermentation is the difficulty to control the size of the pellet. As the pellet size increases, the diffusion of substrate within the pellet decreases [9], which reduces the productivity of many processes [10].

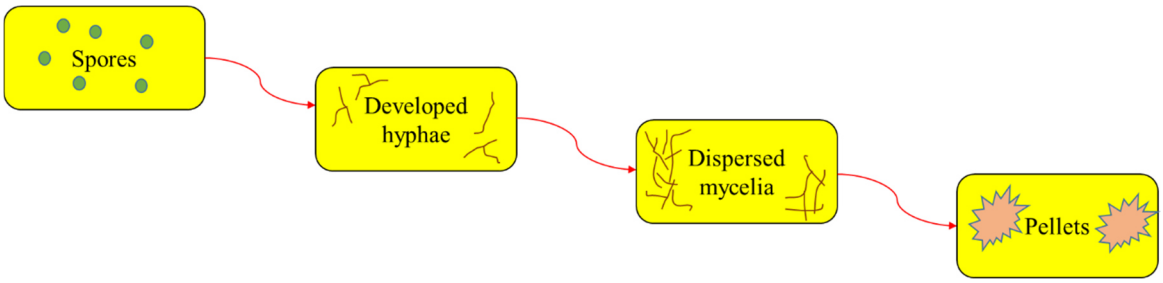


Fig. 5-2 Morphological life cycle of *Aspergillus oryzae* used in this study

As the biomass concentration increases, the mycelial branched network increases the viscosity of the medium and it was leaded to a non-Newtonian fluid behavior. In stirred tank such fluid behavior leads to a non-homogeneous broth, local mixed region near the impellers, where the shear stress is high [11]. Additionally, strong agitation leads to the shorter hyphae length. At high biomass concentration, the presence of slow moving or stagnant broth areas are observed near the fermentor wall [12].

One of the challenges during growing the fungi is formation of vacuoles. In fungal hyphae vacuoles are formed. This will decrease the fungal hyphal activity and reduction in specific growth rate [7].

Investigation on the fungal morphology in turbulent regime in stirred fermenter showed formation of small pellets in fully turbulent flow (less than 0.5 mm) also showed no oxygen diffusion limitation [7]. However, the literature did not mention on the productivity and challenges of shear stress formation. In this study, stirred fermentation was done at low-*Re* regime. Therefore, most of the pellet sizes were relatively large. Moreover, during the fermentation at low *Re*, with increasing the core zone in pellets, the cells faces with depletion of oxygen. Besides, increasing the biomass could be resulted in quicker consumption of the DO.

Regarding to the challenges mentioned above, in this study major motivations were overcoming these obstacles by investigation on the improved mixing condition of biological

fluid. Because improved dispersion in fungal broth is one of the important indirect factors affected on enhancing the morphology behavior in low- *Re* regime.

5-3 The morphology properties of fungi at different power densities during submerged-mixing by the MB and the DRT

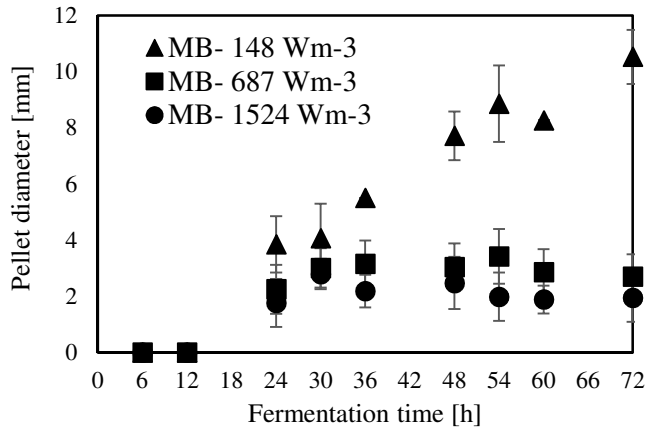
For the production of enzyme by fungi, the pellet is usually reported as being the desired morphology [13, 14]. Free filamentous mycelia give a viscous culture broth as a result of low gas-liquid mass transfer, and high power consumption. Here, the pellet diameter and hyphae length of mycelium were two morphological factors measured at different P_vs to investigate the effect of different impellers on the morphology of the cells (**Figs. 5-3(a), 5-3(b)**). Images of the pellet cell formation during fermentation are shown in **Fig. 5-3(c)** and **5-3(d)**. This indicates that changes in the P_v using the MB impeller caused significant changes in the pellet diameter. The main reason could have been the high $\dot{\gamma}_{ave}$ when using the MB impeller with the method (see **Table 3-2** and **3-3**, chapter 3) established by Metzner et al. [15]. The $\dot{\gamma}_{ave}$ listed in **Table 3-2** show that these greatly influenced the morphology of the cells in the fermenter. According to the results shown in **Table 3-2** and in **Figs 5-4(a)** and **5-4(b)**, by increasing the difference between the mean shear rates of the impellers, the difference between the hyphae lengths of the cultures was also increased. Study on the behavior of mixing by the MB showed that there was a big difference between the $\dot{\gamma}_{ave}$ (133 s^{-1}) at low and high P_vs, which caused a significant decrease in the hyphal lengths and the pellet diameters.

The results of **Fig. 5-3(d)** show that the cells of *A.oryzae* when using MB were more hairy than the case with dense core zone when the DRT was used at low P_v. Pellets mixed by the DRT at high P_v were attached with large hairy zones, but as shown in **Fig. 5-3(c)**, most of the pellet hairs were broken by the high shear stress at the tip of the blades. In particular, the cells were more subject to damage near the turbine tips where the highest speed gradient were

reached. The microscopic image of pellet cells in **Fig. 5-3(d)** also shows that agitation at high P_v prevented optimized growth of the cells when using the DRT, and, therefore, the morphology of the pellets began to form clumps (pellets with a weak core zone and loosely hair zone). The stressed pellets that were agitated by the MB impeller at high P_v were without any branched hairy portions.

Micromorphology studies (**Figs. 5-4(a), 5-4(b)**), have shown that changing the P_v by two impellers did not affect the hyphae diameter but the hyphae length of fungi could be changed at low, moderate and high P_v. Measurement results (**Figs 5-4(a), 5-4(b)**) showed that by increasing the $\dot{\gamma}_{ave}$ during mixing by the MB at low and high P_v the average hyphae length was decreased from (38.37±8.55) µm to (26.3±5.50) µm. **Fig. 5-4(c)** shows that because of a high mean shear rate at the tip of the turbine blade most of the hyphae and mycelium was mechanically damaged and cut. Breakage of the hyphae might result in preventing formation of the active tips during fermentation and in decrease in the enzyme activity. Microscopic images of changing the hyphae length by changing the impeller type and the P_v from low to moderate values are shown in **Fig. 5-5(a)**. Comparison among hyphae lengths in all four cases showed that the hyphae of pellets agitated using the MB at low P_v, were longer than the samples agitated by the DRT. Therefore, MB provided a convenient medium for elongation of hyphae at low mixing intensity. **Figs. 5-5 (a)** and **(c)**, show that by changing the P_v of the MB impeller from 148 to 687 Wm^{-3}, the maximum hyphae length of *A.oryzae* was approximately decreased from (220 ±3.199) µm to (60 ±8.003) µm. Also, increasing the P_v during fermentation when using a DRT impeller resulted in decrease in the maximum hyphae length of from (109 ±2.553) µm to (70 ±2.523) µm, (**Figs. 5-5 ((b)** and **(d)**)).

a



b

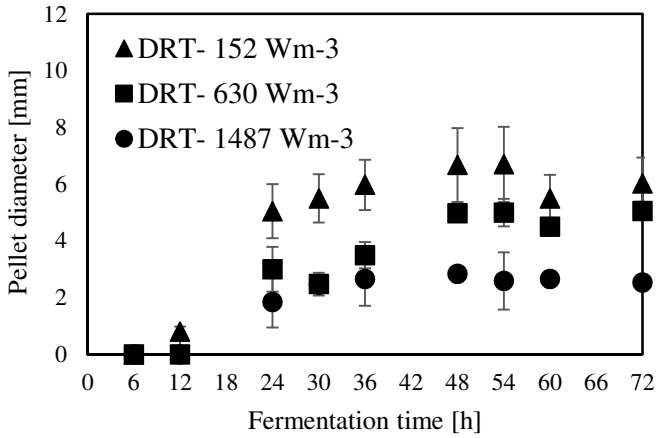


Fig. 5-3 Pellet diameter versus fermentation time at different P_vs, (a): MB, (b): DRT

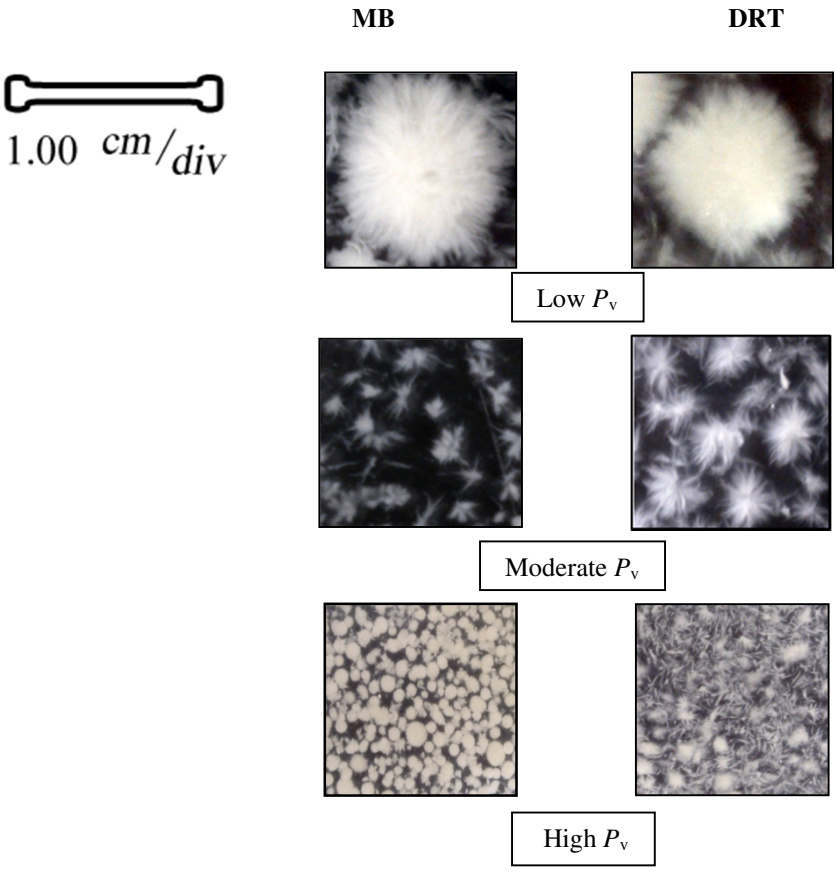


Fig. 5-3(c) Pellet macro morphologies of *A.oryzae* during the fermentation by MB and DRT at low, moderate and high P_vs.

Finally, it can be concluded that changing the power of impellers affects the way of the cells relate to the surrounding environment, and, the growth and product formation rates are influenced. In the present study, at P_v = 148 Wm^{-3} with the MB, the effective shear rate was more than that with the DRT, but because of uniform culture velocity distribution, cells grew like hairy pellets with a big core zone (without high density) could form an appropriate media for mass transfer during fermentation. Also, the simulation results (Chapter 3, **Fig. 3-3**) were compatible with the literature [16], showed that the MB dispersed the bubbles with a high degree of shear between the bottom and the tip of the impeller.

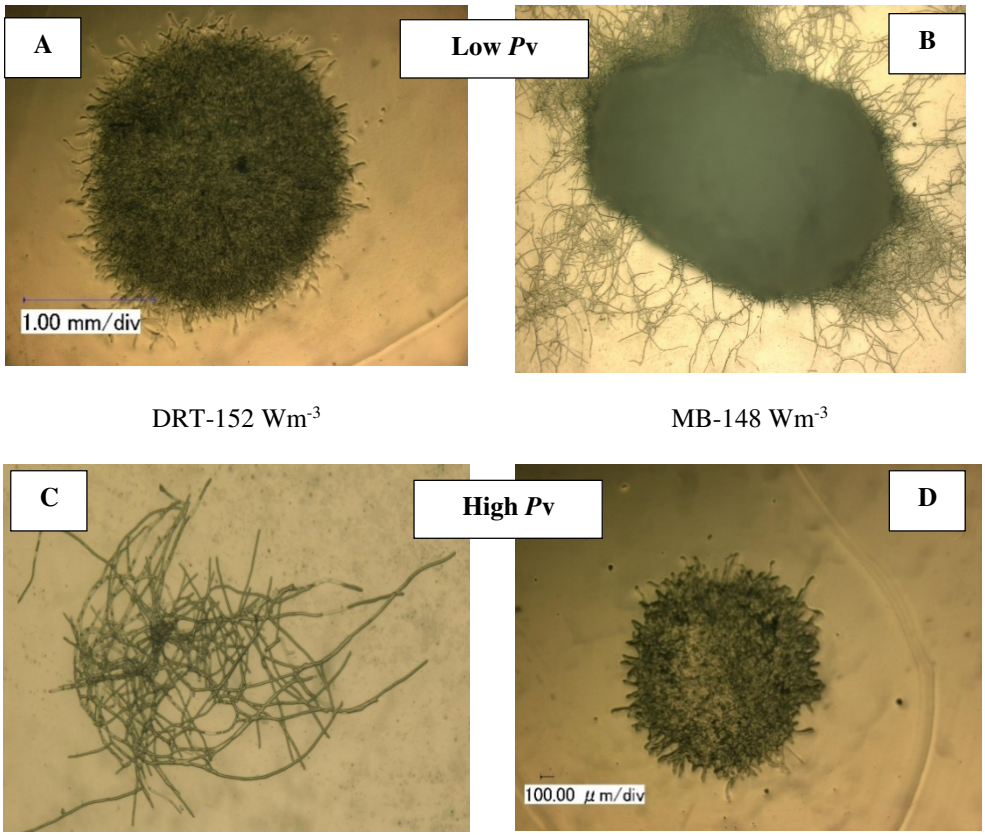


Fig. 5-3(d) Mixing effect at low and high P_v using MB and DRT impellers on macro-morphology

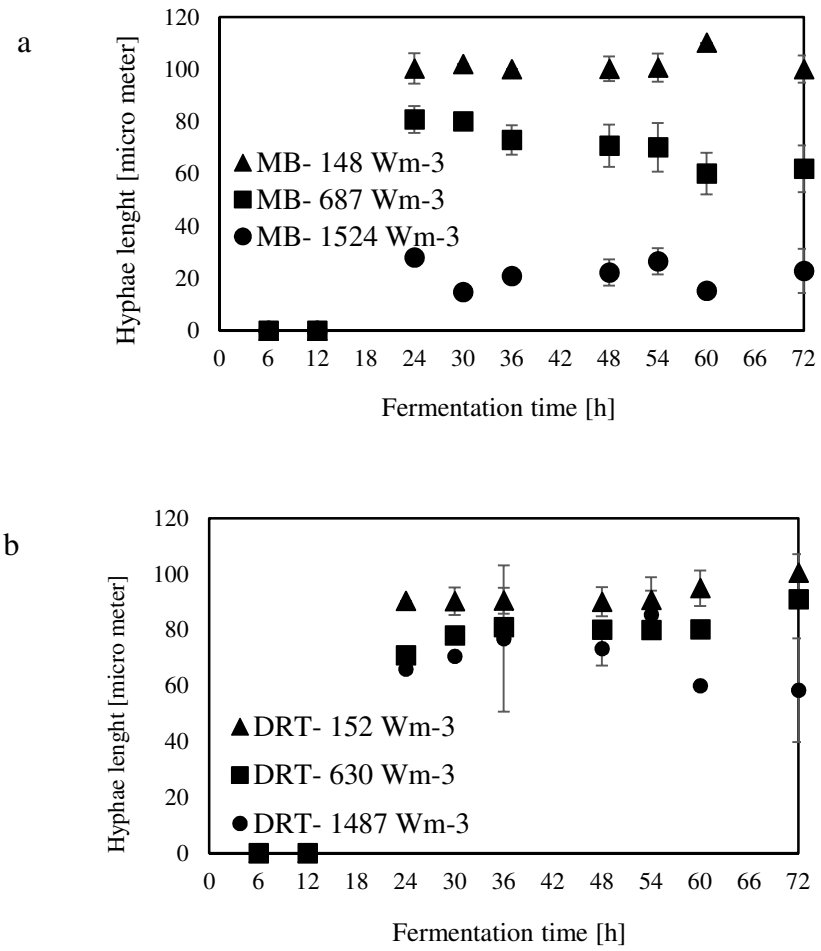


Fig. 5-4 Hyphae length versus fermentation time at low, moderate and high P_vs, **(a)**: MB, **(b)**: DRT

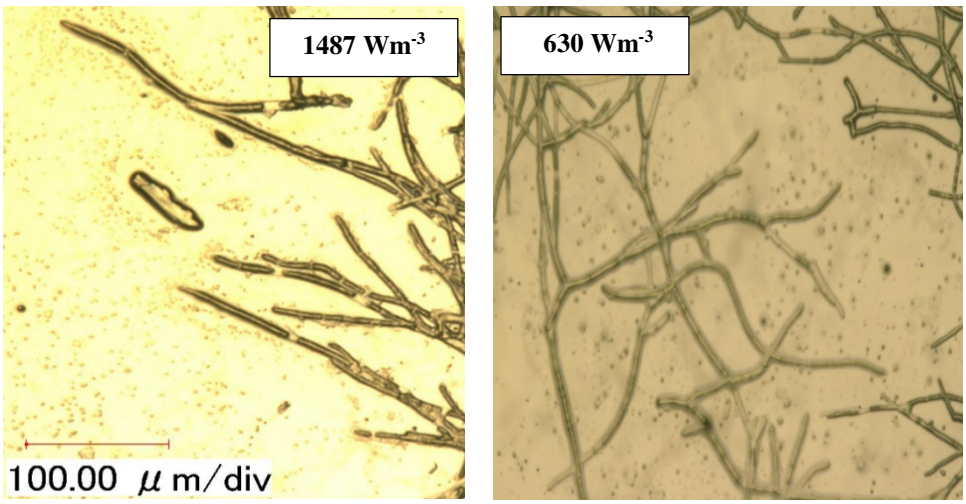


Fig. 5-4(c) Microscopic image of the effect of agitation by the DRT at moderate and high P_v on hyphae fragmentation, (500x)

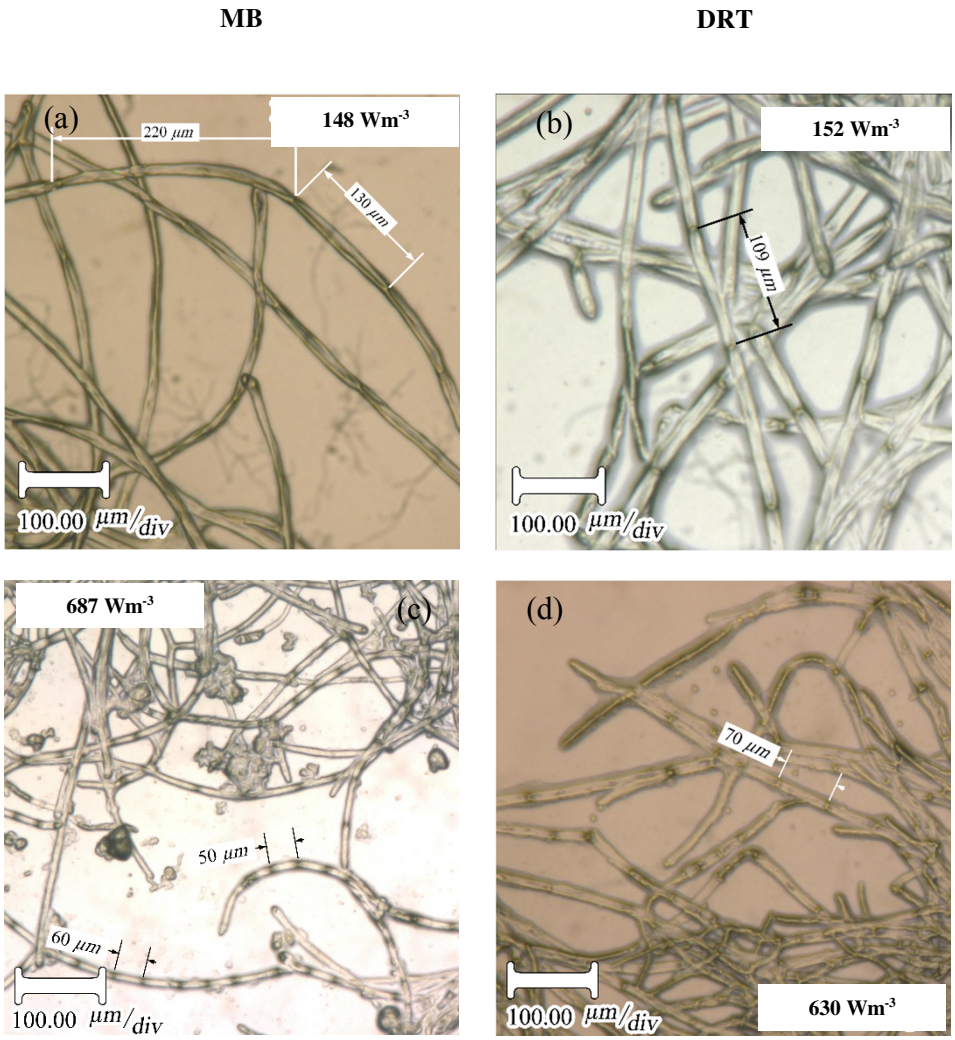


Fig. 5-5 Microscopic image of the effect of agitation by the MB ((a) and (c)), and DRT ((b) and (d)) at low and moderate P_v on hyphae length (1000x).

5-3-1 Effect of an impeller on biomass intensification and alpha amylase activity

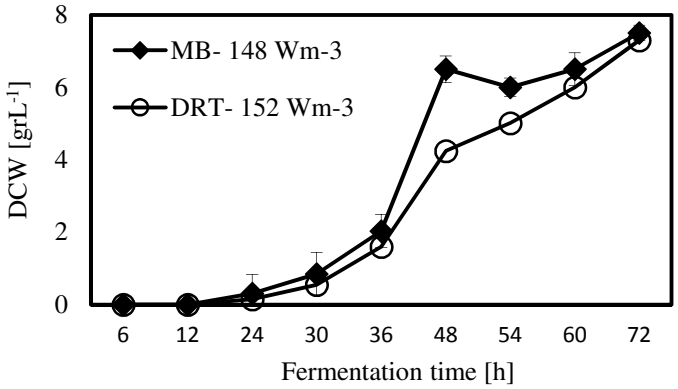
After sampling and extracting the supernatant via filtration of the culture broth, DCW was measured, and the results are shown in **Figs. 5-6(a), 5-6(b)** and **5-6(c)**. As shown in **Figs 5-6(a)** and **5-6(c)**, it was appeared that in all cases increasing the power density (P_v) also increased the DCW. A significant difference between the biomass production by MB and DRT impellers was seen by changing the P_v. Analysis of the results in **Figs. 5-6(b)** and **5-6(c)** clearly show that when using the DRT at a high P_v, the DCW production was lower than under moderate conditions using either MB or DRT. The DCW of cells agitated by the MB impeller

at low and moderate ranges of P_v were higher compared with that when using the DRT. Also, the results show that the P_v of the culture broth and a changing of the radial mixing by MB influenced the growth rate of fungi. In addition, the experimental data clearly show that fermentation via the MB impeller at 1524 Wm^{-3} resulted in the highest growth rate. However, growth rate of fungi when using DRT impeller at high P_v was significantly decreased. The results of DCW versus P_v at t =72 h (**Fig. 5-7**) were also compatible with the results shown in **Figs 5-6(a)** and **5-6(c)**.

The alpha amylase activity of the fermentation culture was measured after filtration of the culture broth during each sampling. These results are shown in **Figs. 5-8(a), 5-8(b),** and **5-8(c)**. As shown in **Figs. 5-8(a) – 5-8(c)**, it is clear that in all cases by increasing the P_v from low to moderate, the enzyme activity was increased, but at high P_v the activity, would be decreased. Diagram of changing *Re* (**Fig. 5-9**) during each fermentation condition showed that the time of cells exposing to the turbulent regime when using MB impeller at high P_v were the highest (*Re* >1000 until t = 42h). These results are important as a validation of exposure time to stress when using MB impeller at high P_v.

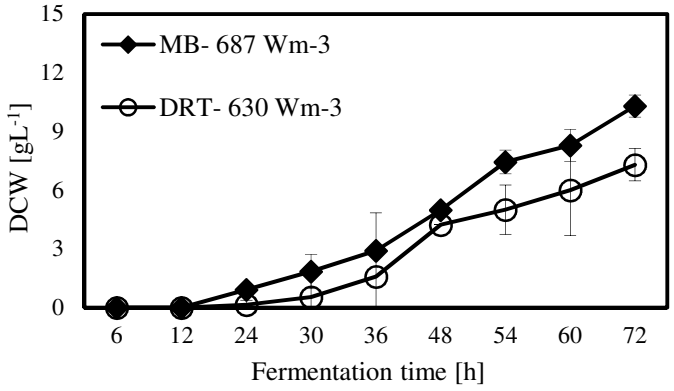
The experimental results of this study showed that increasing the P_v during the fermentation by both impellers had a significant negative effect on both enzyme activity and also the costs of energy consumption. The results of the power density effect on enzyme activity at t = 72 h are shown in **Fig. 5-10**. The data show that at low P_v, the mixing by the MB can enhance the enzyme activity in comparison with mixing by the DRT much more than fermentation at moderate and high P_v. Regarding the results shown in **Fig. 5-7**, biomass production could not be the main reason for the large differences between the enzyme activity of the MB and the DRT impellers at low P_v, because the difference between the DCW of fungi agitated at low P_v was not large.

Low power density



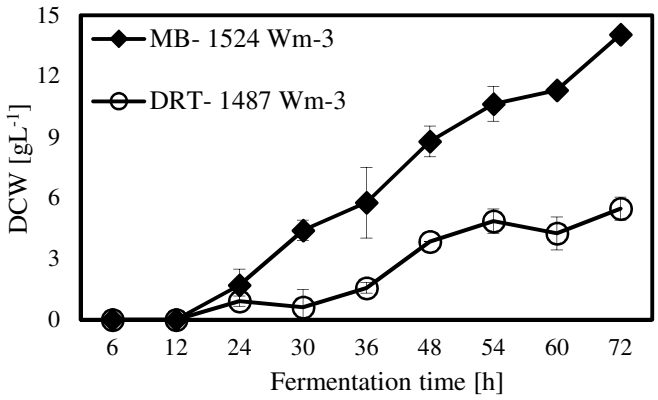
a

Moderate power density



b

High power density



c

Fig. 5-6 DCW versus fermentation time at different P_vs during the use of MB and DRT impellers, **(a)**; low P_v, **(b)**: moderate P_v; **(c)**: high P_v.

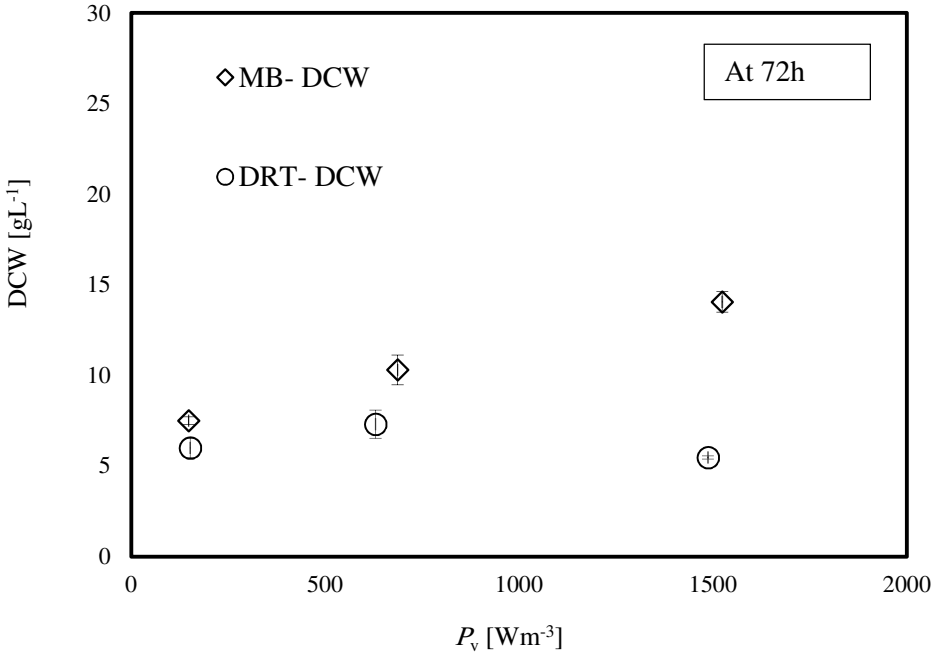


Fig. 5-7 DCW against P_v at t =72 h by the MB and the DRT

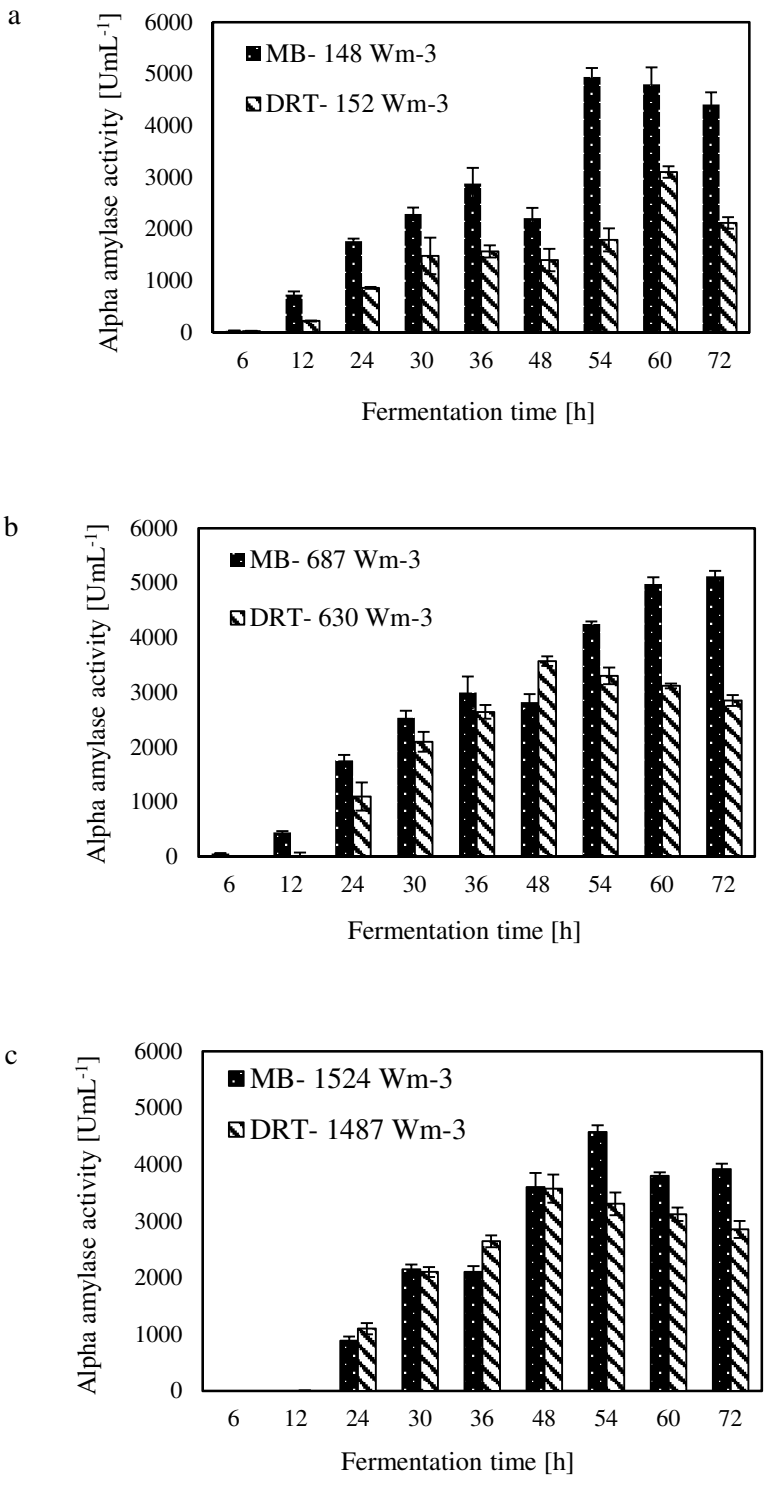
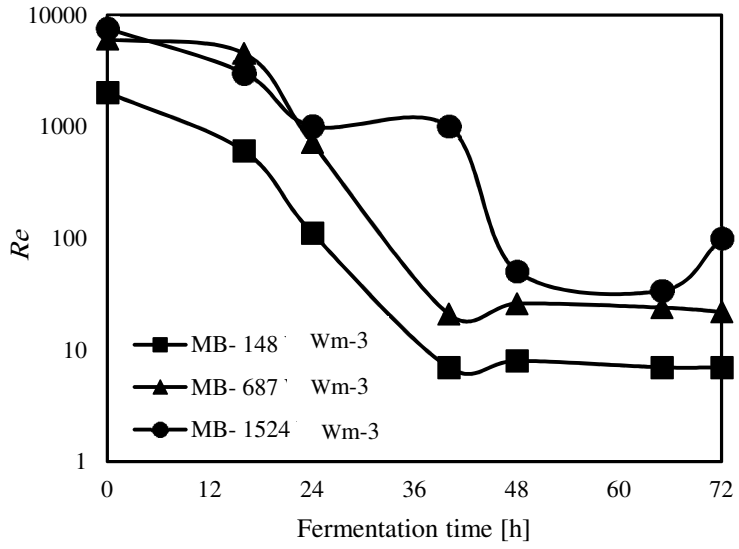


Fig. 5-8 Alpha amylase activity versus fermentation time during fermentation by MB and DRT at **(a)**: low P_v, **(b)**: moderate P_v; **(c)**: high P_v.

a



b

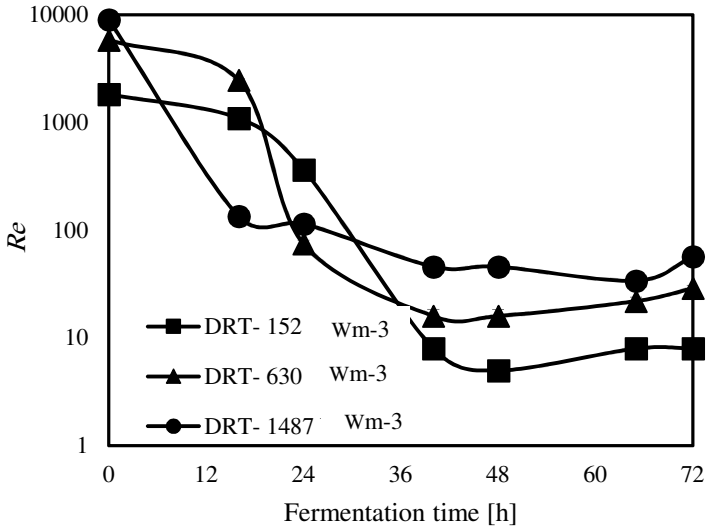


Fig. 5-9 Changing the *Re* in each sampling during the fermentation using MB and DRT impellers.

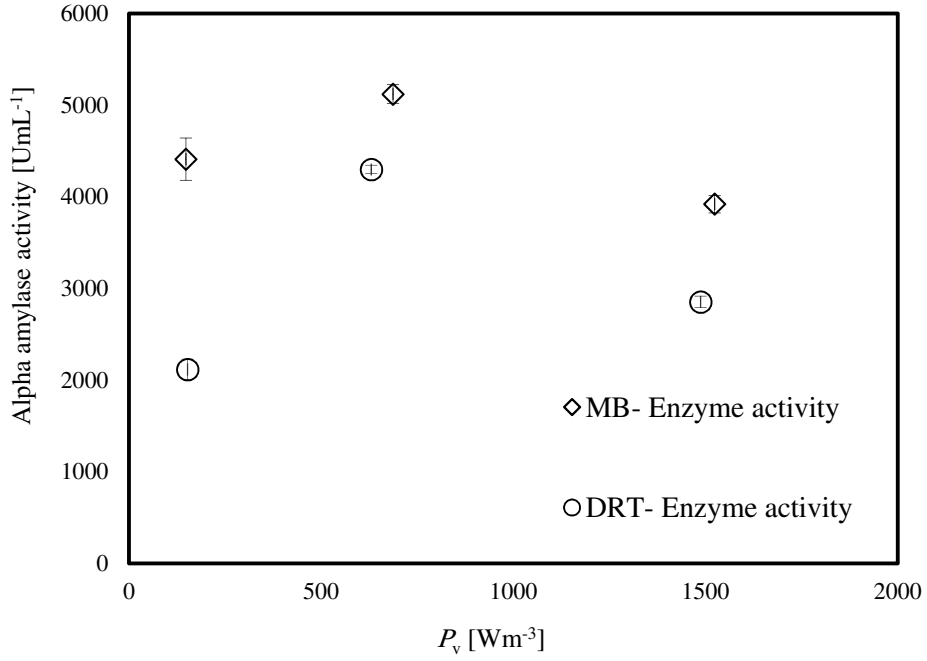


Fig. 5-10 Alpha amylase activity against P_v during fermentation by MB and DRT at 72 h

5-4 Investigation on the effect of multi-large agitator (FZ) on morphology control during submerged fermentation

In addition to the study of DCW, the morphology of fungi can also show the effect of a mixing flow pattern on the fermentation characteristics. For this reason the macro-pellet morphology of *A.oryzae* during the fermentation was studied under four different mixing conditions. Here, the morphological study showed that the overall morphology of the cells during agitation by the two types of impellers was determined by the pellet morphology. From the perspective of processing economy, the pellet morphology has some advantages over the filamentous growth form [17].

In the present study, the macro morphology of cells during fermentation at low and high P_{in} was illustrated in **Figs. 5-11(a) and (b)**. Also, the measurement results are shown in **Figs. 5-11(c) and (d)**. An overall view of the cell morphology indicates that with increasing the P_{in} using two types of impellers, the size of the pellets was decreased. During fermentation at low

and closed P_{in}, the pellet size of the culture agitated by the FZ impeller was larger than that agitated by the DRT. Pellet formation (**Figs. 5-11(a) and (b)**) observed in cultures grown at high and same P_{in} indicates that the size of the cells from the fermenter agitated by the DRT impeller were smaller and looser than the cultures grown by the FZ impeller. In addition, the turbine blades easily can break most of the weak and loose cells. Therefore, the activity of broken and damaged cells toward growth and enzyme production was decreased. Measurement results indicate that at a high P_{in} of 1.80 W by the DRT, the average pellet diameter was reduced by 38% compared with the pellet size at a low P_{in}. Furthermore, at high P_{in} values of 1.14 and 1.80 W by the FZ and DRT, respectively, the average pellet diameter produced by the DRT was decreased by 49%. At 1.80 W (500 min^{-1}), when using the DRT, the highest decline in pellet diameter was detected. The results shown in **Figs. 5-11(a)- (d)** indicate that at a high P_{in} when using DRT impellers not only the size of the pellets was reduced by comparison with use of the FZ impeller but also most of the pellet hairs were broken by the high shear rate at the tip of the blades. Finally, it could be concluded that use of the FZ impeller provided a convenient culture for the growth of pellets without either mycelium fragmentation or any cut damage at low and even at high P_{in}.

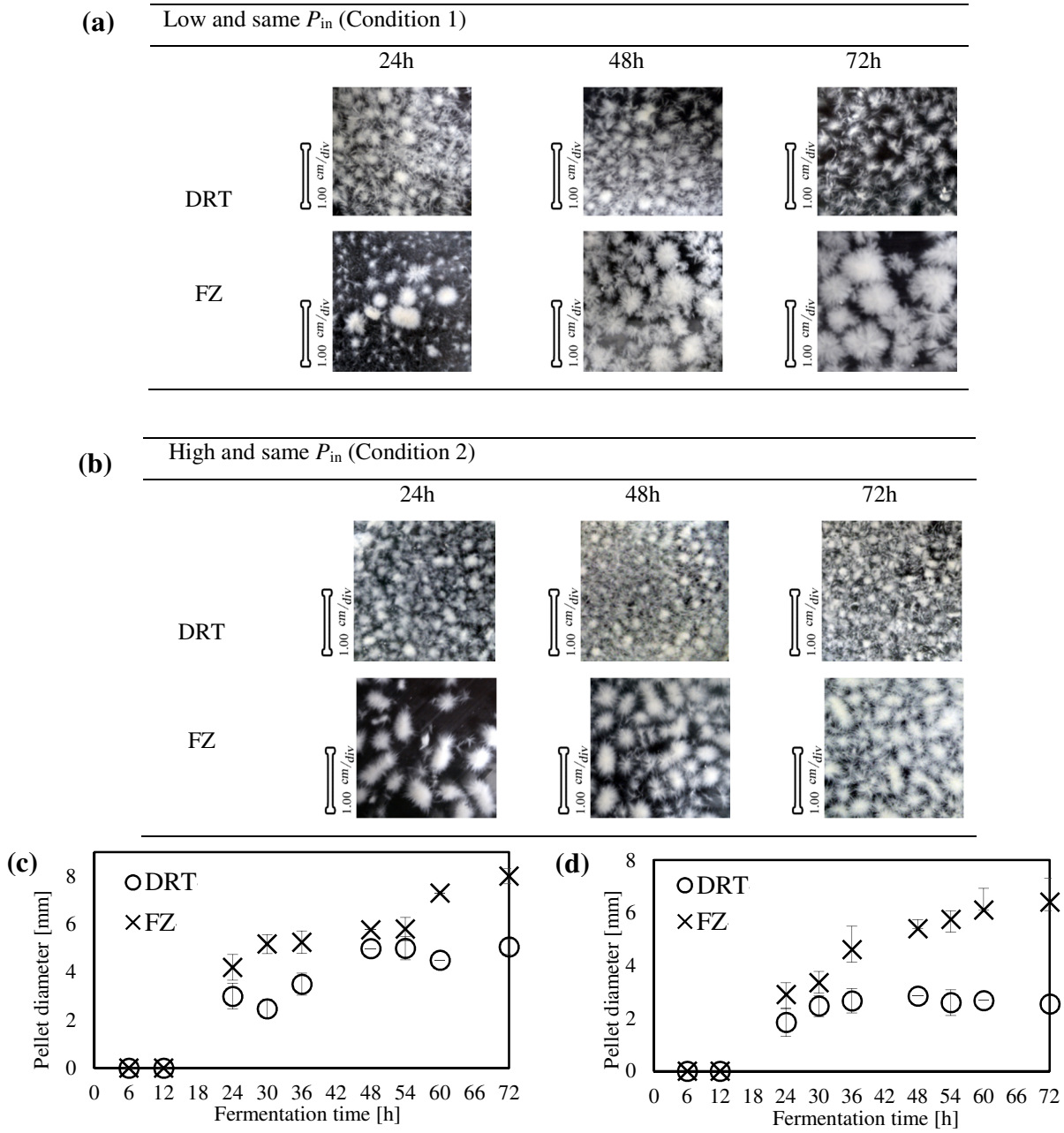


Fig. 5-11 Changing the macro-morphology of cells during agitation by DRT and FZ impellers at (a) low P_{in}; (b) high P_{in}; Changing the pellet diameter during fermentation using FZ and DRT impellers at (c): low P_{in}; (d): high P_{in}.

5-5 Effect of flexible-shaft agitator on the fungal morphology during the batch - stirred fermentation

According to the previous findings, one of the difficulties during mixing the fungal cells with pellet morphology is to control the morphology and enhance the mass transfer. As the

pellet size increases, the diffusion of substrate within the pellet decreases [9] which reduces the productivity of bioprocesses. Among the large size of pellets, substrate diffusion of the cells with large hairy zone and small core zone was lower than that the cells with short hairy zone. This is because the thickness of hairy zone prevents from reaching the glucose, oxygen and other substrate to the center of the cells [9]. In this study, as can be seen in **Fig. 5-12**, fluffy pellets with a loose structure during agitation with DRT exhibit a lower productivity than dense pellets of agitated with Swingstir®.

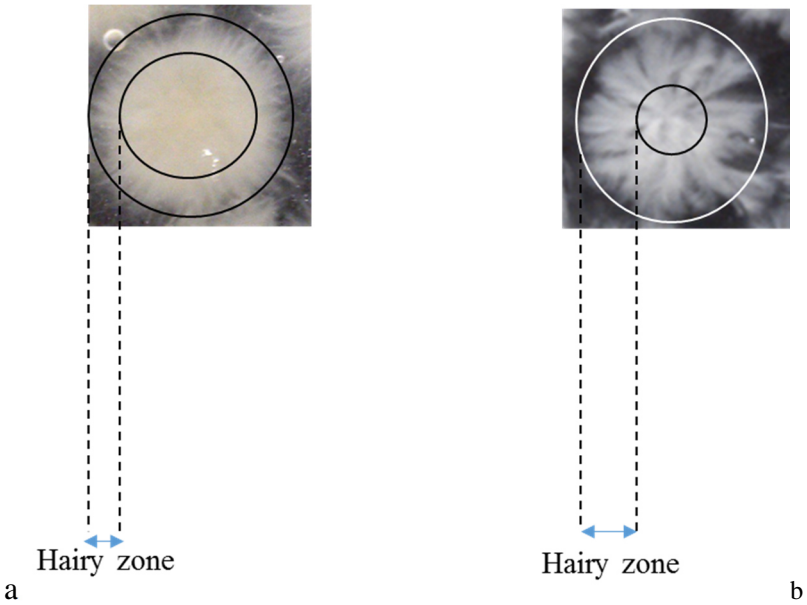


Fig. 5-12 Comparison between cell morphology agitated using (a) Swingstir® and (b) DRT impeller

In this study, the average amylase activity of the cells agitated by Swingstir® was 2.5 times more than that of the DRT. The results of alpha amylase activity during each sampling were also agreed with these findings (**Fig. 5-13**). It meant that using Swingstir® at low P_v could have positive effect on forming the pellets with controllable activity [18]. According to the previous findings, one of the difficulties during mixing the fungal cells with pellet morphology is to control the morphology and enhance the mass transfer. Pellet morphology is a result of environmental conditions and pellet age has an important influence on the effective diffusion coefficient and penetration depth of oxygen into the pellet [19, 20]. As the pellet size increases, the diffusion of substrate within the pellet decreases [21] which reduces the productivity of

bioprocesses. Among the large size of pellets, substrate diffusion of the cells with large hairy zone was lower than that the cells with the dense core zone. This is because the thickness of hairy zone (such as cells were growth by mixing with DRT in this study) that were leaded to a delay in reaching the glucose, oxygen and other substrate to the center of the cells [21]. In Addition, high viscosity condition is leaded to mass transfer limitation and mass transport limitations lead to a depletion of nutrients at the pellet core [22]. Deformation of the pellet structure when using radial agitator was found to have significant effect on penetration depth of substrate. Diffusion limitation of pellets was found to be mainly a function of size with an influence of advection in the outer zone of pellets that is supplied with oxygen [20]. During the formation of pellet morphology problems might arise with transport of nutrient into pellet cores thus reducing productivity [23, 24]. In this study, as can be seen in **Fig. 5-12**, fluffy pellets with a loose structure during agitation with DRT exhibit a lower cell activity than dense pellets of agitated with Swingstir®. It was in agreement with the previously study which mentioned that fluffy pellets with a loose structure were shown to be penetrated with oxygen, they exhibit a lower productivity than dense pellets [22].

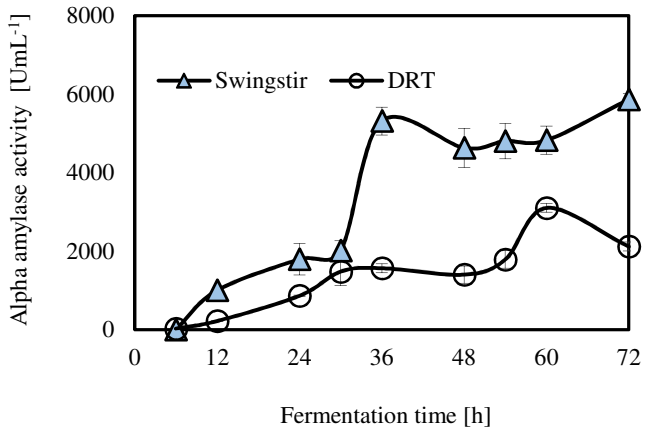


Fig. 5-13 Alpha amylase activity versus time during the fermentation of *A.oryzae* using DRT and Swingstir®

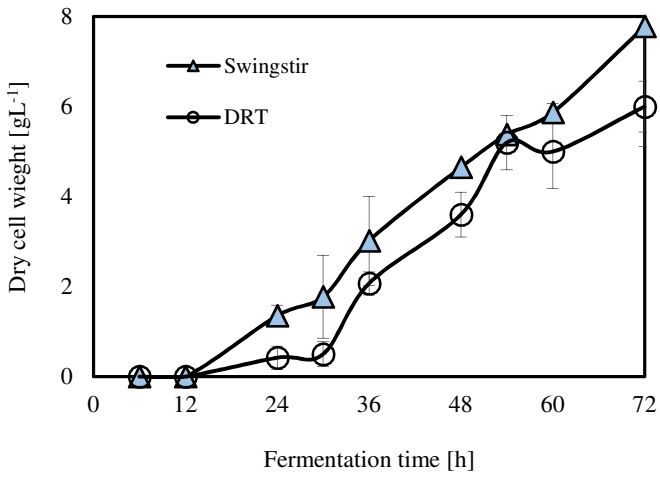


Fig. 5-14 Dry cell weight versus time during the fermentation of *A.oryzae* using DRT and Swingstir® impeller

Microscopic morphology has other, indirect effects on productivity. Studying the **Figs. 5-15(a) and 5-15(b)**, was shown that one of the negative effect of low K_La during agitation with the DRT impeller is reducing the intensity of hyphal branches during growing the hyphae. Previously, Katz et al. [25] suggested that the reduced formation of branches in a medium unfavorable for growth results from a natural selection. Wongwicharn et al. [26] and Rahardjo et al. [27] have shown that a decrease of DO tension reduces the branching intensity of *A.oryzae*, however increases the number of vacuoles in the hyphae (**Fig. 5-16(a)**) and decreases the percentage of active length. Due to this reason, branching intensity reduction could be a representative of decreasing the DO concentration in boundary between the pellets. Here, during mixing with the DRT impeller the presence of ballon-like region in the hyphae was observed, as well as the presence of vacuoles (**Fig. 5-16(b)**). Indeed, this phenomenon enables the fungi to stretch for areas richer in nutrient, with the formation of a minimum amount of biomass. Results of DWC measurements (**Fig. 5-14**) were agreed with formation of vacuoles and ballon-like region in the pellets agitated by DRT impeller and it leads to an increase of medium viscosity, which in turn decreases the oxygen transfer.

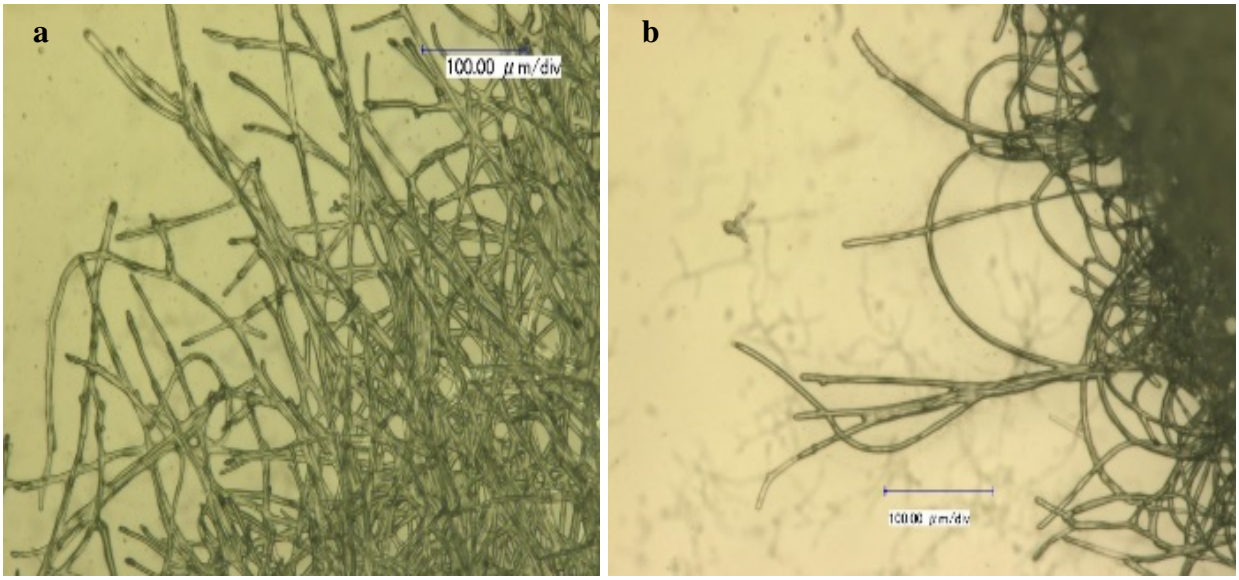


Fig. 5-15 Effect of DO concentration on the branching intensity of *A. oryzae* at the same P_v during agitation by **(a)** Swingstir® and **(b)** DRT impeller (at t =72 h - 500x)

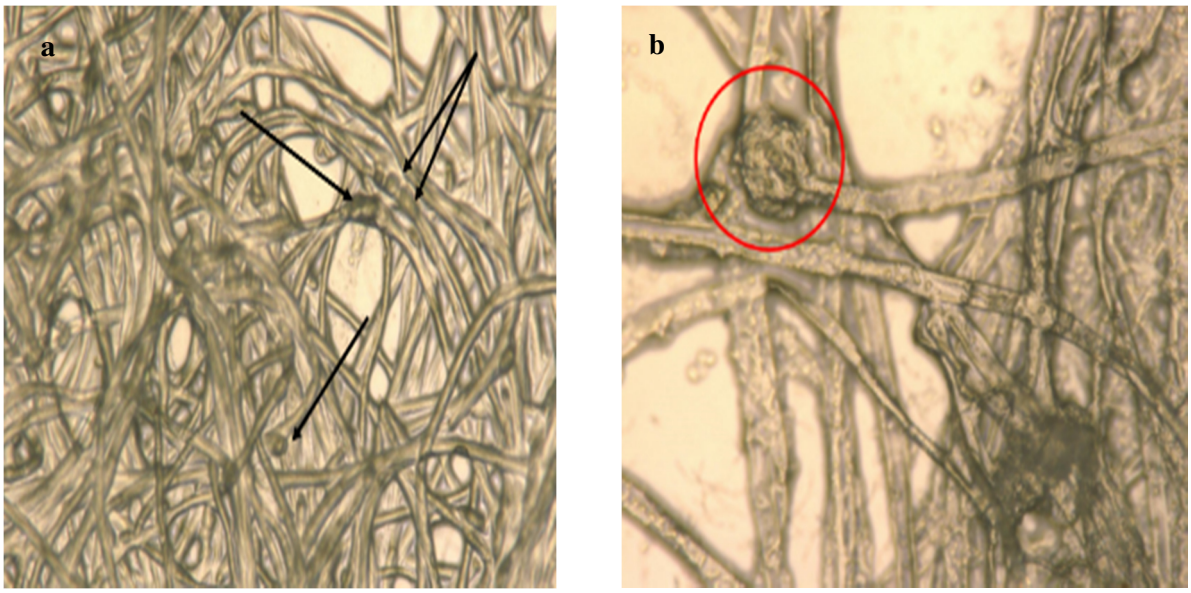


Fig. 5-16 Increasing the number of vacuoles in the hyphae during agitation by DRT impeller at t = 54h, 1000x **(a)**; Presence of ballon-like structures in the hyphae during mixing by DRT impeller at t = 48h, 500x **(b)**.

5-6 Evaluating the morphology improvement of flexible agitator with multi-large blade agitator

However, measurement results of pellet diameter, hyphae thickness (diameter) and hyphae length (**Table 5-1**) showed the flexibility of agitator did not have any effect on the macro- and micro- morphology size, but visualization of cell culture in this study (**Fig. 5-17**) indicates when using FZ impeller, the accumulation of cells was more than the cells agitated by

Swingstir®. Therefore, in the case of using Swingstir® there was enough space between the cells for oxygen and nutrient mass transfer. It was presented that when cells were agitated at a lower K_{app} and n_{app} (**Table 4-5**, chapter 4) when using the Swingstir® the ε_p which was used as a representative of ratio of hairy to core zone of pellets ($\varepsilon_p = 0.97$) was higher than the mixing with the FZ ($\varepsilon_p = 0.86$). Large ε_p value meant that the oxygen and nutrient could penetrate easily into the cells when the pellets with loose cores. Pellets with a more compact surface structure displayed a larger diffusion barrier for substrates than pellets with an open and porous structure [27]. Thus, when pellets had an open surface and porous characteristics in combination with a high K_La, the alpha amylase activity was positively affected.

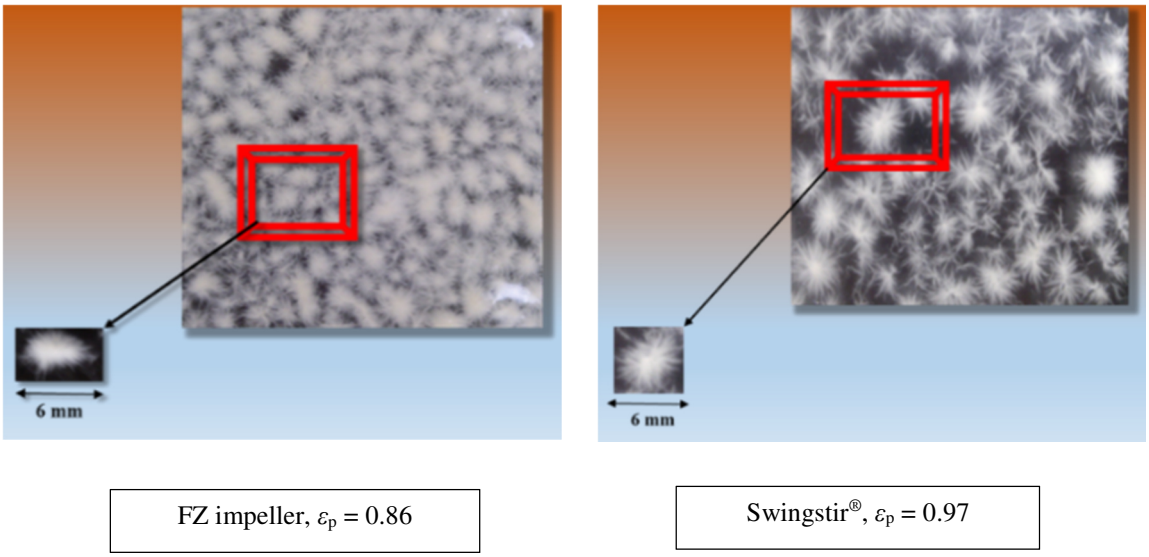


Fig. 5-17 Effect of FZ and Swingstir® on accumulation and pellet porosity at t = 72h

Table 5-1 Effect of Swingstir® on mocro- and micro morphology of *A.oryzae* at the same energy consumption

Fermentation time (h)		Pellet diameter (mm)	Hyphae thickness (µm)	Hyphae length (µm)	N_p	DCW (gL^{-1})	Re	$Y_{P/DCW}$ ($UmL^1g^{-1}L^{-1}$)	$Y_{P/E}$ (UmL^1W^{-1})
48	Swingstir®	5.7±0.50	4.1±0.20	49.00±3.50	7.0	4.45±0.95	6.90	1123	4830
	FZ	5.4±0.42	4.0±0.57	48.00±6.60	6.0	7.37±1.59	54.33	542	3864
72	Swingstir®	6.4±0.40	4.3±0.50	49.90±6.00	30	10.61±1.56	3.76	752	7714
	FZ	6.3±0.56	4.6±0.57	47.56±4.20	7.5	10.78±1.25	32.30	527	5494

$Y_{P/DCW}$: Yield production (alpha amylase activity) based on DCW

$Y_{P/E}$: Yield production (alpha amylase activity) based on power consumption

Macro and micro-morphology of *A.oryzae* have been studied to show the interaction between morphology and rheology. Microscopic image of **Fig. 5-18** has been showed to investigate the effect of mixing by Swingstir® on hyphae branch intensity and quality of uniform hyphae elongation. In addition, size of pellet diameter, hyphae thickness and hyphae length as a morphology parameter were shown in **Table 5-1**.

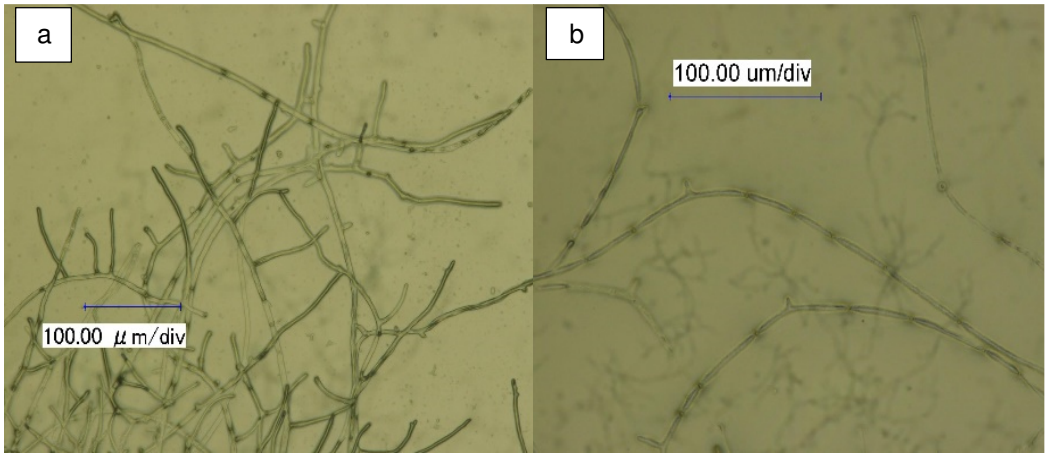


Fig. 5-18 High intensity of branch formation at t = 48 h (500x) **(a)** and uniform hyphae elongation at t = 72 h, (800x) when using Swingstir® in fermentation **(b)**.

5-7 Effect of fermentation by Maxblend on morphology of A.oryzae in comparison with Swingstir®

In this section, the main aim is to found that what the advantage of Swingstir® is in submerged fermentation of *A.oryzae* to intensifying the production comprising with single-large blade agitator. Based on this goal, investigation on the results of **Fig. 5-19** indicates during the fermentation by 2D axial agitator (MB) the size of pellets and DCW were lower than that of the mixing using flexible shaft agitator at the same P_v. It might be for the reason of low stress production during mixing by Swingstir® (See chapter 3 **Fig. 3-5** and **3-9**) comparing with the mixing condition by MB at the same P_v.

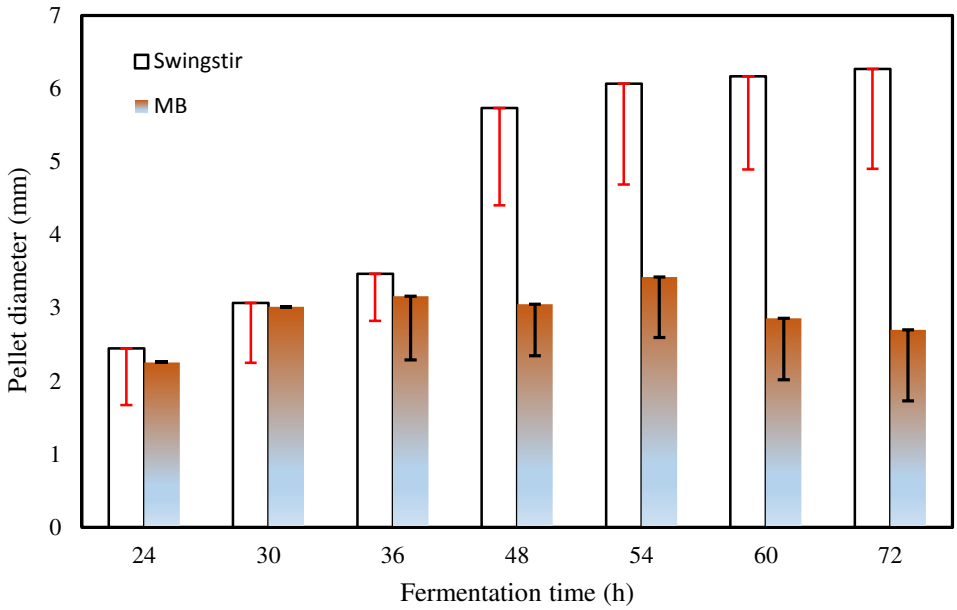


Fig. 5-19 Comparison between pellet diameters agitated by Swingstir® and MB impellers at the same P_v (= 690 Wm^{-3})

Results of **Fig. 5-20** showed that by increasing the biomass production by flexibility of agitator activity of cells also have been increased. Finally, it could be concluded that at the same P_v, and also relatively the same biomass production, the enzyme activity (**Fig. 5-21**) of pellets fermented in the condition using Swingstir® were higher than that of the MB.

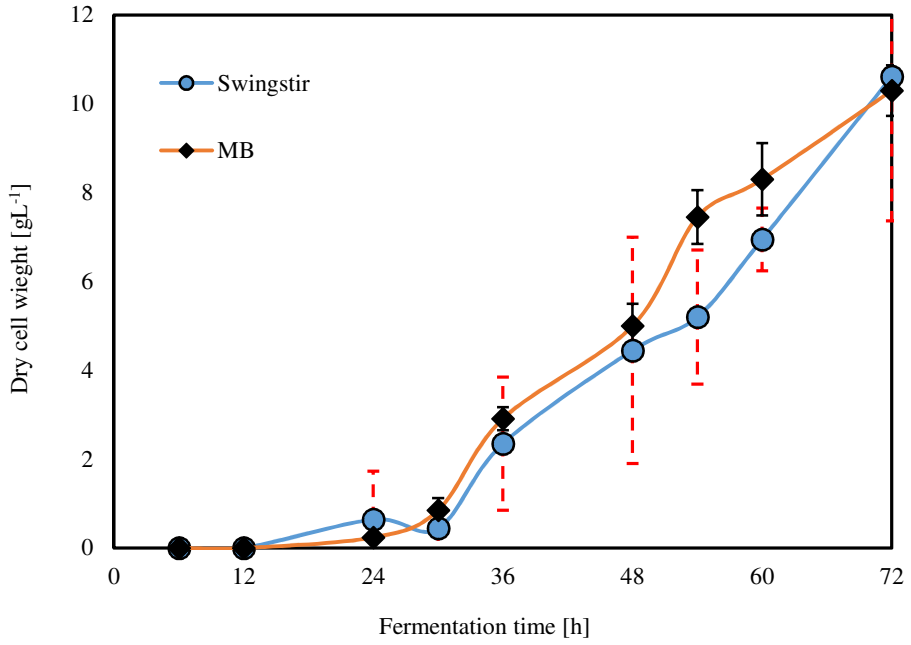


Fig. 5-20 Comparison between DCW agitated by Swingstir® and MB impellers at the same P_v (= 690 Wm^{-3})

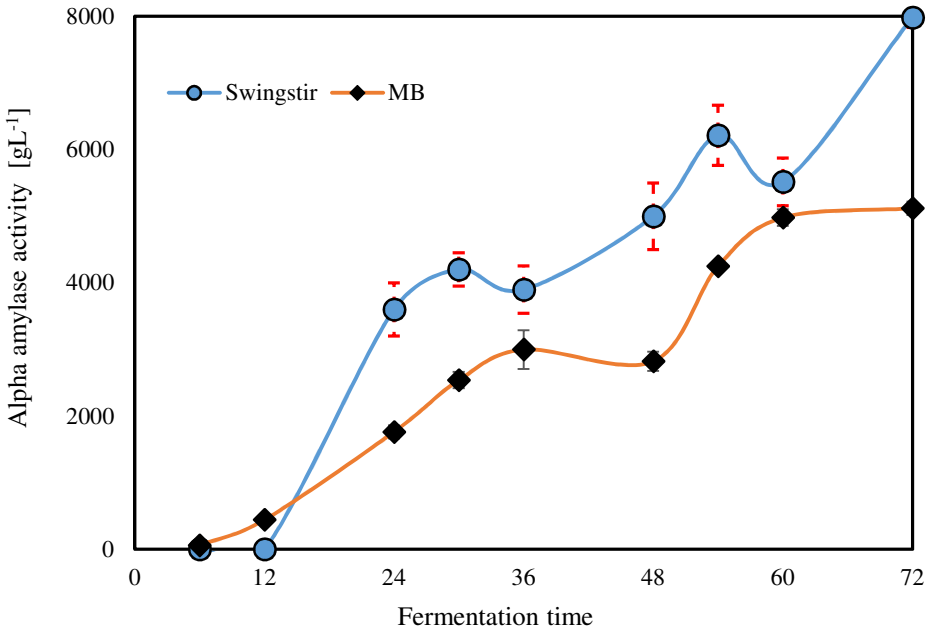


Fig. 5-21 Comparison between alpha amylase activity agitated by Swingstir® and MB impellers at the same P_v (= 690 Wm^{-3})

5-8 Correlation between pellet diameters, hyphae length and energy dissipation rate in stirred fermentation of A.oryzae

At the same agitation intensity pellet size is hardly affected by DO tension. According to the pervious researches [7], particles with a density close to water will follow the stream line

of liquid flow, which diminishes the collisions of the particle-particle or impellers [7]. In this study the wet densities of fungal was closed to that of water (996 ~ 1010 kgm^{-3}), therefore, during fungal fermentation the most important mechanism of cell damage is considered to be the interaction of pellet with circulated liquid. One of the most important factor affects on liquid circulation was the method of applied shear rates and energy to the liquid for circulation in stirred fermenter. Here, energy dissipation rate (EDR) was used as a representative of agitator which effected on the liquid circulation. Results of **Table 5-2** indicates how EDR using different agitator was affected on the P_d (macro-morphology) and H_L (micro-morphology) in determined sampling time.

Table 5-2 Correlation between energy dissipation, macro- and micro fungal morphology using different kind of impellers during the submerged fermentation of *A.oryzae*

Impeller	Fermentation time (h)	P_d - EDR	H_L - EDR
FZ	24	$P_d = 2.90\ \varepsilon^{-0.375}$, $R^2 = 0.91$	$H_L = 2.90\ \varepsilon^{-0.375}$, $R^2 = 0.82$
	48	$P_d = 40.70\ \varepsilon^{-0.201}$, $R^2 = 0.61$	$H_L = 40.78\ \varepsilon^{-0.201}$, $R^2 = 0.62$
	72	$P_d = 5.49\ \varepsilon^{-0.458}$, $R^2 = 0.93$	$H_L = 45.42\ \varepsilon^{-0.244}$, $R^2 = 0.82$
MB	24	$P_d = 2.13\ \varepsilon^{-0.300}$, $R^2 = 0.98$	$H_L = 37.24\ \varepsilon^{-0.588}$, $R^2 = 0.89$
	48	$P_d = 2.82\ \varepsilon^{-0.503}$, $R^2 = 0.97$	$H_L = 37.25\ \varepsilon^{-0.588}$, $R^2 = 0.98$
	72	$P_d = 2.39\ \varepsilon^{-0.743}$, $R^2 = 0.97$	$H_L = 39.87\varepsilon^{-0.567}$, $R^2 = 0.71$
DRT	24	$P_d = 2.30\ \varepsilon^{-0.479}$, $R^2 = 0.96$	$H_L = 79.54\varepsilon^{-0.567}$, $R^2 = 0.99$
	48	-	$H_L = 74.5\varepsilon^{-0.252}$, $R^2 = 0.75$
Swingstir®	24	$P_d = 2.11\varepsilon^{-0.398}$, $R^2 = 0.99$	$H_L = 27.16\varepsilon^{-0.296}$, $R^2 = 0.99$
	48	$P_d = 5.57\varepsilon^{-0.073}$, $R^2 = 0.99$	$H_L = 45.37\varepsilon^{-0.196}$, $R^2 = 0.99$
	72	$P_d = 6.02\varepsilon^{-0.103}$, $R^2 = 0.99$	$H_L = 55.35\varepsilon^{-0.277}$, $R^2 = 0.99$

Correlation between P_d and H_L and EDR were totally exponentially, and it was consisted of exponential value and constant value. Results of **Table 5-2** presented that the fungal hyphae length was found to be proportional to the energy dissipation rate. This implies that mechanical force can deactivate cells or decrease the specific growth rate by affecting on the pellet size and hyphae size. According to the results of **Table 5-2** the below results could be extracted;

- The constant values in both correlations for P_d and H_L during fermentation using MB relatively were kept constant, and at the beginning hours (t = 24h) of fermentation, P_d had less dependency to EDR when agitation was done by MB impeller.
- The biggest exponential values at the end of fermentation were related to the agitation with MB. It means that at high viscosity fermentation culture P_d and H_L had the highest dependency on EDR during the fermentation using MB.
- The smallest exponential values in both correlations for P_d and H_L were related to the fermentation culture agitated by Swingstir®, (low dependency of P_d and H_L on the EDR). In addition, by growing the cells from t = 48 to t = 72h, EDR had low effect on the P_d when agitating by Swingstir®. It could show that the mechanical force had not a big impact on cell deactivation or cell damage.
- Usually the constant value in each correlation have been increased by increasing the fermentation time and growing the cells. The largest constant value in H_L correlation was belonged to the mixed pellets when using DRT. At the beginning of fermentation, the biggest constant value for P_d and H_L were related to the fermentation culture agitated by DRT.

5-9 Summary

Investigation on the interaction between mixing characterization of submerged fermentation fluid and morphology of *A.oryzae* in this chapter showed important results as follows;

- The negative effect of low K_La during agitation with the DRT impeller is reducing the intensity of hyphal branches, increasing the number of vacuoles, and decreasing the percentage of active length. In addition, the turbine blades easily can break most of the weak and loose cells. Therefore, the activity of broken and damaged cells toward growth and enzyme production was decreased.

- Changes in the P_v using the MB impeller caused significant changes in the pellet diameter. The main reason could have been the high $\dot{\gamma}_{ave}$ when using the MB which caused a significant decrease in the hyphal lengths and the pellet diameters.
- Use of the FZ impeller provided a convenient culture for the growth of pellets without either mycelium fragmentation or any cut damage at low and even at high P_{in}. During fermentation at low P_{in}, the pellet size of the culture agitated by the FZ impeller was larger than that agitated by the DRT. It indicates that the size of the cells from the fermenter agitated by the DRT impeller were smaller and looser than the cultures grown by the FZ impeller.
- Fluffy pellets with a loose structure during agitation with DRT exhibit a lower productivity than dense pellets of agitated with Swingstir®. By increasing the hairy zone of pellets the gradient of oxygen between the culture and core zone of the pellet was decreased and it would be resulted in enzyme activity reduction. Thinning the boundary layer between the oxygen and cells is one of the advantage of Swingstir® for mass transfer intensification. Using Swingstir® at low P_v could have positive effect on controlling the cell morphology by saving the energy. When using FZ impeller, the accumulation of cells was more than the cells agitated by Swingstir®. Therefore, in the

case of using Swingstir® there was enough space between the cells for oxygen and nutrient mass transfer.

Symbols;

Nomenclature

H_L	Hyphal length	[µm]
K	Consistency index	[Pa s^n]
K_La	Volumetric mass transfer coefficient	[h^{-1}]
N_P	Non-dimensional power number	**-**
n	Flow index	**-**
P	Power consumption	[W]
P_d	Pellet diameter	[mm]
P_v	Power density	[Wm^{-3}]
Re	Reynolds number	**-**
$Y_{P/DCW}$	Yield production (alpha amylase activity) based on DCW	-
$Y_{P/E}$	Yield production (alpha amylase activity) based on power consumption	-

Greece symbols

ε_p	Pellet porosity	-

Abbreviations

DCW	Dry cell weight	[gL^{-1}]
DO	Dissolved oxygen	[ppm]
DRT	Double Rushton turbine	**-**
EDR	Energy dissipation rate	-
FZ	Fullzone®	-
MB	Maxblend®	**-**

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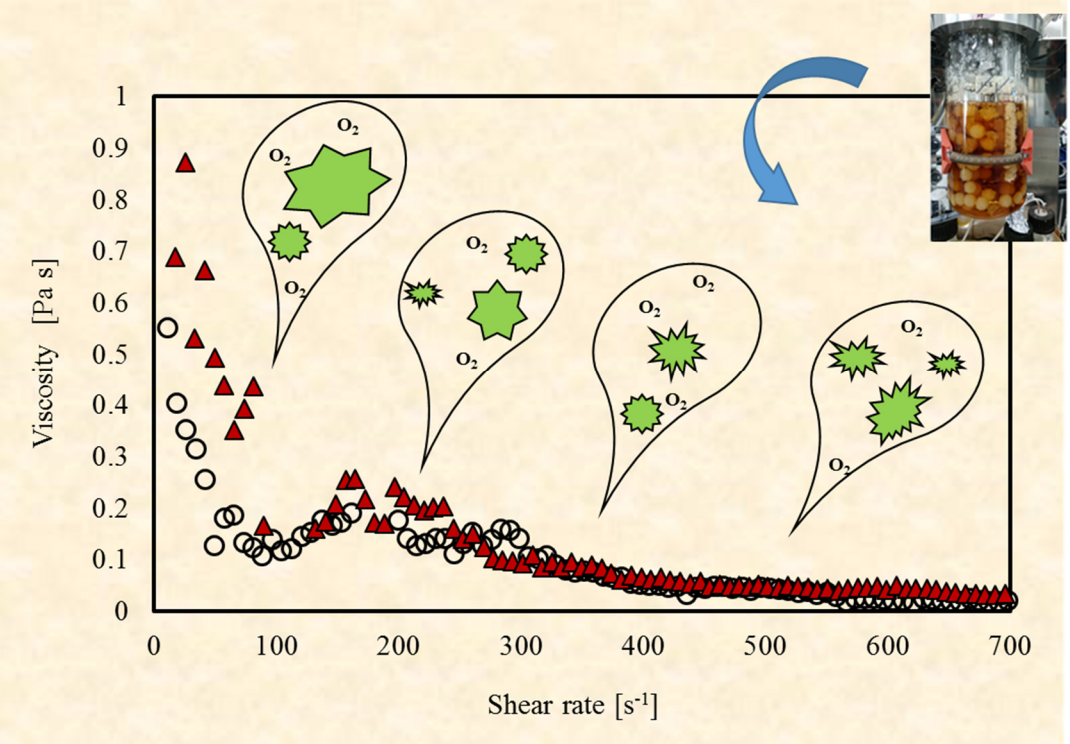
Chapter 6: Effect of flow pattern of mixing on rheology of complex fermented biological fluid



Graphical abstract of chapter 6: [This picture was prepared by Narges Ghobadi, 2016]

Chapter 6 in brief; Results of this chapter showed how viscosity, rheological model and thixotropic behavior of fermentation culture would be controlled and enhanced using mixing enhancement in a stirred fermenter.

6-1 Introduction

Knowledge about rheology of submerged fermentation has been mostly one of the complex and difficult concepts to understand because undesired phenomena such as settling of biomass, phase separation, inhomogeneity of the fermentation fluid, are often resulted in inadequate results. Desired morphology resulted in low power consumption, efficient mixing, homogene rheological properties, and sufficient mass transfer for nutrient dispersion. In a batch fungal fermentation the apparent viscosity (μ_{app}) of broth usually changed from 1mPa.s at the beginning of the fermentation to 1 Pa.s until the end of process [1]. By changing the biomass concentration and fungal morphology during the submerged fermentation pattern of changing the viscosity is changed and consequently, the rheological model has been changed. Controlling the rheological properties of mycelial fermentations may be difficult because of existing many factors influencing mycelial development and hyphal-hyphal interactions [2]. The performance of a bioreactor containing a filamentous fermentation broth is greatly influenced by the rheological properties of the broth. These properties are determined mainly by the concentration of biomass, its growth rate and morphology.

Due to this reason, finding a significant knowledge about the rheology of submerged fluid could not be clearly achieved. The main aim of this chapter is to show how changing the mixing flow pattern could facilitate the way of finding knowledge about rheology of fermentation culture and also present the improvement of mass transfer by decreasing the culture viscosity.

6-2 Study the apparent viscosity of fermentation broth in different hydrodynamic mixing conditions

In this part, however finding the real value of viscosity of fermentation culture was very difficult but finding a significant trend on changing the μ_{app} during the fermentation could be a useful tool to predict the mass transfer quality by growing the cells. Because, the high viscosity could be a representative of low or local mass transfer in stirred tank during the fermentation.

Results of μ_{app} measurement were shown in **Fig**. **6-1**. In each measurement, standard deviations of experimental data were up to 2%.

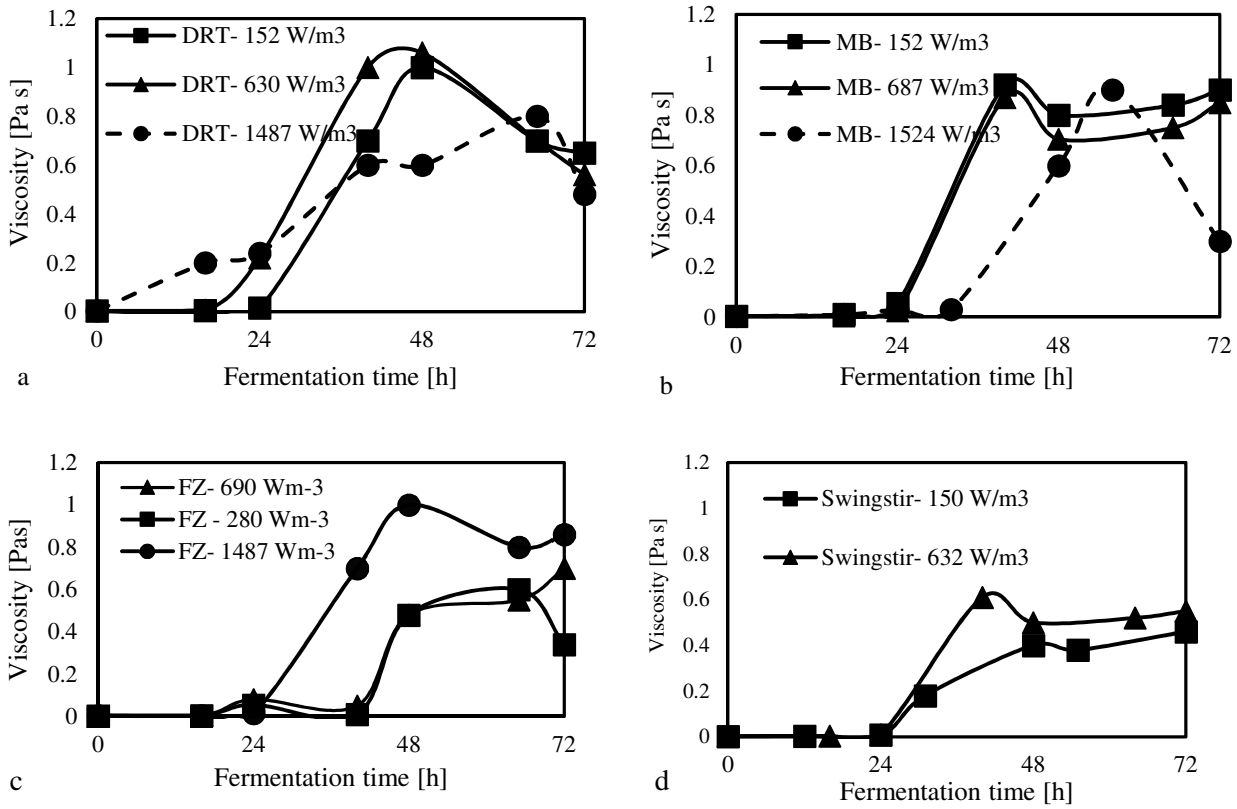


Fig. 6-1 Experimental results of apparent viscosity versus fermentation time using (a) DRT, (b) MB, (c) FZ, and (d) Swingstir® impellers

According to the results of **Fig. 6-1** and **Table 6-1** at the same and low P_v the media agitated by Swingstir® and particularly, FZ were fermented at low viscosity. This is one of the advantage of using flexible-shaft and large global axial multi-blade impeller for fungal fermentation. Experimental measurements provide that, viscosity of fermentation culture during mixing by MB after 36 h were controlled and was kept relatively constant but unfortunately it was quite high. Due to this reason, the improved oxygen mass transfer between the cells could not be expected. **Table 6-1** and **Fig. 6-1** showed that the final μ_{app} of fermentation broth by FZ was kept at low value in comparison with the mixing condition using DRT and MB impeller. Results of literature [3], was agreed with the viscosity measurement in this study.

Table 6-1 Effect of FZ impeller on apparent viscosity of fermentation broth in comparison with typical radial impeller.

Impeller		Medium	Cultivation time (Day)	Apparent viscosity (Pa s)	Reference
FZ	Low P_v	Fermentation of *A.oryzae*	3	(0.34±0.03)	This study
	High P_v			(0.55±0.05)	
DRT	Low P_v	Fermentation of *A.oryzae*	3	(0.56±0.08)	This study
	High P_v			(0.48±0.06)	
FZ		Fermentation of *tacrolism*	5	2.00	[3]
DRT		Fermentation of *tacrolism*	5	7.00	[3]

6-3 Theoretical study on the effective viscosity of fermentation culture in mixing condition using MB impeller concerning on Metzner and Otto's method

Effective viscosity calculation using Metzner and Otto`s method [4], is one of the methods of viscosity prediction when using stirred tank with determined agitator. Here, the results of the effective viscosity, (μ_{eff}), according to Metzner and Otto's method for each of the $\dot{\gamma}_{ave}$ of the impellers are listed in **Table 6-2**, and was showed that using the MB at low P_v (148 Wm^{-3}) could result in a lower μ_{eff} (0.171 Pa s), and was compared with using the DRT at moderate P_v, (630 Wm^{-3}), 0.147 Pa s. For example, the μ_{eff} of fermentation when using the MB (152 Wm^{-3}) was 0.080Pa s, and when the DRT impellers was used at 630 Wm^{-3} the μ_{eff} reached 0.103 Pa s. This is because increasing the viscosity reduces the circulation that is induced by the DRT impeller [5]. One of the disadvantage of theoretical viscosity study in biological fluids is that effective viscosity of the biological fluid could not be calculated by growing the cells. Therefore, using Metzner and Otto method could not show changing the culture viscosity or controlling the viscosity during the fermentation. Due to these limitations, this method was not extensively used in this thesis. Finally, it could be concluded that this method could not describe the viscosity change during the fungal fermentation.

Table 6-2 Mixing parameters of submerged fermentation using DRT and MB impellers

Power	Low		Medium		High	
Impeller	DRT	MB	DRT	MB	DRT	MB
P_v **(Wm^{-3})**	152	148	630	687	1487	1520
μ_{eff}^{*} **(Pas)**	0.316	0.171	0.147	0.08	0.103	0.056

*: μ_{eff} was measured using *Metzner* and *Otto* method

6-4 Study the dependency of viscosity and shear stress on fermentation time (Thixotropic behavior of fermentation broth)

After doing extensive study for finding adaptable rheometer, using viscometer of HAAKE™ viscometer-550 (Thermo scientific, USA), the shear-thinning behavior of fermentation culture using four kind of agitators have been confirmed. According to the results of **Fig. 6-2**, the culture broth was a Newtonian fluid at 6~18 h first from the fermentation, but after 24h, it was strongly presented that the culture was a non-Newtonian culture with shear-thinning (power-law) model. These results indicate that after 24 h from the fermentation the behavior of culture broth has been changed from Newtonian to shear-thinning (It was shown as some examples. **Figs. 6-2~ 6-6**).

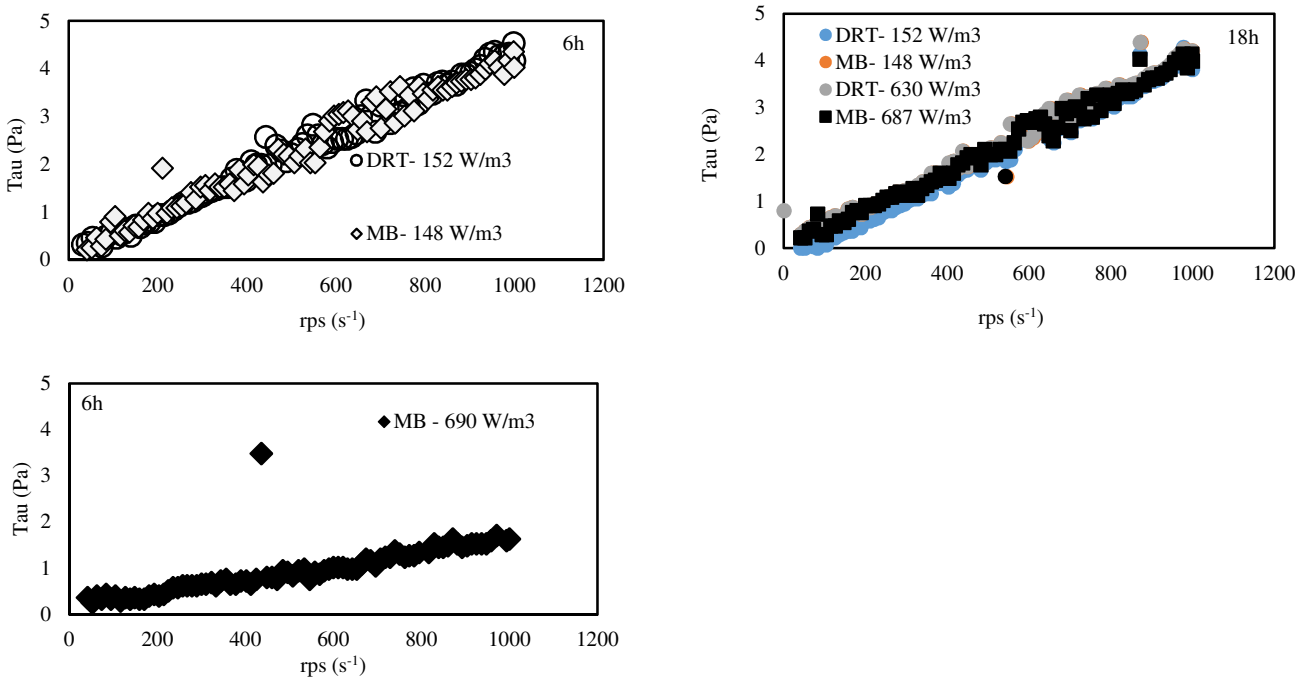


Fig. 6-2 Newtonian behavior of fermentation culture at first hours of cultivation during agitation with DRT and MB impeller

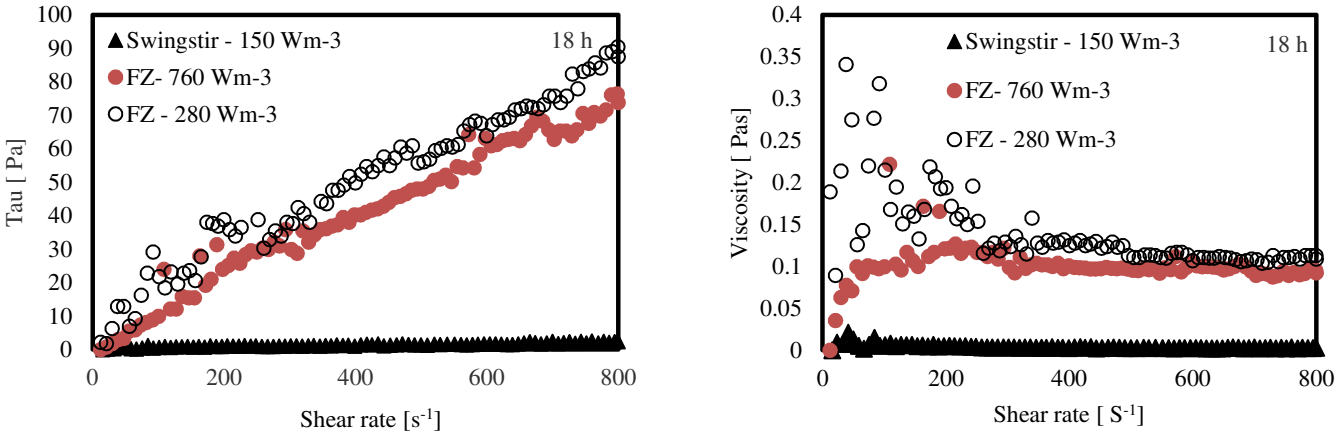


Fig. 6-3 Newtonian behavior of fermentation culture at first hours of cultivation during agitation with FZ and Swingstir®

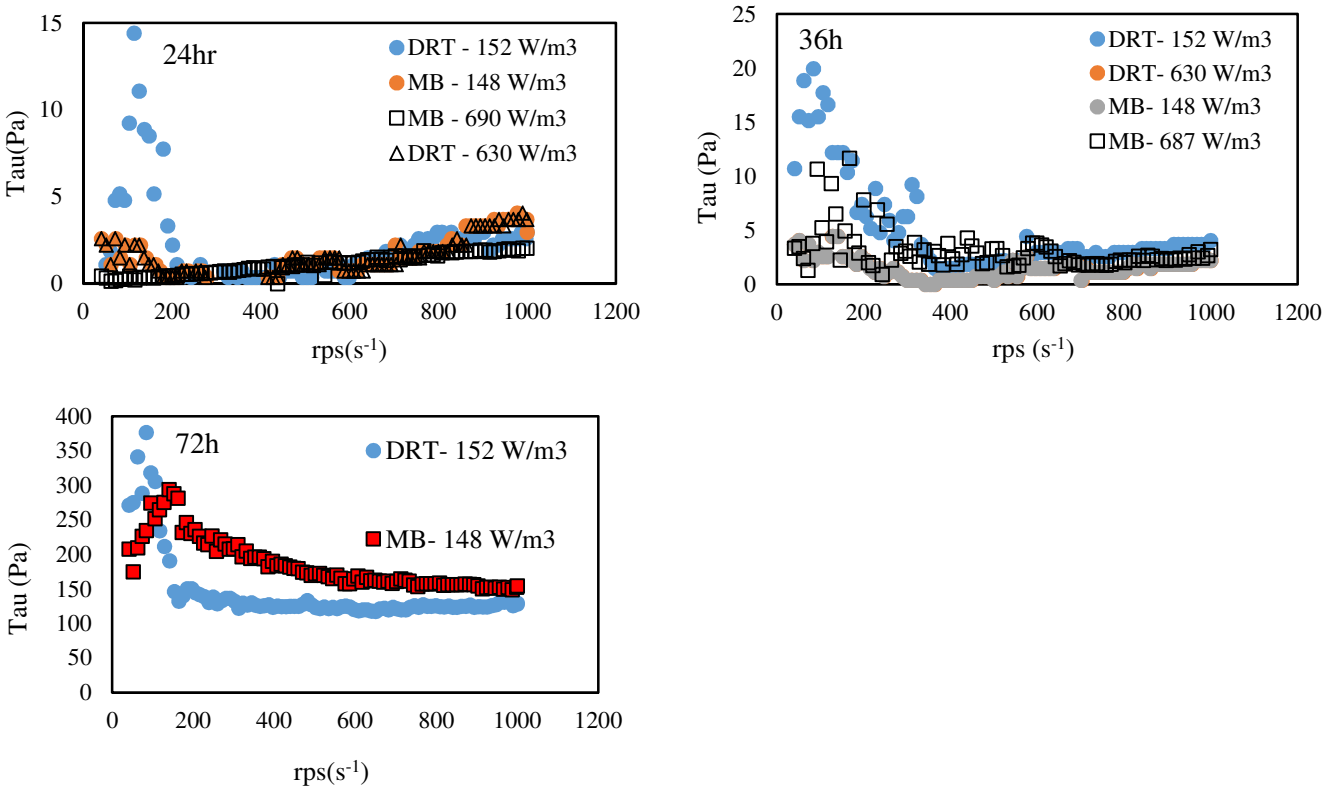


Fig. 6-4 Non-Newtonian behavior of fermentation culture during agitation with DRT and MB impeller

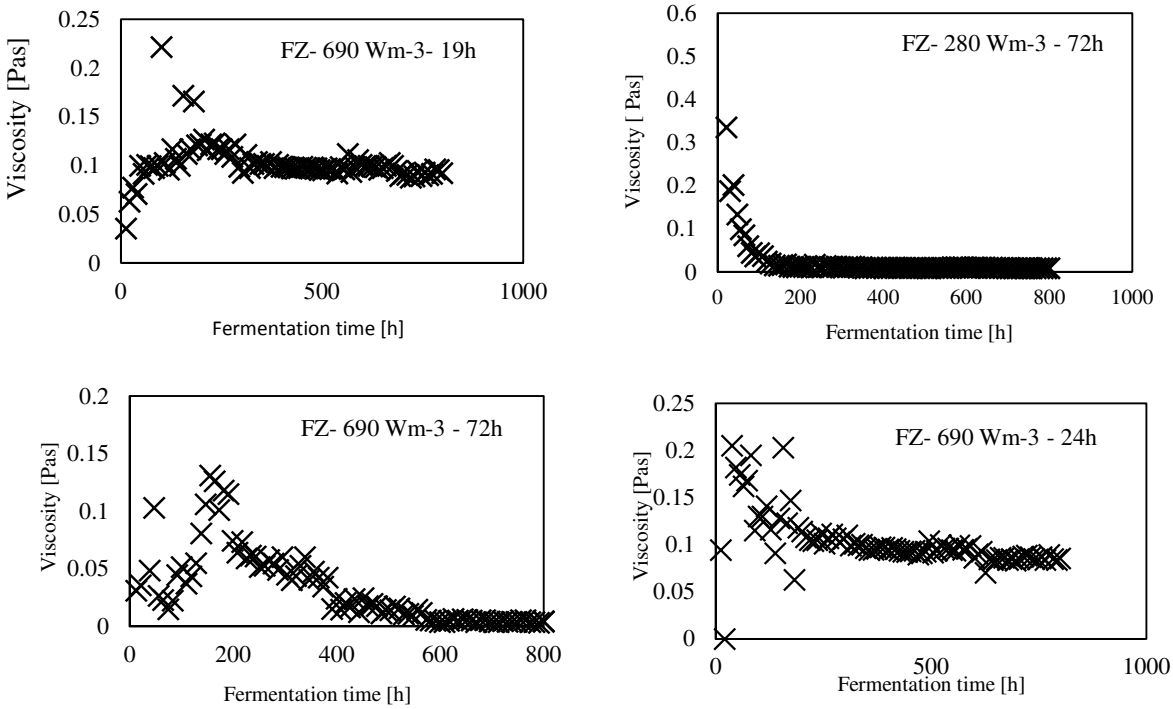


Fig. 6-5 Non-Newtonian behavior of fermentation culture during agitation with FZ impeller

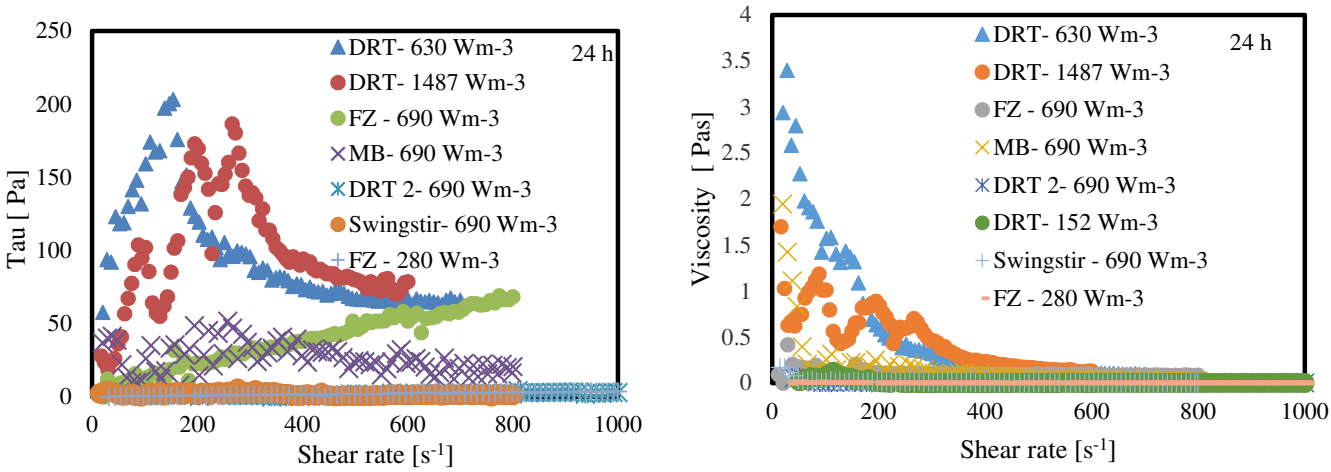


Fig. 6-6 Non-Newtonian behavior of fermentation culture during agitation with four kind of impellers

6-5 Investigation on the effect of mixing on the thixotropic behavior of fermentation culture

After measuring the apparent viscosity of fermentation broth in each sampling, the relation between apparent viscosity (Pa s) and fermentation time (h) in non-Newtonian condition was modeled to determine which mixing condition was resulted in formation of cell-suspension with less viscosity dependency on time or finding a controllable condition (weak thixotropic property). Results of modeling was shown in **Table 6-3** and shown at low P_v, Swingstir® had weak thixotropic property. At moderate and high P_v, the MB had the lowest viscosity dependency on the time respectively. However, mixing by MB could keep the viscosity at constant values, but based on the **Fig. 6-1** the viscosity of broth was high.

Based on the results of shear stress and viscosity versus shear rate, Swingstir® could be an accepted agitator candidate for controlling the viscosity. Therefore, in the next section rheological behavior of fermentation broth using Swingstir® will be investigated.

Table 6-3 Dependency of apparent viscosity on fermentation time by modeling

Impeller	P_v (Wm^{-3})	Model	R^2	fermentation time Interval (h)
DRT	152	$\mu_{app} = -5.10^{-5}t^3 - 0.0027t^2 + 0.184t - 3.019$	0.960	(24 ~ 72)
	630	$\mu_{app} = 2.10^{-5}t^3 - 0.0047t^2 + 0.273t - 3.962$	0.920	(24 ~ 72)
	1487	$\mu_{app} = -2.10^{-5}t^3 - 0.0021t^2 + 0.060t + 0.686$	0.920	(24 ~ 72)
MB	148	$\mu_{app} = 4.10^{-5}t^3 - 0.0066t^2 + 0.342t - 4.931$	0.980	(24 ~ 72)
	960	$\mu_{app} = 5.10^{-5}t^3 - 0.0073t^2 + 0.367t - 5.247$	0.970	(24 ~ 72)
	1524	$\mu_{app} = -6.10^{-5}t^3 - 0.0083t^2 - 0.317t + 3.753$	0.980	(24 ~ 72)
FZ	286	$\mu_{app} = -4.10^{-5}t^3 - 0.0053t^2 - 0.203t + 2.425$	0.990	(40 ~ 72)
	690	$\mu_{app} = 0.0001t^3 - 0.0187t^2 + 1.027t - 18.320$	1.00	(40 ~ 72)
Swingstir®	150	$\mu_{app} = 2.10^{-5}t^3 - 0.0038t^2 + 0.203t - 3.144$	1.00	(31 ~ 72)
	632	$\mu_{app} = -7.10^{-5}t^3 - 0.0112t^2 - 0.616t - 11.460$	1.00	(31 ~ 72)

6-6 Investigating the rheology of fermentation liquid during mixing by Swingstir®

One of the positive hydrodynamic behavior of Swingstir® was due to the formation of un-steady vortices. It was leaded to decrease the consistency index in power-law model (5.95 Pas^n), in comparison with the condition (at the same P_v and sampling time) using DRT (27. 4 Pas^n). Also, the vortices produced by eccentric mixing inducing periodic oscillations in the flow. These periodic oscillations are like flow instabilities [6].

Table 6-4 showed the values of power-law model parameters and μ_{app} at t = 48 and 72h when Swingstir® and FZ were used as an agitator at the same P_v. Besides, for the reason of showing the effect of rheology on K_La, and alpha amylase activity (as a one of the representative of cell activity for biological production) K_{app} and n_{app} were presented in **Table 6-4**. The results of listed in **Table 6-4** showed that the rate of K_{app}, was increased from t = 48 h to t = 72 h, when using the FZ, which was significantly faster than when using the Swingstir®. Also, the n_{app} of fermentation fluid when using the Swingstir® were lower than those when the

FZ was used. This result shows that the dependencies of both shear stress and viscosity on the shear rate when using a flexible impeller were lower than when using the FZ. Besides, shear rate influences the average μ_{app} of non-Newtonian fluids. During agitating by Swingstir® both center and wall of the tank could have similar velocity by periodically change of the circular high-velocity region. The velocity distribution near the shaft and blade of the Swingstir® was axial and uniform. Lack of axial symmetry was leaded to an inclination of the vortices. Indeed, when using the FZ, μ_{app} of the culture was increased from 0.48±0.06 Pa s (at t = 48h) to 0.70±0.08 Pa s (at t = 72h). However, when using the Swingstir® the μ_{app} was relatively kept constant from t = 48h to t = 72h ($\mu_{app} \cong$ 0.5 Pa s).

In a previous report [8] increasing the broth viscosity presented a challenge to cultivation. In some investigations, the K has been used as a single indicator of the viscosity of filamentous cultivations, because the n is an exponent change that has a greater effect on shear stress than a similar change in K [9]. In the present study, however, not only the K_{app} value was higher during mixing with the FZ but the n_{app} also was larger than the n_{app} value in the case of fermentation using a Swingstir®. The results of **Table 6-4** show how enzyme activity might be affected by high shear stress and viscosity when using a FZ. Also, due to the lower rheological parameters of mixing conditions when using a Swingstir® the viscosity distribution in the stirred tank was lower and more homogenous in comparison with fermentation using a FZ. Recorded data in **Table 6-5** indicates the effect of agitator type on rheology of fermentation fluid. As shown in **Fig. 6-7**, when using the FZ, the dependency of the cell-culture viscosity on fermentation time using the FZ was greater than it was when using the Swingstir®. Therefore, fermentation cultures agitated using the Swingstir® were non-Newtonian fluids with a low level of thixotropic behavior.

Table 6-4 effect of Swingstir® on rheological behavior and enzyme activity of submerged culture in comparison with FZ impeller at the same P_v (= 690 Wm^{-3})

Fermentation time (h)		K_{app} (Pas^n)	n_{app}	K_La (h^{-1})	Enzyme activity ($Um^{-1}L^{-1}$)	Shear rate (S^{-1})	μ_{app}(Pa s)	Average stress (Pa)
48	Swingstir®	8.16	0.20	168	5000±500	4.1	0.50±0.06	10
	FZ	36.60	0.36	101	4000±340	5.0	0.48±0.06	119
72	Swingstir®	49.40	0.20	131	7984±150	4.1	0.55±0.05	65
	FZ	299.00	0.30	60	5687±167	5.0	0.70±0.08	754

One of challenges in mass transfer during the fermentation is thixotropic behavior of fluid. Due to this reason, the effect of using Swingstir® on thixotropic behavior of fermentation culture has been investigated. Therefore, viscosity of fermentation culture versus shear rate (s^{-1}) was measured after sampling at t = 48h and 65h, (**Figs. 6-7(a)** and **6-7(b)**).

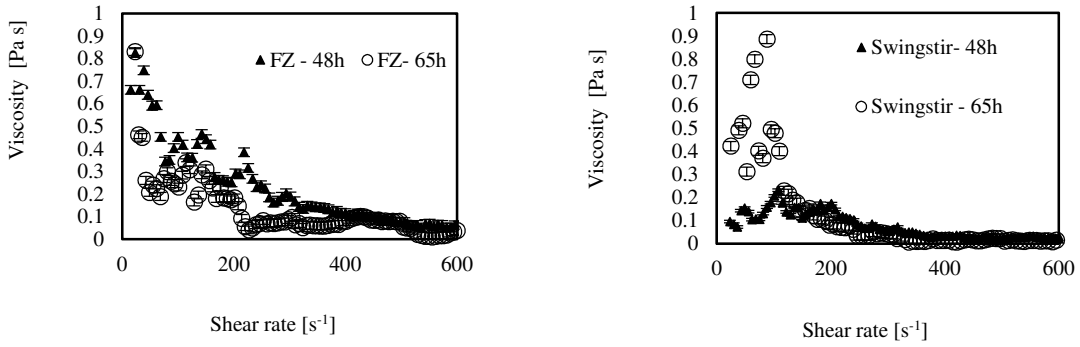


Fig. 6-7 Decreasing the thixotropic behavior of fermentation cultures during agitation by (a) FZ in comparison with (b) Swingstir® at the same P_v

Indeed, when using FZ impeller the viscosity of culture had been increased until end of fermentation (t = 72h). However, using Swingstir® the viscosity was remained lower than that of the FZ. It has been reported that increasing the broth viscosity makes the cultivation challenging [16]. Also due to the lower rheological parameters of mixing condition by Swingstir® the viscosity distribution in stirred tank was more homogenous and low in comparison with fermentation using FZ. Therefore, fermentation cultures agitated using Swingstir® were non-Newtonian fluids with low thixotropic behavior (**Fig. 6-7**).

Table 6-5 Quantitative validation of shear stress and viscosity simulation comparison with literature in stirred tank bioreactor.

Impeller type	Strain of fungi	Fermentation time (h) /Flow regime	Average simulated shear stress (Pa)	Average simulated Apparent viscosity (Pas)	Reference
Dual twin RDTs (475 min^{-1})	*A.oryzae*	Turbulent Power-law fluid (n =0.3, K = 12 Pas^n)	-	0.6	[1]
Dual B2-30	*A.oryzae*	Turbulent Power-law fluid (n =0.25, K = 10 Pas^n)	-	0.37	[1]
A-315	Carbopol ($\rho = 2\ kgm^{-3}$)	Transient (n =0.26, K = 34.6 Pas^n)	27.1	-	[2]
	Xanthan Gum ($\rho = 35\ kgm^{-3}$)	Transient (n =0.18, K = 34.8 Pas^n)	20.7	-	[2]
3RT	*A.niger*	96h /Turbulent (n = 0.3, K = 2.5 Pas^n)	-	0.5	[3]
3WHu	*A.niger*	96h /Turbulent (n = 0.3, K = 2.5 Pas^n)	-	0.40	[3]
Swinstir®	*A.oryzae*	(48h), Laminar	10	0.64	This study
Swinstir®	*A.oryzae*	(72h), Laminar	65	0.41	This study
FZ	*A.oryzae*	(48h), Laminar	119	1.00	This study
FZ	*A.oryzae*	(72h), Laminar	754	0.80	This study

6-7 Results of rheological modeling of fermentation culture by power-law model; effect of mixing condition on apparent values of K_{app} and n_{app}

Rheological measurement of fermentation culture confirms that after passing 18~24 h from the fermentation the behavior of fermentation culture was changed from Newtonian to a shear-thinning fluid but finding the consistency index (K) and flow index (n) for each fermentation culture was very difficult and complicated. Experimental measurement showed that these parameters was not constant during each sampling particularly the values of K_{app} were faced with some big fluctuations by changing the fermentation time. According to the results of **Table 6-6**, the values of n_{app} when fermentation was done using radial impeller (DRT) were in the range of 0.1 ~ 0.43. From the experimental data, a unique value for K_{app} during each fermentation condition could not be found. In addition, by increasing the fermentation time

n_{app} values were relatively decreased but K_{app} values were increased. In addition, it was showed that by increasing the P_v, the apparent K_{app} values have been decreased.

Table 6-6 Calculation of K_{app} and n_{app} for fermentation culture agitated by radial agitator (DRT impeller)

rpm (min^{-1})	P_v (Wm^{-3})	K_{app} (Pas^n)	n_{app}	Sampling time (h)
100	152	27.4	0.28	30
		110	0.40	48
		330	0.13	52
		379	0.20	72
300	630	8.58	0.30	30
		3.97	0.4	35
		665	0.29	40
		-	0.21	53
		544	0.28	58
		304	0.10	72
500	1487	-	0.43	40
		-	0.41	48
		121	-	65
		-	0.27	72

$\mu_{eff} = K_{app} \gamma^{n}{}_{app}{}^{-1}$

Experimental results of **Table 6-7**, showed that when mixing was done by MB impeller by axial flow pattern of mixing the n_{app} values was changed from 0.1 to 0.46. Moreover, by increasing the P_v, n_{app} and K_{app} values have been increased. The K_{app} values were increased by increasing the fermentation time. In addition, at the same P_v and sampling time the K_{app} values of culture mixed by MB were lower than that of the DRT.

Table 6-7 Calculation of K_{app} and n_{app} for fermentation culture agitated by MB impeller

rpm (min^{-1})	P_v	K_{app} (Pas^n)	n_{app}	Sampling time (h)
100	148	2.26	0.14	24
		36.60	0.10	33
		40.44	0.12	48
		544	0.10	54
		134	0.10	60
		244	0.25	56
		610	0.20	72
300	690	90	0.20	24
		-	0.24	40
		2.03	0.46	48
		134	0.23	64
		812	0.31	72
500	1524	-	0.46	24
		7.4	0.14	30
		270	0.46	40
		228	-	48
		-	0.30	54
		403	0.42	72

In the case of using FZ impeller (**Table 6-8**), n_{app} values were change from 0.11 to 0.39 (lower than that of the MB and DRT). However, in this study a clear analysis could not be done on the K_{app} values.

Table 6-8 Calculation of K_{app} and n_{app} for fermentation culture agitated by FZ impeller

rpm (min^{-1})	P_v	K_{app} (Pas^n)	n_{app}	Sampling time (h)
100	286	209	0.11	48
		79	0.39	54
		-	0.20	72
300	690	299	0.36	48
		36.6	-	65
400	1326	2.23	0.88	48
	1480	-	0.26	60
		2.23	0.88	72

Investigation on the results of **Table 6-9** showed the lowest K_{app} values were belonged to the culture agitated by Swingstir® (among the other three kinds of agitator). In addition, at moderate P_v the n_{app} value were low (were kept constant) and at low P_v, n_{app} values were high and controllable.

Table 6-9 Calculation of K_{app} and n_{app} for fermentation culture agitated by Swingstir®

Impeller	rpm (min^{-1})	P_v	K_{app} (Pas^n)	n_{app}	Sampling time (h)
Swingstir®	85	150	5.95	0.56	24
			-	0.59	55
			20	0.20	72
	247	690	5.92	0.21	24
			6.48	0.25	30
			8.16	0.10	48
			27.11	-	64
			49.4	0.26	72

In both Newtonian and power law fluids, the $\dot{\gamma}_{ave}$ in laminar flow are dependent on the impeller rotational speed. In turbulent flow in both Newtonian and non-Newtonian medium, the average shear rate is shown to depend on $N^{3/1+n}$ [10]. Therefore, by increasing the n_{app} value the dependency of shear rate on the rotational speed of impeller has been decreased. In this

study, when fermentation was done at turbulent regime (just in a few hours of fermentation in moderate and high P_{in}), according to the results of n_{app} measurement the highest average n_{app} value was calculated in the condition using Swingstir® ($n_{app,ave}$ = 0.45 at P_v = 150 Wm^{-3}), it means that the average shear rate during the fermentation using Swingstir® had the lowest dependency on agitation rate. Besides the fermentation condition with the highest dependency on shear rate was done when using MB impeller at P_v = 150 Wm^{-3}, (n_{ave} = 0.14).

6-8 Summary

- Keeping the viscosity of fermentation culture at high and moderate P_v using MB impeller at high value could be a challenge for mass transfer. Existing a strong viscosity fluctuation during fermentation by DRT impeller was resulted in local mass transfer, local cell growth, stagnant zone preparation, and finally decrease the enzyme activity.
- Agitation with flexible shaft could control the viscosity at low *Re.* Dependency of shear rate on rotational speed in the condition using Swingstir® was the lowest. At low P_v, using Swingstir® the weak thixotropic behavior was shown. Besides, at high and moderate P_v the culture agitated by MB showed a significant thixotropic behavior.
- Viscosity of culture mixed by FZ was relatively low but was not controllable.
- The low K_{app} values were belonged to the culture agitated by Swingstir®.
- K_{app} was one of the important factors that affected on the shear stress. At relatively closed n_{app} values, the larger K_{app} was resulted in higher shear stress applied to the microorganism.

Symbols;

Nomenclature

K_{app}	Apparent consistency index	[Pa s^n]
$K_L a$	Volumetric mass transfer coefficient	[h^{-1}]
N	Rotational rate	[s^{-1}]
n_{app}	Apparent flow index	-
P_v	Power density	[Wm^{-3}]

Greece symbols

μ_{app}	Apparent viscosity	[Pa s]
μ_{eff}	Effective viscosity	[Pa s]

Abbreviations

DO	Dissolved oxygen	[ppm]
DRT	Double Rushton turbine	-
FZ	Fullzone®	-
MB	Maxblend®	-
STR	Stirred tank reactor	-

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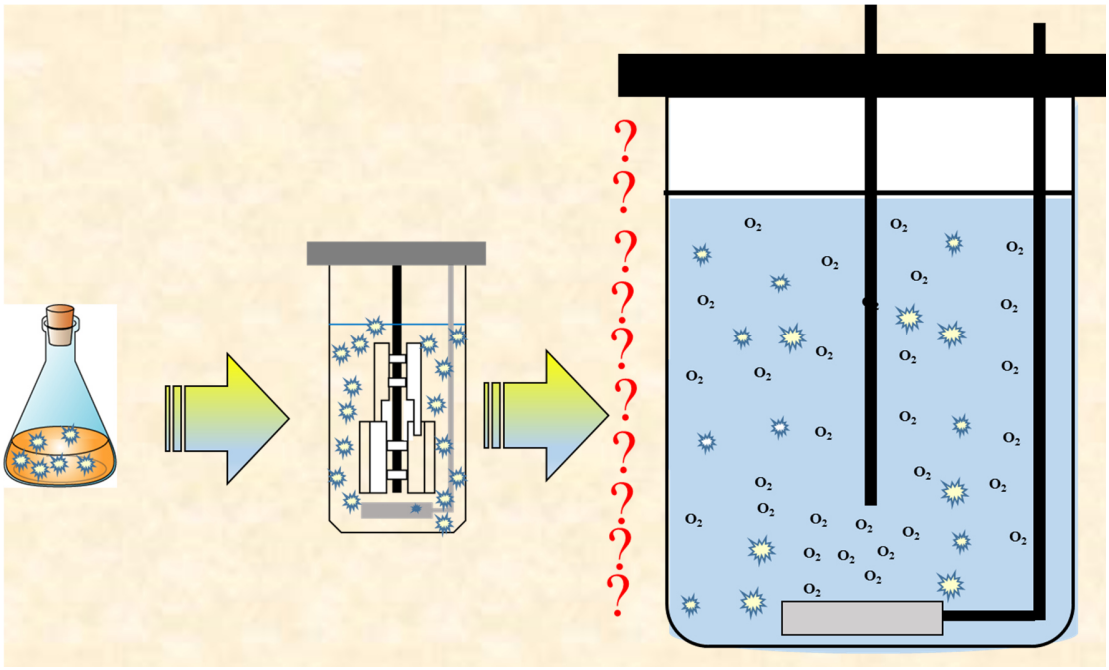
Chapter 7: Suggestion and review on the scale-up of stirred- batch fermentation of *Aspergillus oryzae*; from mixing aspects of view



Graphical abstract of chapter 7: [This picture was prepared by Narges Ghobadi, 2016]

Chapter 7 in brief; In this chapter optimal criteria for scale up of the *A.oryzae* fermentation were proposed. Besides, important effects of mixing on the design of a stirred batch fermenter using bio mechatronic design methodology were shown.

7-1 Introduction

Recently, one of the most important investigation before doing any scale-up for a process is to investigate a clear definition for intensification of related process. It is useful to find the best method for increasing the process volume [1]. Most of the processes intensification (PI) which were related to the mixing phenomena were associated with the intensification of transport processes such as heat and mass transfer. One of the ideas behind PI is that each molecule will experience the same processing environment, thereby providing a uniformity to the process [2].

Results of PI was mostly leaded to decreasing the equipment size, energy consumption, or waste production [3]. Here, important goal of PI in fermenter was remove limitation of fermenter by giving each fungal pellet the same process experience, optimizing the driving force used for growing the cells and maximize the specific surface area to which this forces applies [4]. The intensified batch fermenter should be adapted for cultivation of suspension and fungal cells at very high densities and also avoid cross-contamination risks.

Definition of PI for submerged fermentation can be based on the various parameters. This section was concentrated on how PI is defined for submerged fermentation of *A.oryzae* in batch condition, and also how the bio mechatronic design methodology could analyze the requirements of intensified-batch fermentation of *A.oryzae* during the fermentation. One of the main engineering tasks in the field of biotechnology is an effective transfer of a biological process from the laboratory to the industrial or semi-pilot scale. Because, during a scale-up, both physical and biological processes should be taken into account. The modified hydrodynamic should have the positive effect on mass transfer enhancement. It means that the enhanced mixing flow pattern could be able to give each of fungal cells the same processing experience, and finally decreased the energy per volume of culture during the fermentation.

Fermentation intensification by approaching on mixing can be classified into two sections; macro-mixing and micro-mixing. PI of stirred fermentation by focusing on macro-mixing give energy to each pellet-shaped fungal cells, the same processing experience (the same mass transfer medium with the same exposed shear stress). But, PI of stirred fermentation was focused on micro-mixing improvement means that giving the energy to bio-molecule the same processing experience for mass transfer in culture media. We have motivated to focus on the macro-scale. Our motivation was finding the mixing condition adaptable to control the challengeable transport phenomena during the fermentation.

According to the previous studies [5], to reach the high amount of productivity, not only the level of impeller power is important, but also relevant is how this power is applied. Method of applying the power could be optimized by changing the flow pattern of mixing. Bulk mixing improved with increased impeller power but productivity of fermenter is decreased. Due to this reason, improvement the mixing and dispersion of fungal cells without increasing the mixing energy is desirable [5].

7-2 A review on the intensified methods for scale-up of aerated bioprocess with suggestions for fermentation of A.oryzae

Scale-up is still an art and not just a technical science. Traditional scale-up is empirical, particularly if the system is only reaction or only transport controlled. Common scale-up of stirred fermenter are the maintenance of constant parameters that were shown in **Table 7-1**.

Table 7-1 Common scale-up criteria for stirred batch bio-reactor due to the literature

Bioprocess	Scale- up criteria	Volume	Impeller	Reference
Biotransforming benzaldehyde to l-phenyl acetyl carbinol (l-PAC)	Same K_La (oxygen transfer rate can control the overall rate of the bioprocess in aerobic bioreactors)	From 100 mL shake flask to 5 L fermenter	Disc turbine (DT) and pitched blade turbine down flow (PTD) impellers	[6-8]
Tryptophan fermentation/ L-glutamic acid fermentation	constant oxygen concentration constant oxygen transfer rate	-	-	[9, 10]
Linolenic acid production from *Mortierella ramanniana*	Constant impeller tip speed	30 L/ 10000L	-	[11]

According to the literatures [12], results of using constant parameters are more favorable with Newtonian broths than with non-Newtonian systems. Besides, results of previous chapters showed fermentation culture of *A.oryzae* was done mostly in non-Newtonian condition. Therefore, finding an appropriate constant parameter is an important step before increasing the production volume. Here, a detail about each constant parameter for scale-up of submerged fermentation have been investigated.

7-3 Investigation on the adoptable methods for scale up of stirred-batch fermentation of A.oryzae

Scale-up can be done according to four different approaches, as follows: fundamental methods; semi-fundamental methods; dimensional analysis; and rules of thumb [12].

Fundamental methods; They are those based on the application of mathematical models for showing the influence of operational conditions and geometrical design of the bioreactor on the flow pattern in the bioreactor. It is helpful tool for scale-up and determination of the optimal conditions at the production scale [12].

Semi-fundamental methods; in this method equations (are applied to obtain a practical approximation to the bioprocess operation) have been simplified. The parameters obtained will be scale dependent. Therefore, the impact of size on the process can be examined by simulations [12].

Dimensional analysis; This is based on keeping the values of dimensionless groups of parameters constant during the scale-up. The dimensionless groups used are the ratios of rates or time constants for the different mechanisms involved in bioprocess. In this method dimensionless groups are kept constant, the relative importance of the mechanisms or phenomena involved in the process will not change during scale-up. It is often impossible to keep all the dimensionless groups constant during scale-up, therefore determination of the most important groups is inevitable.

The rule of thumb method; This is the most common method. The scale-up criterion usually used in the fermentation industry are: constant P_v, (30% of use); constant K_La (30%); constant impeller tip speed of the agitator or shear rate (20%); and constant DO concentration (20%) [13]. Based on the successful results of using this method for fermentations, the rule of thumb method is suggested for scale-up of fermentation of *A.oryzae* .

7-3-1 Aerated bioprocess scale up based on constant P_v

One of the mostly used scale-up method is to keep the volume per volume aeration (vvm) and the P_v constant [14]. Conservation of constant vvm is a stoichiometric approach, as the amount of oxygen available in the reactors preserved. However, scale-up according to the constant vvm is quite questionable from the viewpoint of hydrodynamics. Besides it was reported that scaling up by constant vvm will produce different hydrodynamic conditions in different scale and is not recommended [15]. It will be shown that change of scale has a profound influence on the fluid dynamics in mixing vessels and constant superficial gas velocity but P_v is a more convenient

criterion for scale-up of bioreactors. It was reported that scale-up based on constant P_v will increase the maximum shear rate by 28% however using a constant *Re* value is not a good scale-up criterion, because a very low P_v values [12].

7-3-2 Constant tip speed of impeller

Agitator tip speed has some advantages in bioprocesses with sensible micro-organisms as follows [12]; 1) Shear stress produced by stirrer could be predictable, tip speed determines the maximum shear stress, 2) Determining the possible cell damage, 3) Influences the stable size gas bubbles. However, its use is resulted in a reduction in P_v and in the stirrer speed, which causes a remarkable reduction of the rate of oxygen transport. Therefore, it seems that the best criteria for scale up is to maintain the P_v [6, 16].

In viscous mycelial fermentations, a constant impeller tip speed is often used as a scale-up criterion, because of the shear sensitivity of the used micro-organisms for this kind of fermentation. Some authors [8] note that not only the impeller shear, but also the impeller pumping capacity is important. Others [17] used only the impeller shear as a criterion. Scale up criteria for a dispersed growth form of *Penicillium chrysogenum* was studied in 10 and 100 L stirred bioreactors at various aeration rates and stirrer speeds in the transitional region of *Re*. It was found that equal energy dissipation rate, as well as equal impeller tip speed scale-up criteria, were not valid as a measure of hyphal damage and production rate [18]. However, the results were successfully correlated with a correlation based on the circulation rate through the impeller zone [18].

7-3-3 Scale-up based on constant Mass transfer factors

One of the most commonly used criteria in aerobic fermentations is constant K_La. Because, supply of oxygen for growing cells is usually the limiting operation in industrial fermentation. Moreover, oxygen supply is one of the most important operational costs in fungal cultivation. During the fermentation, oxygen demand is high (fast growing microorganisms, high cell concentrations) also, the rheological properties of the broth offer a high resistance to the mass transfer [18]. Scale-up by mentioning to a constant K_La, is often referenced [16]. However, it should be mentioned that what really matters is not the transfer capability (K_La), but the oxygen transfer rate (OTR), which is the product of K_La and the mass transfer potential (C^*-C_L). Due to this reason, constant OTR instead of constant K_La as scale-up criterion was proposed [6, 19-24]. However, microorganism growth and oxygen consumption should be scale independent, the growth rate in a bioreactor is scale dependent because of the scale dependency of OTR. The turbulence intensity, and thus local shear forces are exacerbated with the increasing scale of bioreactors; under these conditions, cellular stress can affect growth [20]. For the scale-up of toyocamycin production by a shear-sensitive mutant of *Streptomyces chrestomyceticus* the OTR constant method could not use and thus scale-up was done at the lowest possible tip speed for the geometrically similar larger vessel [25]. Besides, the OTR into the microbial cell in aerobic bioprocesses strongly affects growth and product formation by influencing metabolic pathways and changing metabolic fluxes [18]. When K_La is relatively high, no influence of the oxygen uptake by the microorganism can be detected because the enhancement is very small, and the DO concentration is dependent on the OTR [20]. Aeration and agitation are important variables in order to produce an effective OTR into the medium.

7-3-4 Scale up of stirred fermenter based on constant mixing time

Mixing is an important function of bioreactors, it would seem desirable to keep the mixing time constant on scale-up. Large scale reactors are poorly mixed compared with small scale reactors. This is a common cause for a changing of regime, however this method can give problems for mass- and heat-transfer, particularly in viscous broths. To estimate the mixing time, a lot of correlations can be used. To keep the mixing time constant, the velocity of fluid in the tank must be increased in proportion to the size. Due to this reason, before scale-up of fungal fermentation based on the constant mixing time, predicting the shear stress and maximum shear rate to prevent from cell damage will be necessary.

7-3-5 Combination of different operating variables as a constant criteria for scale- up

Another strategy for scale- up is to keep a combination of different operating variables at the same value at the different scales. Same tip speed, P_v, and aeration rate at different scales are often used combination. When the scale-up based on three above constant criteria is needed some conditions should be satisfied in stirred vessels. They are listed as below [30];

- Tip speed velocity should be larger than 3 ms^{-1}
- *P/V* should be around 2 kWm^{-3}
- Gas flow rate should be about 0.5 vvm

Regarding to the above conditions, scale-up of fermentation of *A.oryzae* in this study based on the constant three criteria were not possible because the flow velocity, P_v, and vvm were out of range. According to Wang et al.[8], a comparison between different constant criteria for scale-up of 100 L- gassed stirred fermenters (constant P_v, K_La, shear (*ND*), mixing time) showed that the agitation rate when using the mixing condition with constant mixing time is dramatically high

(1260 rpm) and it could lead to damage the cells by producing very high shear stress. The condition of constant shear was done at low rotational rate (50 rpm) that was not a good condition for aeration. It was shown using the constant P_v (85 rpm), was relatively appropriate because the agitation rate was relatively at accepted value (for keeping the K_La) and prevented from the high stress production in fermentation liquid.

7-3-6 Importance of morphology change for possibility of scale-up

According to the literature [34], filamentous growth characteristic creates a number of process engineering problems attributed to the morphological during the fermentation in large scales. Therefore, one of the effective method in this way is doing scale-down to investigate that whether changing the scale could be resulted in changing the macro- and micro- morphology or pellet size during the fermentation. Morphology analysis might be different when fermentation was done at large volume because morphology of fungi is a volume-based parameter. Therefore, prediction of this factor before fermentation of *A.oryzae* is complex before doing the process at large scale.

7-3-7 Necessity of scale-down for investigation on scale up potential of fermentation of A.oryzae

It is important to know whether a process will work properly before it is constructed in full size. Scale-down is an effective method to determine the effect of vessel scale on the morphology, rheology and mass transfer during the submerged culture because most of these parameters cannot be predicted through CFD. But some information about the morphology characterization could not be achieved using the scale-down experiment. For example, according to the investigation on the fermentation of *Aspergillus Awarromi* in large-scale of fermenter pellets had a larger hairy length than in the smaller [32]. Finally, it could be concluded that before scale-up of batch fermentation of *A.oryzae* considering to the scale-down experiment is a useful way for getting the information about impact of scale change on the mass transfer and culture rheology.

7-3-8 Propositions for scale up of fermentation of A.oryzae using FZ and Swingstir® agitator

Same specific energy dissipation or P_v, is usually used to scale up of fermentation [30]. In this method, the geometrically similar systems have been considered. It implies that all vessel dimensions have a same dimension ratios. In this study, if scale-up will be done based on constant P_v, similar geometrical ratio should be considered. Therefore, according to the characteristics of Fullzone® and Swingstir®, in a laboratory experiment the below geometrical ratio parameters have been proposed for scale-up of batch submerged fermentation (**Table 7-2**).

Table 7-2 Similar geometrical ratio proposed for scale-up of submerged fermentation using FZ and Swingstir®.

Impeller	Tank height/Tank diameter	Impeller diameter /Tank diameter	Liquid height/tank height	Impeller blade width/ impeller diameter	
FZ	1.97	0.53	0.78	Upper blade: 0.31	Lower blade: 0.38
Swingstir®	1.97	0.2	0.78	Upper wing-side:0.22	Lower wing-side:0.66

7-4 Application of principles of bio mechatronic design on finding the requirement of scale-up in batch stirred fermentation of A.oryzae

Design methodology in mechanical engineering has been applied to biotechnology products [8, 17]. The term bio mechatronic design indicates the biotechnology products combining the biological, mechanical and electric systems into one product where the complexity is considerable compared to other engineered products. Main aim of bio mechatronic is comparing the interdependence of the biological subsystems functions with the other systems of the bioreactor in a systematic way [33]. In this study, the importance and effect of mixing techniques and mixing equipment on design methodology of stirred fermenter will be shown from the aspects of bio mechatronic. Because, the technical requirements of the fermenters are both complex and diverse [33].

7-4-1 Principle of the bio mechatronic design methodology

The methodology based on bio mechatronic is mainly based on two previously well-known methodologies;

(a); the Ulrich–Eppinger [36] and (b); the Hubka-Eder [35-37] design approaches. The methodology of Hubka-Eder [35] relies on a description of the transformation that shall be carried out by the designed machine, where inputs of materials and energy are processed and where outputs are created. The transformation bio-process is divided into sequential or parallel steps which all contribute to its realization [33]. Different systems are needed for doing the transformation which can be classified as biological, technical, and human, information and management systems. These systems cause effects on the transformation process. The biological systems have key roles in the transformation, also their interactions with the other systems are crucial for efficient operation [38, 39]. One of the key factors affected on the transformation is mixing. Here, the roll of mixing on each needed system has been investigated.

To design a fermentation process by the methodology at first; the user needs should clarify the type of process and microorganisms that are concerned.

7-4-2 Procedure for applying the methodology in fermentation of A. oryzae

The procedure for applying the methodology has been described as follows;

1) The requirements of the user on the fermenters are identified. The user need in this study was shown in **Table 7-3**. In this step the star needs indicate the requirements of batch-fermentation affected by mixing.

2) The requirements shown in **Table 7-3** are converted to target values. These targets can either be quantifiable or qualitative.

3) Based on the target specifications a Hubka- Eder map is worked out. The transformation

process in the Hubka-Eder map can adapted to a transport phenomenon caused to enzyme and cells. The cell culture is an active biological system and the fermenter with pumps and impeller are the technical systems (**Fig. 7-1**). Electrodes and flow meters are included in information systems, the control software with the operator was included in the management systems and the operators the human systems are main factions of Hubka Eder map.

4) Subsequently, or in parallel with the Hubka-Eder map, a basic conceptual design structure is generated where the functions necessary for the realization of the product are investigated. These factions for submerged fermentation of *A.oryzae* were not investigated in this study.

5) From this conceptual structure, different design solutions are generated in a concept generation chart. The functional analysis in the Hubka-Eder map and concept generation chart are supported by an evaluation of the interactions of the functions and sub-functions of the systems and the steps in the transformation process. It was noticed that in this study, the concept generation and chart interaction matrix were not mentioned and they are as a future approach of this research. Here effect of mixing until step of three have been studied.

In this thesis, the considered fermentation mode is batch, and due to this reason, the substrate limitation can be ignored. Fungal fermentation is important by three different aspects of view. The first is intensification of homogeneity. The second is oxygen transfer intensification and the third is based on high productivity at low stress medium. Regarding to these concepts, the requirements and target values of critical parameters were shown in **Tables 7-3** and **7-4**. It was mentioned that the target values were extracted based on the results of fungal fermentation in laboratory scale.

Table 7-3 scale-up requirement of batch-stirred tank fermenter containing *Aspergillus oryzae*

User needs	Bioengineering property
Waste biomass formation should not increase* (it should be decreased or be independent of scale-up)	Uniform oxygen, nutrient and pellet-cells mixing
The scale-up should not lead to the cell fluidization with gradient *(give same experience to each pellet for mass-transfer/ cell growth intensification	Homogene fermenter (modified hydrodynamic condition)
Energy consumption per unit volume should not dramatically increase*	Possible to be scale-up at relatively same P_v
Production capacity independent to scale	Keep the improved enzyme activity yield
Incubation temperature should be unaffected of scale-up	Heat transfer efficiency
The large scale fermenter should not reduce mass transfer of media content*	Mass transfer efficiency
Pellet cells should not fragment or exposed to the shear stress due to the aeration and agitation*	Shear stress (mixing pattern with effective shear rate and low stress)
Chemical reagent and mechanical foam breakers should have negative effect on cell growth, activity and morphology (usually anti-foams unaffected the scale-up)*	Additive effect investigation during fermentation and scale change
Growth rate of cells should be unaffected of scale-up*	Specific cell growth
Cells should not be damaged by tip blade of impellers*	Fermenter equipment effect
K_La should be enough for maximum growth (same oxygen up-take rate by fungal cells) *	Oxygen transfer efficiency
The volumetric enzyme activity of fermentation culture should be un-affected by scale-up*	Process efficiency
Micro morphology of pellet cells should be unaffected by scale up*	Geometrical and environmental properties of biological culture

Table 7-4 Target values for the fermentation scale-up of *A.oryzae*.

Attribute	Target value	Unit
Cell density	1.5×10^7	Spore mL^{-1}
Volumetric power consumption	500~700	Wm^{-3}
K_La	140~160	h^{-1}
Average alpha amylase activity	4000	UmL^{-1}
Maximum alpha amylase activity	6000~7000	UmL^{-1}
Scale-up factor	The scale-up ratio is typically about 1:10 for bioprocesses, but lower ratios decrease the risk of unexpected performance on scale-up[12]	
Cultivation temperature	30	℃
Agitation rate	3 ~ 4	s^{-1}
Maximum shear stress Average shear stress	Lower than 40.0 Lower than 3.0	Pa
Bulk culture viscosity range	0.2 ~ 0.4	Pa s
Average pellet diameter	0.006 ~ 0.008	m
Blade tip velocity	0.7 ~0.8	ms^{-1}
Vocuolation/balloon-like formation in fungal hyphae	Very low	-
Cell porosity	0.86 ~ 0.97	-
Energy dissipation rate	0.7	Wkg^{-1}
Rheological parameters of power-law model (K and n)	K : lower than 49 n : (0.2~0.3)	Pa s^n -
PH	5.5	-
Fermentation time	72	h
Sterility	High	-
Aeration	1 ~ 2	v.v.m
Cell type (morphology)	Pellet	-
Sampling (on-line)	Moderate	-
Control instrumentation	High	-
Software equipment	Yes	-
Automation	Yes	-
Disposable	No	-

7-4-2-1 Transformation process

According to the fundamental of bio mechatronic design the most important part of fermenter design is transformation process (TrP) [40]. The block diagram of TrP during fermentation of *A.oryzae* was shown in **Fig. 7-1**. The systems and subsystems necessary for carrying out the TrP in a stirred fermenter were illustrated in **Fig. 7-1**. The Hubka-Eder mapping is used to analyze the interactions between the systems in TrP [40, 41], also, it displays all functional actions of the biological systems.

The main purpose of a fermenter is to control the biological transformations that take place in it thus, many of the key components of the fermenter are the functions of the biological systems and the transformations. The TrP is classified into sub-process; preparing, executing, and finishing phases. All of the functional actions that affected on these three phases display in Hubka-Ede mapping (**Fig. 7-1**).

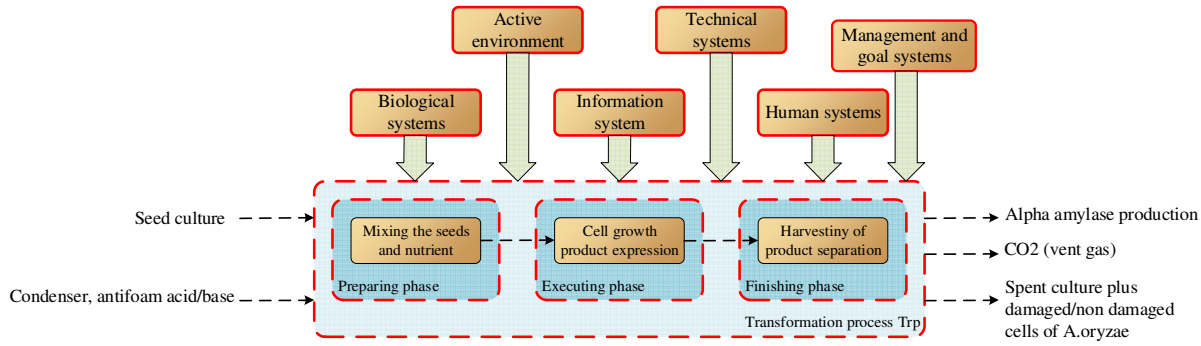


Fig. 7-1 Trp of submerged fermentation of *A.oryzae* from aspect view of bio mechatronic design.

Regarding to the **Fig. 7-1**, biological systems, active environment, information system, technical systems, human systems and management systems have direct effect and interaction with Trp.

7-4-2-2 Preparing phase

Among the three steps of TrP, preparing and executing phase are affected by performance of agitator (flow pattern of mixing) more than that of the finishing phase. The most effective agitator for preparing phase (**Fig. 7-2**) should resulted in higher K_La, high enzyme activity and lower mixing time at the same P_v among the other agitators. Due to this reason, hydrodynamic condition using Swingstir® could be useful for applying a modified preparing phase. Besides using the experimental results (in chapter 4 and 6) in preparing phase the fermentation fluid is a Newtonian fluid and mixing energy of Swingstir® in this condition was the lowest.

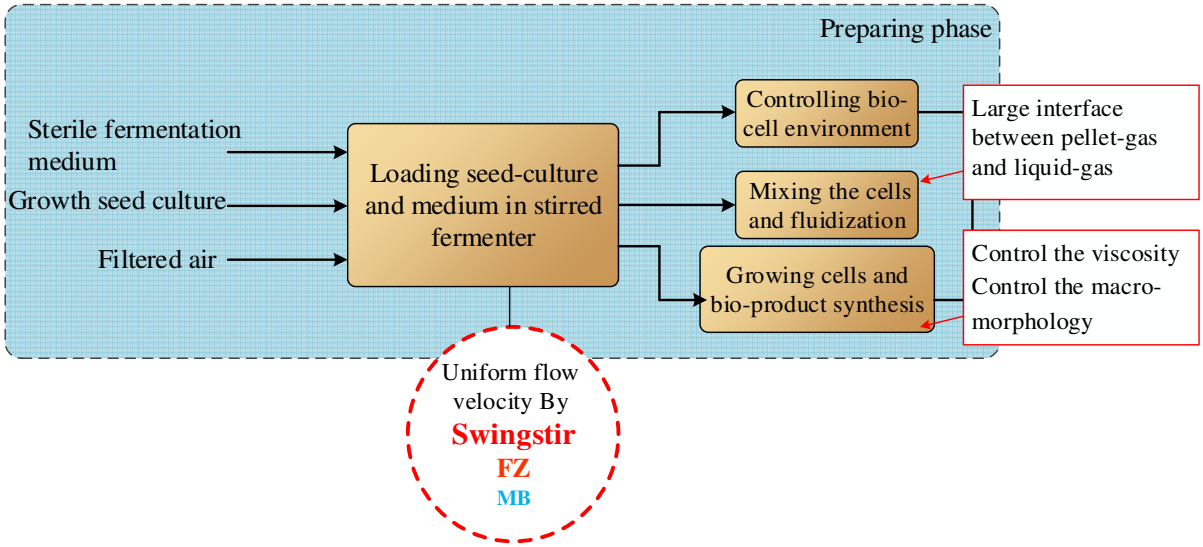


Fig. 7-2 Diagram of preparing phase during batch fermentation of *A.oryzae*.

7-4-2-3 Executing phase

One of the parameters effects on the executing phase (**Fig. 7-3**) are viscosity and rheology. Besides, OTR is a function of rheological properties and the power input. Generally, high power inputs are required to obtain high transfer rates. This is the reason of using stirred tank bioreactor with improved mixing agitator for submerged cultivations of fungal cells. The quality of bulk mixing in viscous mycelial fermentations, especially in large bioreactors (> 500 L) is an important design and scale-up parameter. Therefore, it could be concluded that scale up of executing phase is usually associated with the rheological characteristics of the broth. According to the results of previous chapters, agitation intensity, mean shear rate and shear stress of all four impellers affected on the cell growth rate and final enzyme activity. Based on the experimental and simulation results, in this thesis fermentation and stirred condition using an agitator with flexible-shaft could be leaded to the preparation of a shear-thinning fermentation fluid with low viscosity, low K_{app} and n_{app}, and consequently, formation a biological media with high enzyme activity and K_La.

The other factor influenced on improving the executing phase is fungal morphology. Because from the biological point of view, physiological similarity must be taken into account [18] during the scale-up design. Therefore, the conditions to ensure the same morphological characteristics in large-scale production are usually investigated. It was reported that FZ installed into the scale-up fermenter for FR901379 production, reduced the mycelium damage due [42]. It was shown that viscosity of culture when using FZ impeller in large-scale fermenter was lower than that of the fermentation condition when using DRT impeller. Results of viscosity measurement in this study was agreed with using FZ impeller at large scale to decrease the viscosity and improve the rate of mass transfer. Finally, it could be concluded that besides of using Swingstir® in stirred fermentation, using FZ in large scales has positive effect on executing phase.

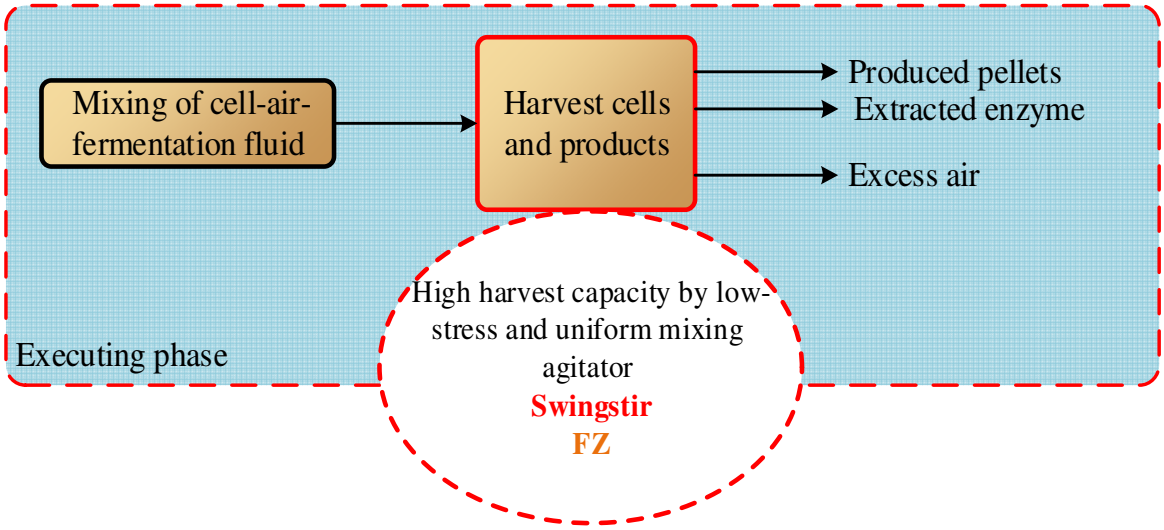


Fig. 7-3 Diagram of executing phase during batch fermentation of *A.oryzae*.

7-4-2-4 Finishing phase

One of the important issue in finishing phase is harvesting the biomass and separation process easily. Therefore, the best mixing condition in finishing phase is the condition with low biomass

waste. Also, there should be low biomass adherence to the impeller and internal sensors, to enhance the separation process in fermentation of *A.oryzae*. Using Swingstir® showed the lowest cell adherence to the internal equipment in stirred vessels (Appendix 5, **Fig. 26**). Diagram of mixing roll on finishing phase was shown in **Fig. 7-4**.

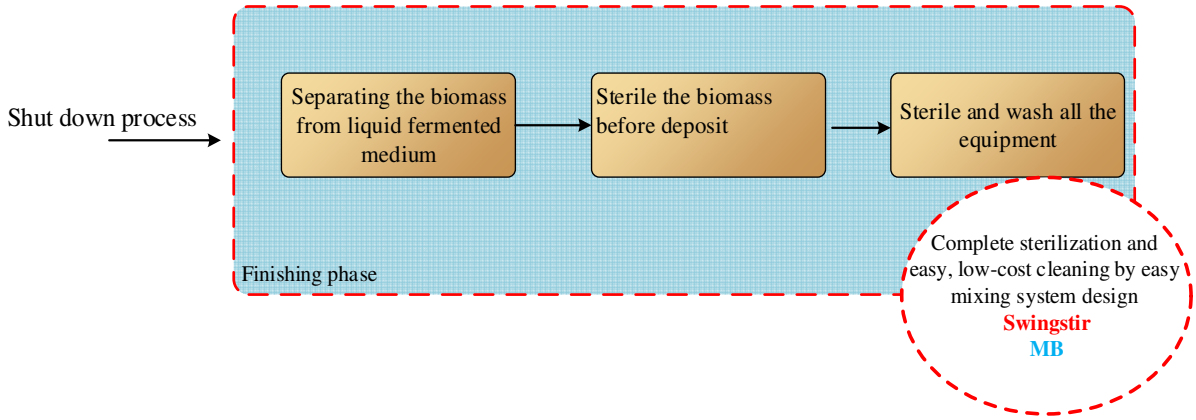


Fig. 7-4 Effect of mixing techniques on the finishing phase of fermentation of *A.oryzae*.

7-4-2-5 Technical systems

In this section the equipment and technical factors which affected on the design of fermenter during increasing the liquid volume have been investigated (**Fig. 7-5**). Among these technical parameters most of them were related to the stirring equipment. The identification of which parameters are dependent on the fermenter volume and which remain constant are essential for the development of any fermentation process at industrial level [43]. Some important application of analyzing the mixing technical factors on production in fermenter are mentioned as follows:

(a) Basically, a minimum height of the fermenter is necessary to achieve enough agitation power for perfect mixing in the reactor. This minimum height has importance in the process, since the presence of heterogeneity in the system may have a substantially negative effect on the productivity, mainly due to the appearance of different local rates. Additionally, many researchers have assumed that the power consumption depends principally on the tip speed and projected area

of the blades. The projected area depends on variables such as impeller diameter and blade height. However, it was showed that blade thickness also has a significant effect on N_p for the RT [44] and later it was confirmed by the results of Rutherford et al. [45].

(b) The power consumption of a stirred tank equipped DRT in both single and gas-liquid phase conditions were predicted by Taghavi et al. [46], and showed in turbulent regime, N_p approximately remained constant. Data resulted from two phase case showed a reduction in power consumption values comparing with single phase case which was decreased as gassing rate increased. The power consumption of upper and bottom impeller was measured and it was showed that the bottom impeller consumes more power than the upper one. These technical effects could be leaded to non-uniform energy distribution in stirred biological fluid and consequently non-uniform biological growth, mass transfer and low level of productivity were seen [47].

(c) The efficiency of a mixing process can be determined from the power consumption and mixing time, which are the two parameters that define homogenization energy [1].

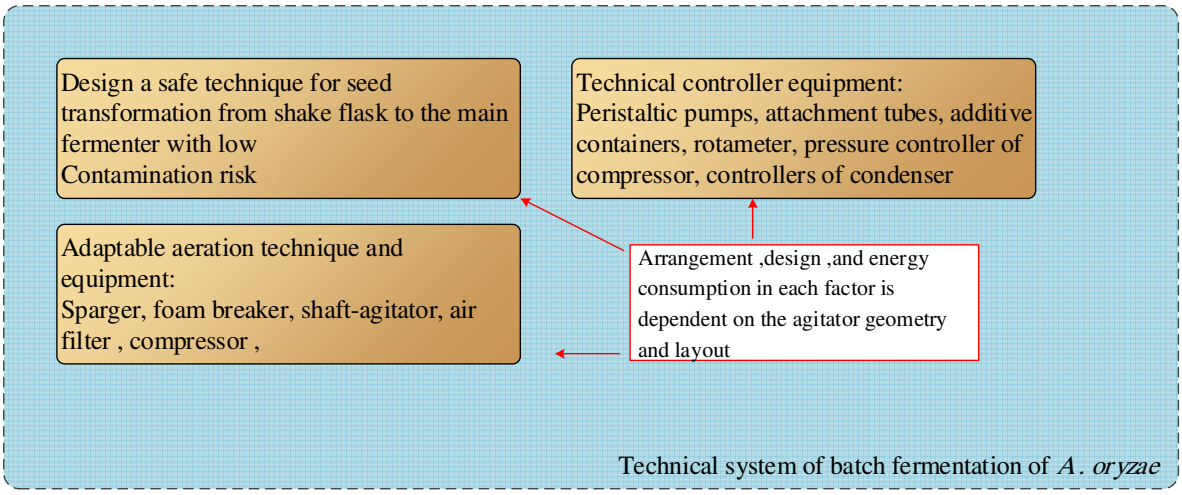


Fig. 7-5 Effect of mixing analysis on the technical system of fermentation of *A.oryzae*.

7-4-2-6 Biological systems

Mixing investigation during submerged fermentation of *A.oryzae* could have a direct and clear effect on all of the sub-functions of biological system. Biological system was classified into several sub-functions as follows; (1) enzyme production, (2) fungal cell growth, (3) nutrient supply, (4) harvesting the production and biomass. According to the results of chapter 4-6, movement or flow pattern produced by each kind of mixers had major impact on all of the sub-functions, also there is a complex relation between biological system and mixing condition of stirred fermentation system.

7-4-2-7 Active environment

This approach pays more attention to the conditions the fungus will meet on its way through the reactor. It is based on small scale simulation of the environmental conditions in the production scale fermenter. Young et al. [48] summarized the environmental factors, which can influence the activity of biological environment as follows:

Chemical variables: carbon and nitrogen source, oxygen concentration, product formation, pH, antifoam.

Physical variables: temperature, viscosity, liquid substrate/product distribution, power input, shear, morphology.

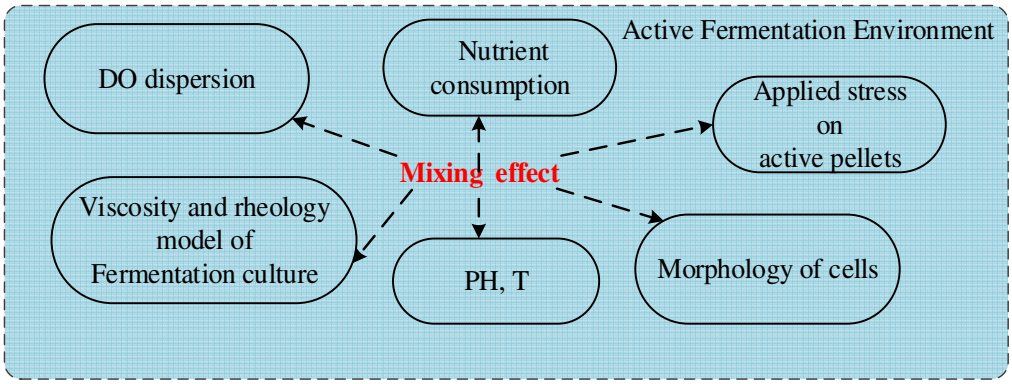


Fig. 7-6 Relation between fermentation factors and activity of fermentation environment.

According to the results of previous chapters, mixing pattern has a significant effect on each factors of active fermentation medium. Due to this reason, during the evaluation of activity of fermentation fluid all parameters shown in **Fig. 7-6** should be investigated.

7-4-2-8 Management and goal systems

According to the **Fig. 7-7**, mixing condition during the fermentation could have indirect effect on management system, particularly on operational management.

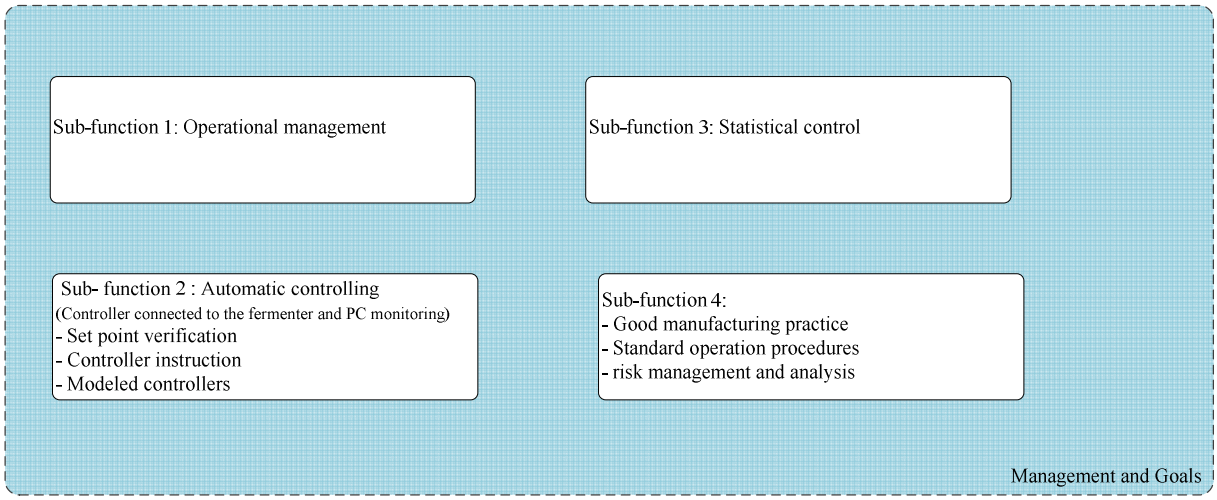


Fig. 7-7 Illustrations of management and goal by sub-functions during batch-stirred fermentation of *A.oryzae.*

7-4-2-9 Human systems

Investigation on the effect of mixing on human system is quite complicated but it was clear that designing a mixed biological system by user friendly and easy maintenance characterization could be resulted in human cost reduction.

7-4-2-10 Effect of mixing on sub-functions of information system

Information of mixing characterization of fermentation process can have effect on information system. The most important sub-function that were related to mixing information were mass transfer, homogenization, heat transfer, avoiding destruction, and harvesting enhancement.

CFD is one of the useful tools to improve the information system. Using CFD, we can examine various parameters relating to various phenomena in a shorter time and with less expense. During the last two decades, CFD has become an important tool for understanding flow phenomena, developing of new processes, and optimizing the existing processes. The capability of CFD tools to predict the flow behavior in several fields is considered as a successful achievement of these methods. Due to this reasons, CFD tools could be a useful tool to enrich the information about behavior of biological system before beginning of process. Yang et al. [49] have described a model to predict mycelial morphology and mycelial growth in the development scale-up strategy to constant energy dissipation, mass transfer coefficient, or impeller tip speed and showed that simulation of mycelial processes can be a valuable tool for developing process and scale-up of such processes [49]. Another tool is using scale-down results. Parameters can be tested more quickly and inexpensively than at the production scale during the scale down experiments.

7-5 Summary

In this chapter various constant criteria for scale-up of the fermentation of *A.oryzae* have been reviewed. Among these criteria, constant P_v, constant OTR (constant K_La or oxygen up-take rate) and constant energy dissipation were suggested for future scale up.

Besides, studying the effect of mixing on fungal fermentation from the aspect of bio mechatronic principles showed the results as follow;

- Mixing using Swingstir® and FZ could be resulted in enhancing the preparing phase and executing phase of transformation process in submerged fermentation.
- Investigation on finishing phase of fermentation indicated that using Swingstir® and MB impellers could satisfy the requirement of finishing phase.

Symbols;

Nomenclature

C	Oxygen concentration in liquid medium	[ppm]
K_{app}	Apparent Consistency index	[Pa s^n]
K_La	Volumetric mass transfer coefficient	[h^{-1}]
N_P	Non-dimensional power number	-
n_{app}	Apparent flow index	-
P	Power consumption	[W]
P_v	Power density	[Wm^{-3}]
v_g	Superficial gas velocity	[ms^{-1}]

Abbreviations

DO	Dissolved oxygen	[ppm]
DRT	Double Rushton turbine	-
FZ	Fullzone®	-
MB	Maxblend®	-
OTR	Oxygen transfer rate	[$h^{-1}gL^{-1}$]
PI	Process intensification	-
PTD	Pitched turbine blade	-
vvm	Volume per volume aeration	-

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Chapter 8: Conclusions and recommendation for future work

8-1 Conclusions

The main aim of the research for PhD study was related to the fermentation intensification in STBR by mixing enhancement. The work conducted can be classified into 7 sections. Major areas were contributed to finding a hydrodynamic pattern to improve the oxygen transfer from fermentation liquid to the cells, viscosity control at low value by control the growth rate of pellet diameter and keep an ideal proportion between hairy zone and core zone of pellet cells (pellet porosity), and also finding agitation condition with low $\dot{\gamma}_{ave}$ and shear stress and also with high enzyme activity. Decrease and control the shear stress applied to the fungal cells in critical fermentation intervals (after accelerating the cell growth until end of the fermentation) was also one of the important finding in this thesis. Based on these scope, the following conclusions could be drawn from above researches.

- Fermentation by DRT at low, moderate and high P_v cause dead mixing zones for the reason of local and non-uniform shear rate distribution, also local-central stress formation was damaged the cells and produced week and loosely pellets. Therefore, intensifying the batch fermentation using single large blade has been investigated to overcome the problems of fermentation using radial multi blades.
- Study the stirred fungal fermentation using MB impeller as a single large impeller, showed that enhancement of mass transfer in bio-culture using MB impeller at moderate and high P_v was not in a good way, because by increasing the P_v the shear rate and stress (particularly near the grid section of blade) dramatically has been increased and morphology of cells were damaged. Therefore, the enzyme production was decreased however the K_La and DO concentration was increased. By increasing the K_La, fungal growth rate and also the thixotropic behavior of fermentation culture have been increased by an un-controlled way when using MB impeller at high P_v. However fermentation using MB impeller at low P_v, was intensified the enzyme

production and also the local stagnant zone could be decreased, but by increasing the P_v the average shear rate, stress and morphology could not be controlled thus, it was concluded that MB might not be flexible candidate impeller for PI of fermentation by changing the energy consumption.

- FZ impeller was used as a multi-large agitator for solve the problem of negative effect of shear stress. Because controlling the shear stress is one of the important issues for overcoming the major obstacles during fermentation of *A.oryzae*. Simulation and experimental results showed that using FZ shear stress exposed to fungal cells could decrease in comparison with the agitation condition by DRT and MB. Due to this effect, the viscosity of culture could relatively be kept at low values however the thixotropic behavior and fluctuation in apparent viscosity of fermentation culture could not be negligible. Mixing using FZ impeller could enhance the enzyme activity in comparison with the stirred fermentation using DRT and MB impellers. High stress at tank wall, high cell accumulation (low porosity of fungal cell), and thixotropic behavior of culture were the problems that were remained when using FZ impeller. Due to this reason to enhancing the mixer movement and overcoming the obstacles when using multi-large blade, investigating on the effect of flexible-shaft agitator has been studied.
- Swingstir® was used as a flexible-shaft mixer. Applied shear rate at low and moderate P_v using flexible-shaft impeller (Swingstir®) was caused a periodically high shear rate in entire fermenter. Concerning to this issue, the stagnant zone during the fermentation was not formed and K_La at low P_v could be kept at desirable value until the end of fermentation. Moreover, viscosity of culture broth was kept at low value to enhance the mass transfer, also the thixotropic behavior of fermentation fluid has been significantly decreased. Using Swingstir® the porosity of pellet has been increased (in comparison with the mixing condition by multi-large impeller(FZ)) and it could be effective for

mass transfer enhancement. Experimental analysis indicates that mass transfer intensification using the flexible-shaft impeller was done by thinning the gas-liquid boundary layer not by breaking the bubbles into small size. Finally, advantages of flexible-shaft impeller for PI of fermentation were controlled the viscosity at low *Re*, PI of mass transfer by improving the surface morphology, controlling the K_La at desirable value until end of fermentation, PI of fermentation by waste reduction, enhance and keep the enzyme activity at high value with the low mixing energy in comparison with DRT, MB and FZ at the same Re_m.

8-2 Recommendation for future work

One of the suggested solution for possibility to calculate and intensify the performance of fermentation, is working with a flexible agitator with the behavior of fungal culture. Based on this point, there will be always a novel approach for further investigations. The following suggestions below are recommended as areas worthy of investigation;

- Intensification of submerged fermentation of *A.oryzae* using Swingstir® by the aim of reduction in fermentation time.
- Intensification of fermentation of *A. oryzae* in a semi-pilot scale using oscillatory baffle fermenter and investigation the optimal fermenter. To investigate the optimal cost, productivity with reduced shear stress environment in cultivated medium using plug flow.
- Finding an efficient and novel method for whole immobilization of *A.oryzae* by the aim of shear damage reduction, controlling the morphology, viscosity, and mass transfer during the batch fermentation when using large blade or flexible movement agitator. Besides investigate the production cost of this method with other conventional methods of submerged fermentation.
- Intensification of fermentation of *A.oryzae* by decreasing the substrate material and vessel size in stirred tank reactor using Swingstir®.
- Design, scale-up and scale-down (before doing scale up) experiments to investigate the efficiency of Swingstir® and FZ in large fermentation volume.

Publications

1) Ghobadi, N., Ogino, C., Yamabe K. and Ohmura, N.: (2016) Characterizations of the Submerged Fermentation of *Aspergillus oryzae* Using a Fullzone Impeller in a Stirred Tank Bioreactor, Journal of Bioscience and Bioengineering. pii: S1389-1723(16)30150-5. doi: 10.1016/j.jbiosc.2016.07.001

2) Ghobadi, N., Ogino, C., Ogawa, T., and Ohmura, N.: Using a flexible shaft agitator to enhance the rheology of a complex fungal fermentation culture, Bioprocess and Bio Systems Engineering Journal, (39) 1793-1801

3) Ghobadi, N., Ogino, and Ohmura, N.: (2016) Intensifying the Fermentation of *Aspergillus oryzae* in a Stirred Bioreactor using Maxblend Impeller, The Open Chemical Engineering Journal, (10) 88-109

4) Ghobadi, N., Ogino, C., Ogawa, T., and Ohmura, N.: Mixing characteristics of fungal submerged fluid in a flexible stirred mixer system, (Under submission to Journal of Chemical Engineering of Japan.)

5) Ghobadi, N., Ogino, and Ohmura, N.: Investigation on the scale-up criteria during fermentation of *Aspergillus oryzae* in shake flask using scale-down method, (Under preparation)

6) Ghobadi, N., Ogino, C., Yamabe K. and Ohmura, N.: Mixing improvement in high-viscosity submerged fermentation using multi large impeller at low energy consumption, (Under preparation)

Selected Conference Papers;

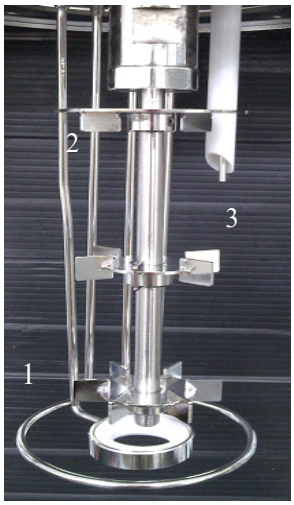
1) Narges Ghobadi, Chiaki Ogino, Tomohiro Ogawa, Naoto Ohmura, Effect of Swingstir® impeller on rheology and morphology of submerged fermentation, Proceeding 5th Asian Conference on Mixing, Tendo, Japan, August (2016).

2) Ghobadi, N., Ogino, C., and Ohmura, N.: Intensification of submerged fermentation of *Aspergillus oryzae* in stirred fed-batch bioreactor by improved mixing, Proceeding 15th Eur. Conference on Mixing, St. Petersburg, Russia, 1, 128-133 July (2015).

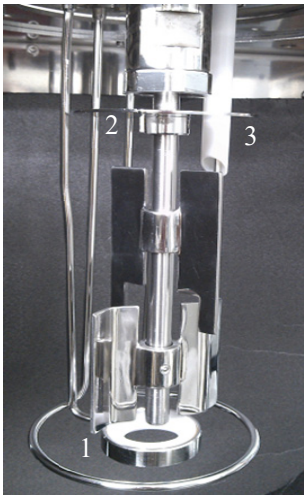
3) Ghobadi, N., Ogino, C., and Ohmura, N.: Effect of large impeller on fermentation of Aspergillus oryzae, 80th Annual Meeting of Chemical Engineering Society of Japan, Tokyo, Japan, March (2015).

Appendix 1; Apparatus

Impellers used in this research



DRT



FZ

Fig. 1 Image of impellers assembled on the shaft of fermenter; (1): ring sparger, (2): mechanical foam breaker, (3): unti-foam sensor.

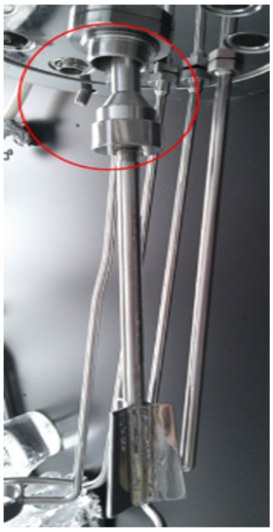
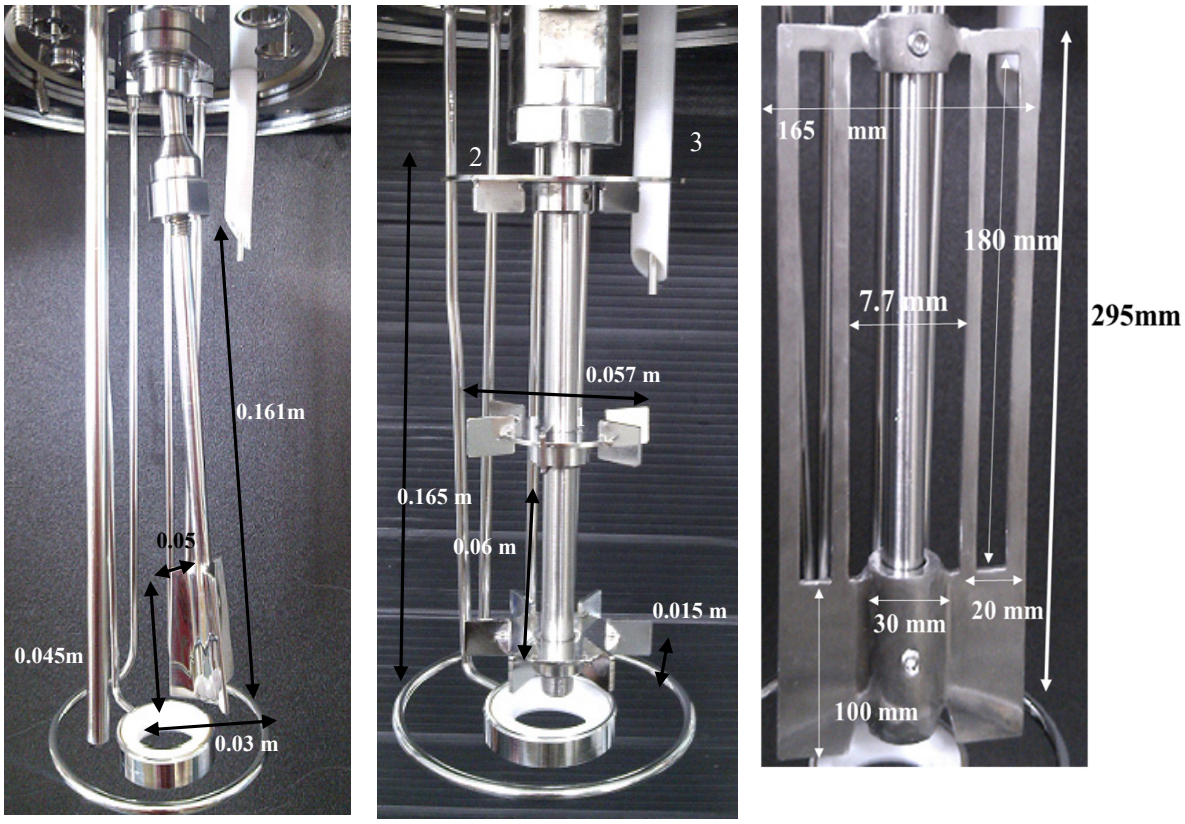


Fig. 2 Special sealing of Swingstir® to the main shaft in stirred fermenter.



Swingstir® DRT MB

Fig. 3 Size illustration of Swingstir®, DRT, and MB impellers assembled on the shaft of fermenter.

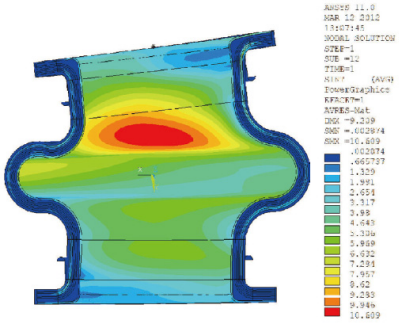


Fig. 4 FEM result of flexible seal of Swingstir® agitator [This figure was provided by Kobe Elco Co., Ltd. Kobe, Japan].

Notice;

In the experimental set-up, adjusting the agitation by both impellers (MB and DRT) at the same power density was not possible because of some limitations. For this reason, the

fermentation study was attempted at roughly closed P_v for three different ranges (low, moderate and high values) with the *Re* number in the same range.

Appendix 2; Rheology

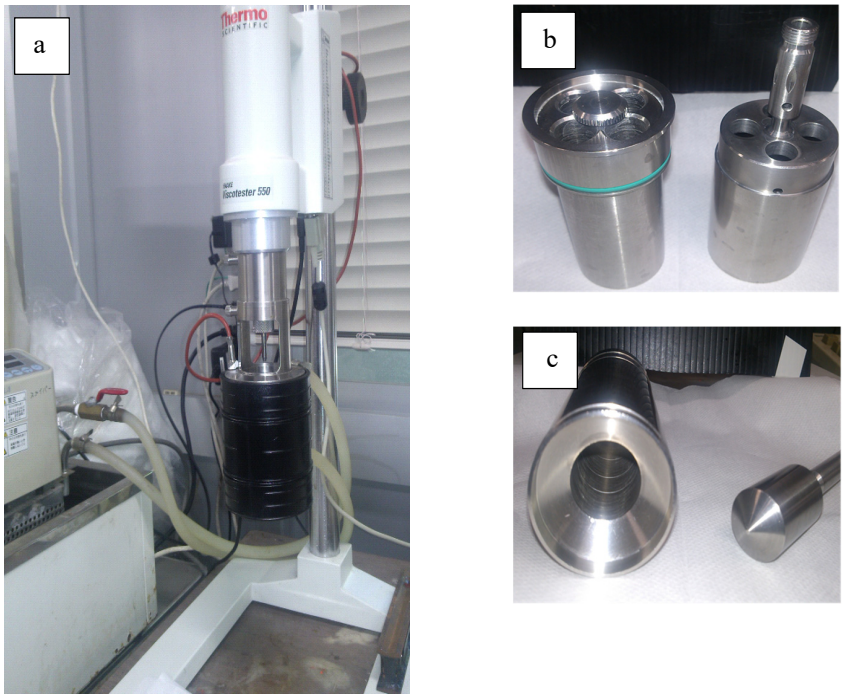
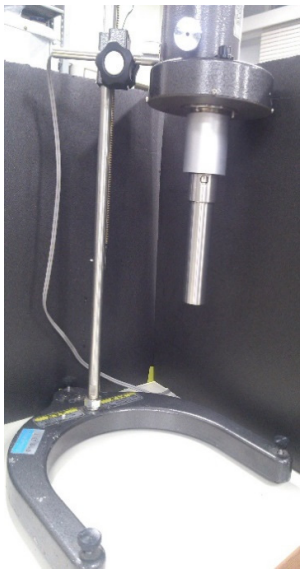
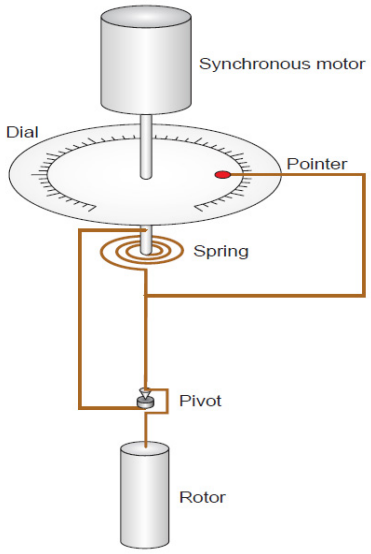


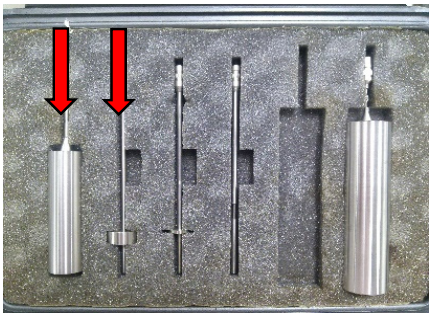
Fig. 5 (a); Illustration of rheometer used for measuring the viscosity and parameters of power-law model, **(b)** Rotor used for fermentation fluid before cell growth, (c); Rotor used for fermentation fluid after cell growth.



a



b



c

Fig. 6 (a) and **(b)**; Image of B-type viscometer used in this study for measuring the apparent viscosity of shear-thinning fermentation culture after each sampling time; **(c)** Red arrows showed the rotor type used for viscosity measurement of fungal suspension.

Details of experimental viscosity and Re measurement in each fermentation condition

Swingstir®-85 rpm

Sampling Time	Density (kg/m^3)	Viscosity (Pa s)	*Re*
0	1013	0.003	430.53
12	1001	0.004	319.82
24	1008	0.01	128.52
31	1003	0.18	7.10
48	1006	0.4	3.21
55	1014	0.38	3.41
72	1015	0.46	2.79
Average	1006	0.21	127.33

DRT-100 rpm

Sampling Time	Density (kg/m^3)	Viscosity (Pa s)	*Re*
0	1013	0.003	1832.12
16	1012	0.005	1098.62
24	1012	0.015	366.062
40	1010	0.4	7.82
48	1006	1	5.46
65	1009	0.7	7.82
72	1007	0.65	8.4
Average	1010	0.40	473.99

DRT-500 rpm

Sampling Time	Density (kg/m^3)	Viscosity (Pa s)	*Re*
0	1000	0.003	9021
16	1011	0.20	135.45
24	1019	0.24	114.91
40	1018	0.60	45.92
48	1012	0.6	45.65
65	1000	0.8	33.73
72	1013	0.48	57.12
Average	1010	0.42	1350.54

DRT-300 rpm

Sampling Time	Density (kg/m^3)	Viscosity (Pa s)	*Re*
0	1013	0.003	5845.40
16	1012	0.006	2741.06
24	1007	0.22	74.93
40	1000	1.00	16.25
48	975	1.06	15.52
65	980	0.7	22.74
72	986	0.56	28.61
Average	996	0.51	1249.21

MB-300 rpm

Sampling Time	Density (kg/m^3)	Viscosity (Pa s)	*Re*
0	1013	0.003	6078
16	1008	0.004	4536
24	1001	0.025	721.29
40	1007	0.87	20.83
48	1012	0.705	25.83
65	997	0.75	23.93
72	1016	0.85	21.52
Average	1008	0.46	1428.43

MB-100 rpm

Sampling Time	Density (kg/m^3)	Viscosity (Pa s)	*Re*
0	1013	0.003	2030
16	1000	0.01	607.21
24	1010	0.054	112.45
40	1009	0.92	6.59
48	1011	0.8	7.58
65	1010	0.84	7.23
72	1009	0.9	6.73
Average	1010	0.50	347.22

MB-500 rpm

Sampling Time	Density (kg/m^3)	Viscosity (Pa s)	*Re*
0	1013	0.003	7586.96
16	1012	0.01	3034.78
24	1012	0.03	1011.99
33	1009	0.03	1008.73
48	1016	0.6	50.8
56	1010	0.9	33.56
72	1002	0.3	100.221
Average	1010	0.27	1832.43
Average(laminar)	1009	0.6	61.53

Swingstir-247 rpm

Sampling Time	Density (kg/m^3)	Viscosity (Pa s)	*Re*
0	1013	0.003	1251.05
16	1004	0.004	929
24	1005	0.01	372
40	1008	0.61	6.23
48	1022	0.55	6.9
64	1012	0.50	1.37
72	1003	0.40	3.76
Average	1009	0.44	367.19

FZ-300 rpm

Sampling Time	Density (kg/m^3)	Viscosity (Pa s)	*Re*
0	1013	0.003	1251.05
16	1006	0.007	2586.54
24	1007	0.008	2265.75
40	1012	0.93	364.32
48	996	0.33	54.33
64	988	0.25	71.13
72	988	0.55	32.30
Average	1001	0.30	897.24

FZ-100 rpm

Sampling Time	Density (kg/m^3)	Viscosity (Pa s)	*Re*
0	1013	0.003	2030.05
16	1010	0.008	759.015
24	999	0.055	108.882
40	1012	0.01	608.653
48	1004	0.48	12.57
55	997	0.6	10.00
72	1005	0.34	17.77
Average	1005	0.25	506.705

FZ-400 rpm

Sampling Time	Density (kg/m^3)	Viscosity (Pa s)	*Re*
0	1013	0.003	6054
16	1010	0.005	4852
24	1009	0.015	1616
40	1010	0.7	346
48	1010	1.0	24
55	1010	0.8	30.3
72	1009	0.86	28.19
Average	1010	0.48	1850

Appendix 3; Torque and power consumption

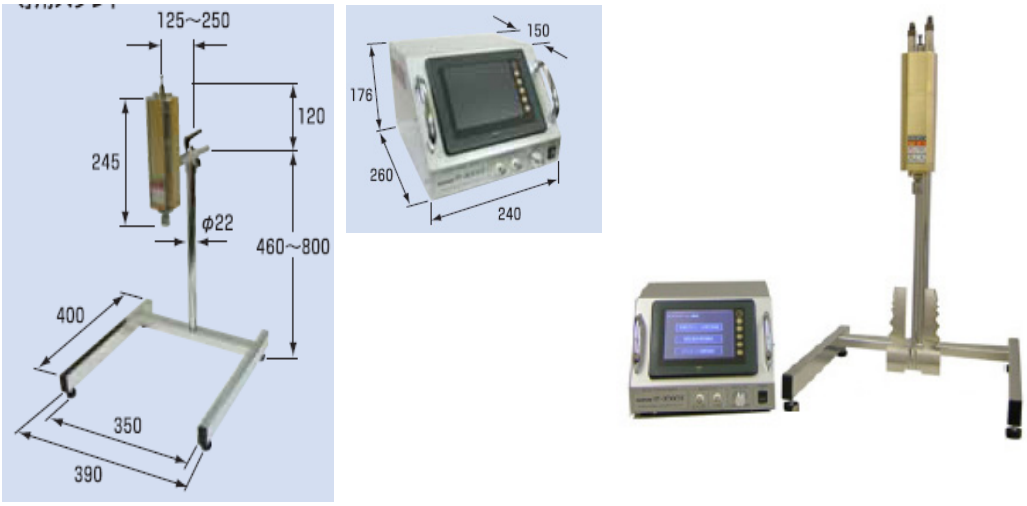


Fig. 7 Illustration of SAKATE torque meter used in this study for final torque measurement.

As an example, the torque value (T), and N_p of fermentation culture using SATAKE torque meter was shown in **Table 1**.

Table 1 T and N_p of fermentation culture using SATAKE torque meter.

Impeller	T (Nm)	N_p	N (min^{-1})
DRT	0.0217±0.0041	76.97	100
MB	0.0212±0.0077	93.23	100
DRT	0.0301±0.0069	11.85	300
MB	0.03282±0.0111	9.6	300
DRT	0.0427±0.0118	6.33	500
MB	0.0437±0.0094	4.23	500

Appendix 4; Simulation

To calculate the 2-D and 3-D velocity and shear stress in stirred fermenters with different impellers, the fluid dynamics software "R-FLOW" (R-flow Co., Ltd., Saitama, Japan) was utilized to solve the Navier-Stokes equations. Using R-Flow software, there are three models for multi-phase flow simulation as below;

- Drift flux model
- Two fluid model
- Gidaspow model (Granular kinetic model)

In this study, the shear stress given by Eq. (1);

$$\tau = \mu \left[(\nabla v) + (\nabla v)^T - \frac{2}{3} (\nabla . v) I \right] \quad (1)$$

In the above equation, μ is the molecular viscosity, and I is the unit tensor. For incompressible fluids, the stress tensor is given by Eq. (2);

$$\tau = \mu [(\nabla v) + (\nabla v)^T] = \mu D \quad (2)$$

where D is the rate of deformation tensor. For multi-dimensional flow of non-Newtonian fluids, the apparent viscosity (μ) is a function of all three invariants of the rate of deformation tensor [1]. However, the first invariant is zero for incompressible fluids, and the third invariant is negligible for shearing flows [2]. Thus, for the incompressible non-Newtonian fluids, μ is a function of shear rate, which is given by Eq. (3). It can be seen that $\dot{\gamma}$ is related to the second invariant of D.

$$\dot{\gamma} = \sqrt{\frac{1}{2}(D:D)} \quad (3)$$

Eulerian multiphase model

Although CFD models have shown to be successful in simulating single-phase flow

generated by impeller(s) in complex reactors [3], the complexity of modeling increases considerably for multiphase flows because of various levels of interaction of different phases. Two widely used modeling methods for multiphase flows are Eulerian-Eulerian and Eulerian-Lagrangian approach. Eulerian-Lagrangian approach can only be used for multiphase systems with a low dispersed-phase volume fraction (≤ 5%) because of the huge computational need. Therefore, in this study the estimated gas volume fraction was not compatible with the Lagrangian condition. In Eulerian–Eulerian approach, the dispersed phase is treated as a continuum. This approach is more suitable for modeling dispersed multiphase systems with a significant volume fraction of dispersed phase (>10%). The coupling between different phases is incorporated in this approach by developing interphase transport models. Each phase is treated as a different continuum which interacts with other phases everywhere in the computational domain. The share of the flow domain occupied by each phase is given by the volume fraction. Each phase has its own velocity, temperature and physical properties. The motion of each phase is governed by averaged *Re* mass and momentum conservation equations. In two-fluid model, the continuous and the dispersed (bubble) phase are separately expressed with the equations of "Conservation of Momentum" and "Mass Continuity". These equations were written for continuous phase and were shown in Eqs. (4) and (5)

$$\alpha_c \rho_c \left(\frac{\partial \boldsymbol{v_c}}{\partial t} + \boldsymbol{v_c} \cdot \nabla \boldsymbol{v_c}\right) = -\alpha_c \nabla \boldsymbol{p} + \alpha_c \rho_c \boldsymbol{g} + \alpha_c \alpha_d \beta (\boldsymbol{v_d} - \boldsymbol{v_c}) + \nabla \cdot (\alpha_c \mu_c \nabla \boldsymbol{v_c}) + \mathrm{F} \tag{4}$$

$$\frac{\partial \alpha_c}{\partial \mathrm{t}} + \nabla \cdot (\alpha_c \boldsymbol{v_c}) = 0 \tag{5}$$

The term including β in Eq. (4) expresses momentum exchanges between the continuous phase and the dispersed phase (in this study β supposed to be zero). Considering to the interaction between the fermentation fluid and gas phase, (The fluid resistance is the most important forces in interactions between the fluid and the bubble.). Recently, influence of

different interphase forces has been studied [4] and reported that the effect of the virtual mass force is not significant in the bulk region of agitated reactors and the magnitude of the Basset force is also much smaller than that of the inter-phase drag force. This force is expressed by Eq. (6).

$$\beta = 3C_D'\rho_c|\boldsymbol{v_d} - \boldsymbol{v_c}|/(4d_p) \tag{6}$$

Where d_p means the bubble diameter, C_D' is the fluid-resistance-coefficient of a bubble-cluster against the fluid, and is approximated with C_D, the fluid-resistance-coefficient of one bubble against the fluid was defined in Eq. (7).

$$C_D' = \frac{C_D}{\alpha_d^{n/2}} \quad (n = 1.5\sim3) \tag{7}$$

According to the Eq. (7), C_D' is bigger than C_D. This is explained that the existence of the bubbles causes the reducing the area of flow pathway and increasing the local flow velocity near the bubbles, hence the flow resistance is bigger than expected. It is noticed that C_D is estimated with the formula of the resistance near the sphere bubble. When using R-Flow software, it is necessary to give terminal velocity during a multiphase flow analysis.

If the input of terminal velocity is omitted (based on default of software), terminal velocity will be automatically calculated.

$$V_{\mathrm{T}}^2 = \frac{4|\rho_{\mathrm{g}} - \rho_{\mathrm{L}}|gd_g}{3C_{\mathrm{D}}\rho_{\mathrm{L}}} \tag{8}$$

In the case of dealing with one bubble in the fluid, it should take into account of the increase of resistance force caused by the deformation of the bubble, the following formula (Eq. (9) and (10)) are used.

$$C_{\mathrm{D}} = max\left\{C_{\mathrm{D0}} \;\; \frac{8}{3}\frac{E_{\mathrm{o}}}{E_{\mathrm{o}} + 4}\right\} \tag{9}$$

$$E_{\mathrm{o}} = \frac{(\rho_{\mathrm{c}} - \rho_{\mathrm{d}}) g d_{\mathrm{p}}^2}{\sigma} \quad \text{(Eötvös number)} \tag{10}$$

Where σ means the interfacial tension, C_{D0} is the same to C_D in the Eq. (9).

In two-fluid model, the dispersed phase is approximated as a continuum and is described by Eqs. (11) and (12).

$$\alpha_d \rho_d \left(\frac{\partial \boldsymbol{v_d}}{\partial t} + \boldsymbol{v_d} \cdot \nabla \boldsymbol{v_d} \right) = -\alpha_d \nabla \boldsymbol{p} + \alpha_d \rho_d \boldsymbol{g} + \alpha_c \alpha_d \beta (\boldsymbol{v_c} - \boldsymbol{v_d}) + \nabla \cdot (\alpha_d \mu_d \nabla \boldsymbol{v_d}) - F \tag{11}$$

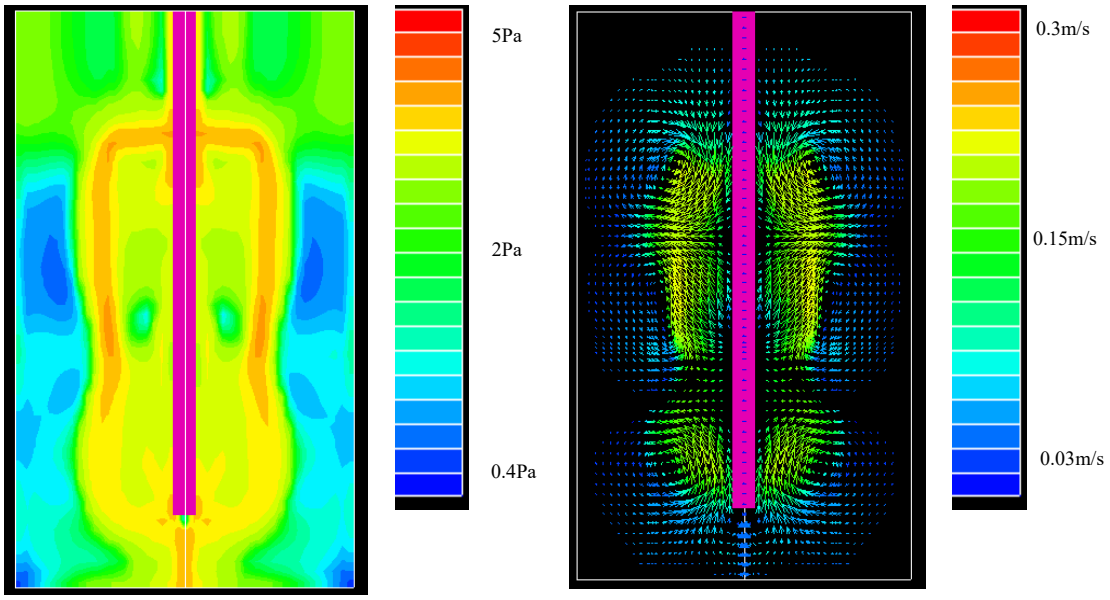
$$\frac{\partial \alpha_d}{\partial \mathrm{t}} + \nabla \cdot (\alpha_d \boldsymbol{v_d}) = 0 \tag{12}$$

where, ρ_d is density of the gas phase. In the term containing μ_d in Eq. (11) expresses the viscous effect caused by the interactions between bubbles. v_{d} in Eq. (11) and (12) is the lattice mean flow velocity. The term including v_{c} expresses the momentum exchange between the fluid and the bubbles.

Validation of shear stress simulation during mixing by FZ

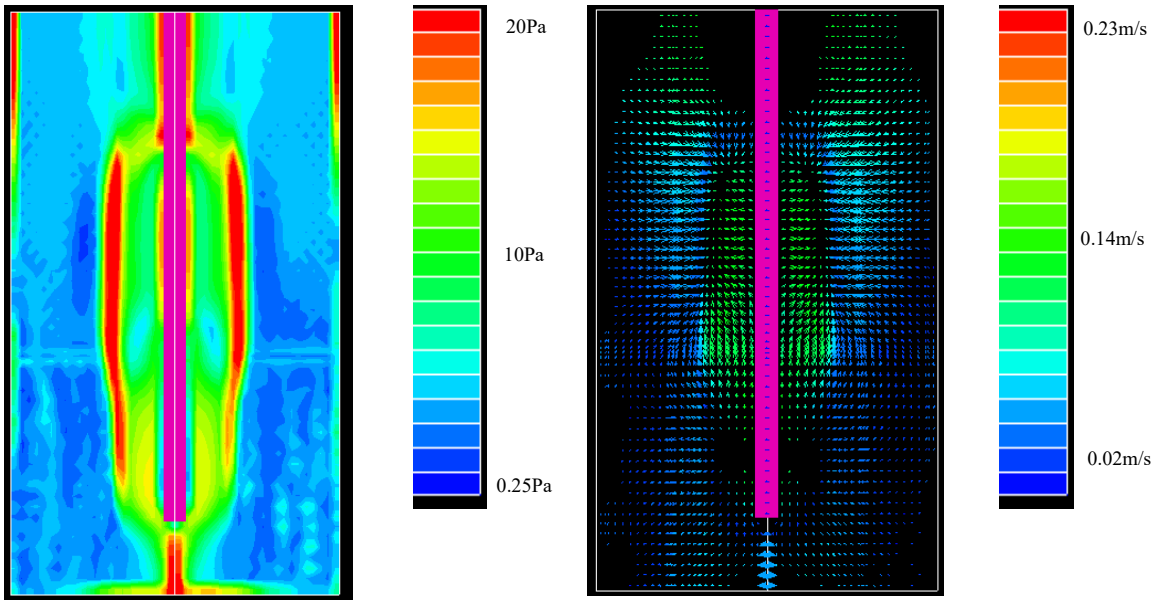
Shear stress simulation during mixing by FZ at low P_{in}

Simulation results of fluid flow velocity using FZ impeller showed that shear rate distribution in tank was strongly dependent on the consistency index, (*K*) which is a parameter of power-law fluid. By increasing the *K* stress near the blade has been increased (**Fig. 8** and **9**).



K= 2 Pa s^n, t = 72h, P_v = 280 W/m^3 K= 2 Pa s^n, t = 72h, P_v = 280 W/m^3

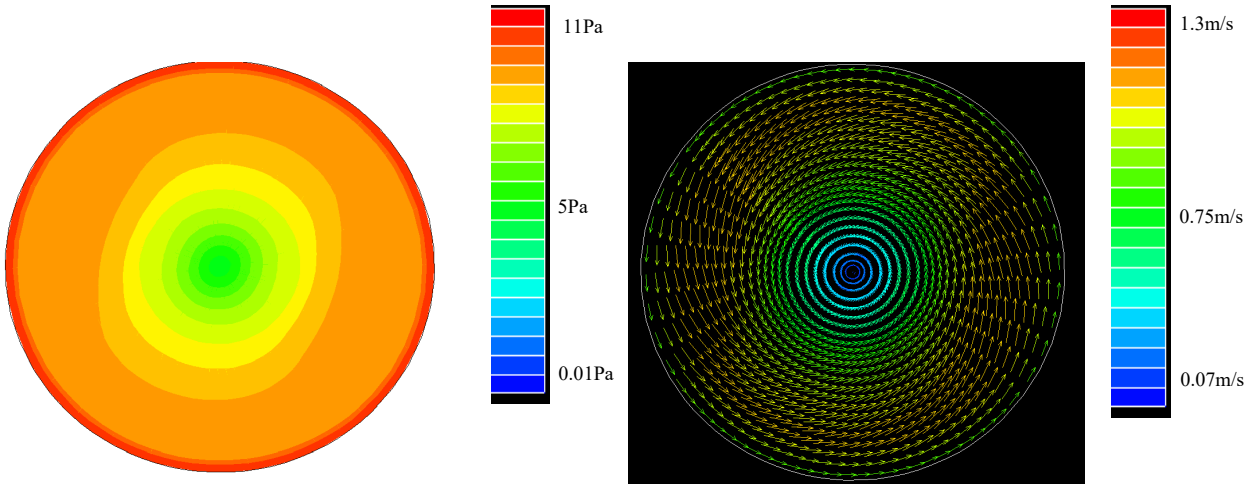
Fig. 8 Dependency of shear stress and shear rate on K value when agitating by FZ impeller at low power consumption.



K= 209 Pa s^n, t = 72h, P_v = 280 W/m^3 K=209 Pa s^n, t = 72h, P_v = 280 W/m^3

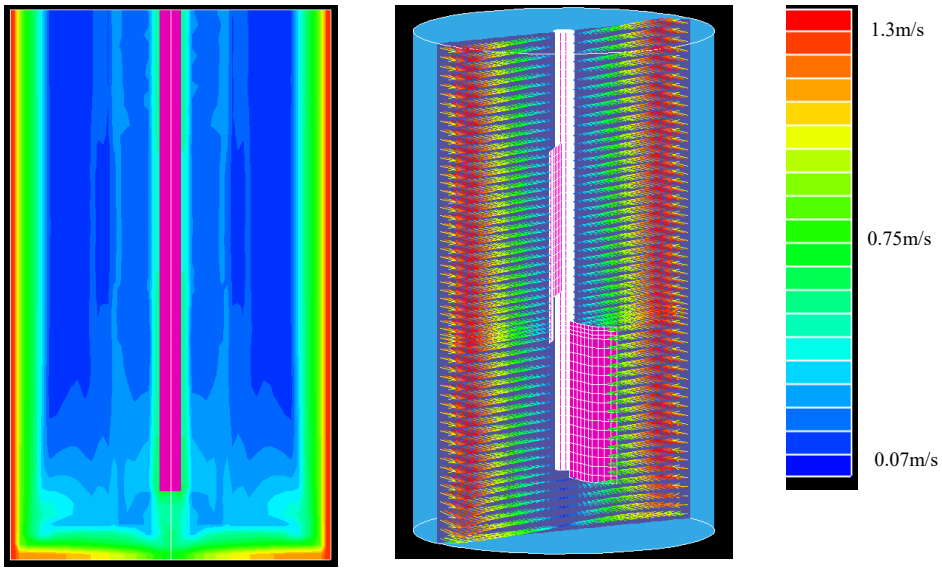
Fig. 9 Dependency of shear stress and shear rate on K value when agitating by FZ impeller at low power consumption.

Shear stress simulation during mixing by FZ at high P_{in}



K= 1.8 Pa s^n, n = 0.3 K= 1.8 Pa s^n, n = 0.3, P_v = 600 W/m^3

Fig. 10 Simulation of shear stress and shear rate when agitating by FZ impeller at high power consumption (X-Y plane).



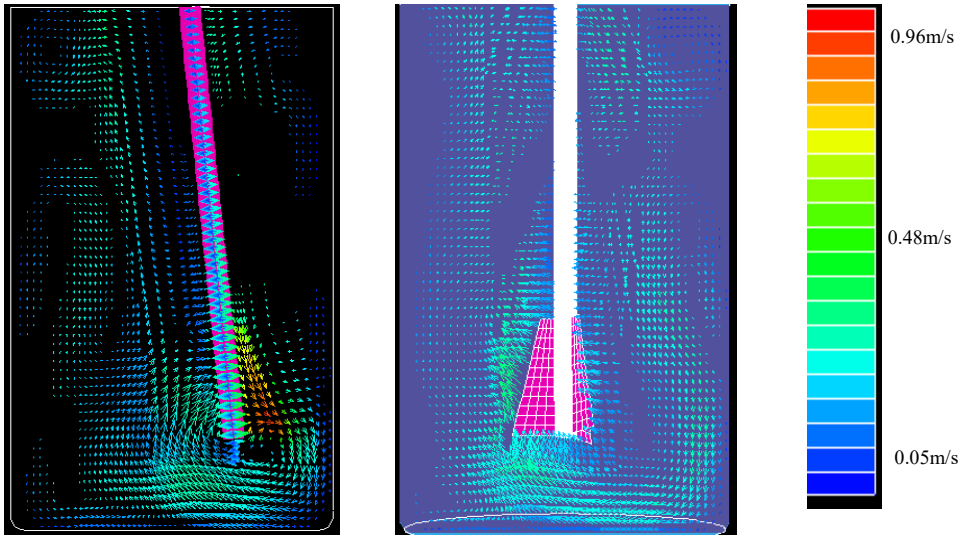
K= 1.8 Pa s^n, n = 0.3, P_v = 600 W/m^3

K= 1.8 Pa s^n, n = 0.3, P_v = 600 W/m^3

Fig. 11 simulation of shear stress and shear rate when agitating by FZ impeller at high power consumption (Z-Y plane).

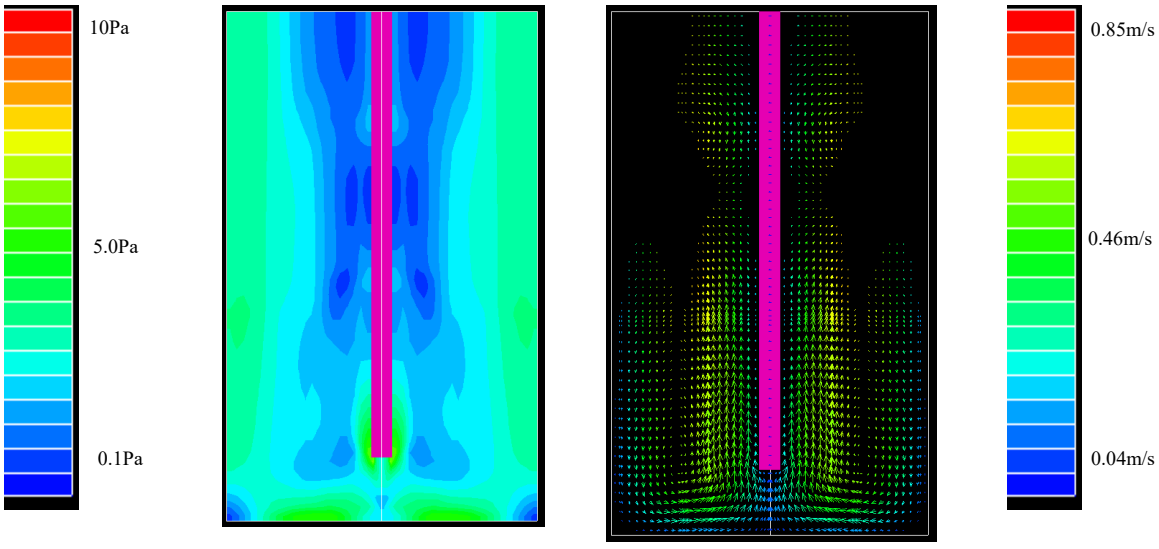
Validation of flow velocity and stress simulation using Swingstir® and FZ using Newtonian fluid

For verification, the simulation of flow velocity during the fermentation using Swingstir®, simulation of two-phase flow using glycerol as a Newtonian fluid was used and was shown in **Fig. 12**. Flow pattern of simulation using glycerol at N = 230 rpm was in agreement with the result of **Fig. 3-8 (b)**.



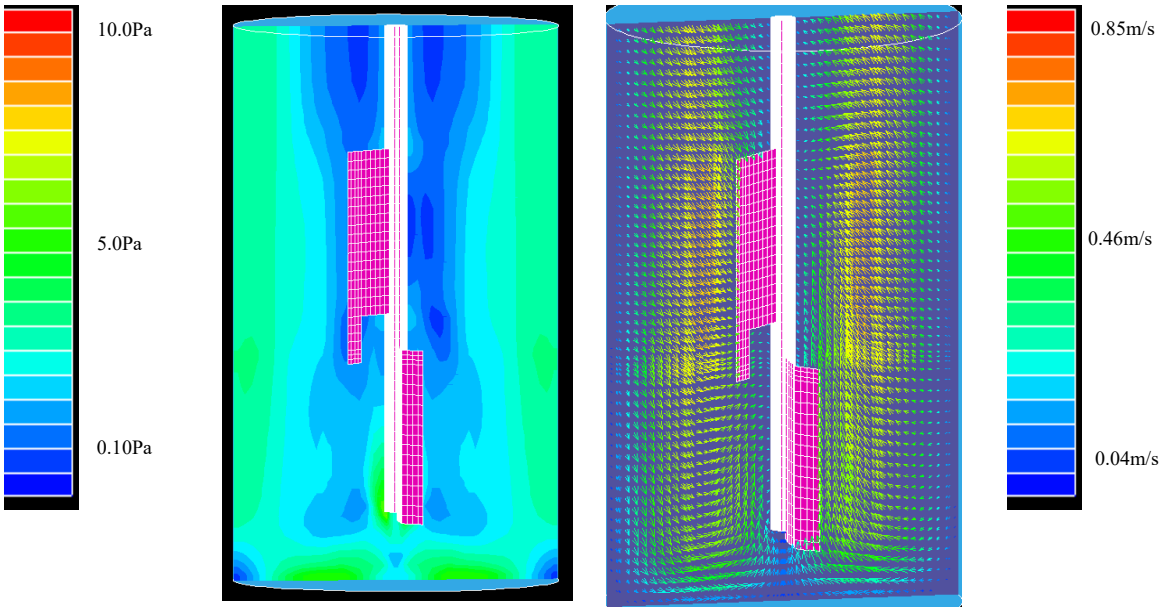
(a) N = 230 min^{-1}, 2D (b) N = 230 min^{-1}, 3D

Fig. 12 Fluid flow simulation using glycerol in 2-D and 3-D.



(a) N = 100 min^{-1}, 2D (b) N = 100 min^{-1}, 2D

Fig. 13 (a); Shear stress and **(b)**; Fluid flow simulation during mixing by FZ at low P_{in} using glycerol in 2-D.



(a) $N = 300$ min^{-1}, 3D (b) $N = 300$ min^{-1}, 3D

Fig. 14 (a); Shear stress and **(b)**; Fluid flow simulation during mixing by FZ at high P_{in} using glycerol in 3-D.

Stirred tank and impeller geometry generation

In this study, the geometry of stirred tank and impellers used in simulation were shown as follows; the shape and arrangement of meshes for stirred tank and each agitator were shown in **Figs. 15-18**.

Table 2 Details of geometrical design of stirred tank fermenter used in simulation

Parameter	Value
1- Height of liquid in tank	0.20145 m
2- Depth of tank	0.005 m
3- Radius of tank bottom	0
4- External diameter of tank	0.118 m
5- Diameter of shaft	0.008
6- Free slip (2) / free surface (10 or 11)	2
7- n (n : 1/n model)	1/1
8- Number of Tank Mesh	204480

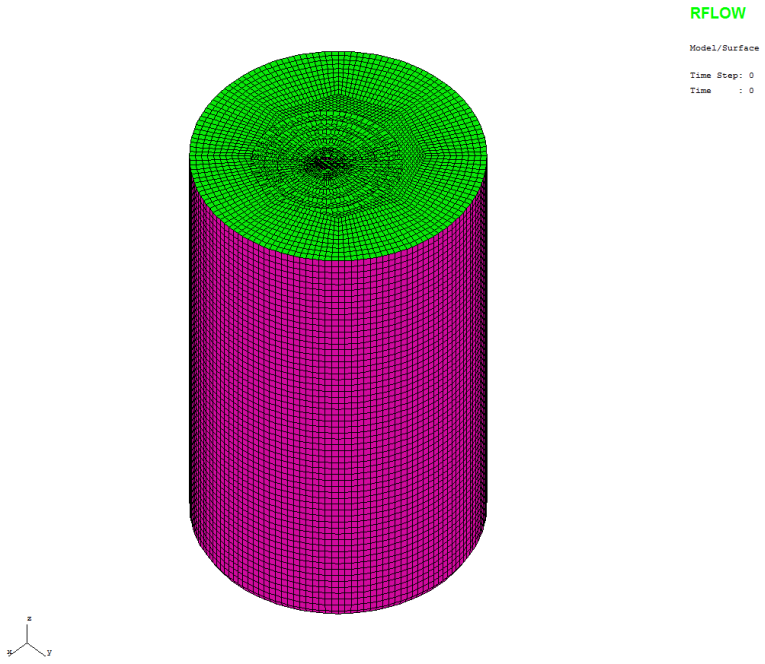


Fig. 15 Mesh generation for stirred tank fermenter at tank walls and liquid surface in mixing condition by Swingstir®.

Details of input data for geometrical generation of stirred fermenter attached with four type of impellers

(a) Details of geometry input data for DRT impeller generation used in simulation

Impeller mesh number; 27000

Table 3 Geometrical design of DRT impeller used in this study.

Parameter	Value
1-Number of Blades	6
2-Height of Blade	0.015 m
3-Outer Diameter of Blade	0.057 m
4-Inner Diameter of Blade	0.03 m
5-Diameter of Disk	0.037 m
6-Diameter of Shaft	0.008 m
7-Rotation Number of Impeller (rpm)	100/300/500 rpm
8-Z-Coordinate of Impeller Center	0.092 /0.022 m
9-Lower-End (0)/Middle (1)on Shaft	1
10-Number of Symmetry(n : 1/n model)	2/4

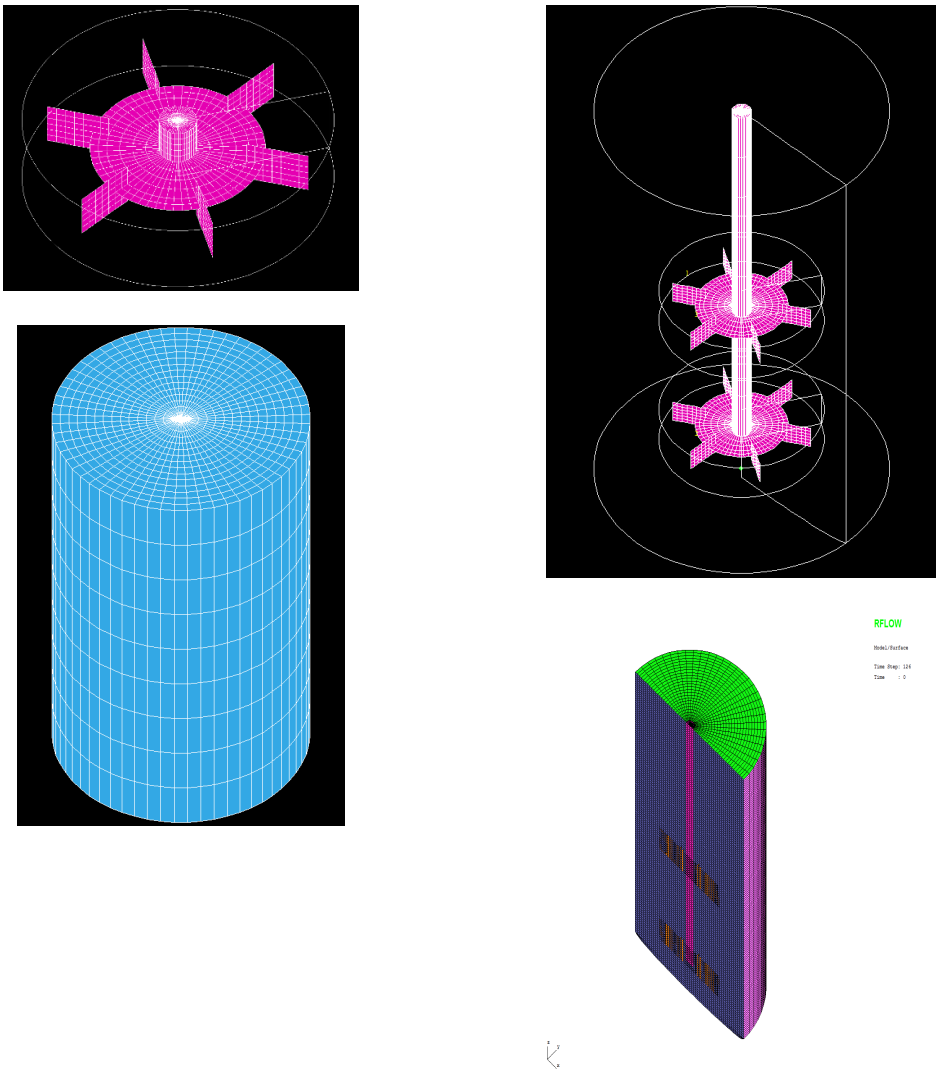


Fig. 16 DRT impeller and mesh generation in stirred fermenter.

(b) Details of input data for MB impeller generation used in simulation

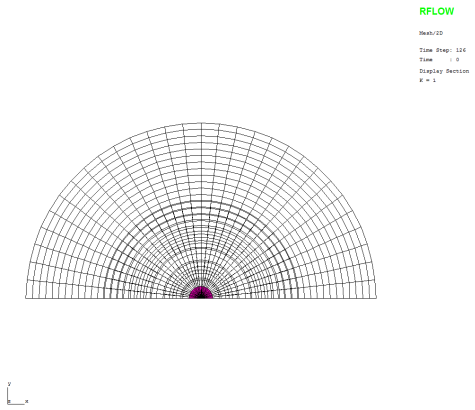


Fig. 17 Mesh generation at liquid surface of fermenter during agitation by MB impeller.

Total mesh number in stirred tank containing MB impeller; 101040

Table 4 Geometrical design of MB impeller used in this study.

Parameter	Value
1-Height of Blade	0.145 m
2-Height of Liquid Surface	0.20145 m
3-Depth of Tank Bottom	0.005 m
4-Radius of Tank Bottom	0
5-Clearance between Blade & Bottom	0.022 m
6-Lower-End Position of Shaft	0.022 m
7-External Diameter of tank	0.118 m
8-Diameter of Blade	0.062 m
9-Diameter of Shaft	0.008 m
10-Diameter of Blade Inner Edge at Shaft Lower-End	0.003
11-Diameter of Blade Inner Edge at Tank Bottom	0.005 m
12-Width of Outer Cavity	0.104 m
13-Clearance between Inner & Outer Cavities	0
14-Clearance between Outer Cavity & Blade Tip	0
15-Height of Lower Cavity	0.005 m
16-Height of Upper Cavity	0.104 m
17-Clearance between Lower & Upper Cavities	0
18-Clearance between Upper Cavity & Upper Edge	0
19-Rotation Number of Impeller(rpm)	100/300/500
20-Number of Symmetry (n：1/n model)	2

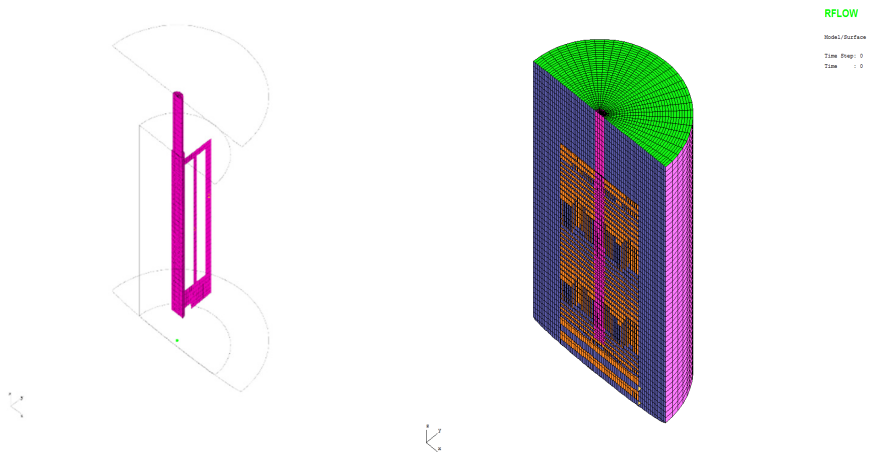


Fig. 18 Mesh generation and design of MB impeller in stirred fermenter.

(c) Details of input data for FZ impeller generation used in simulation

Total mesh number in stirred tank containing FZ impeller; 106200

Table 5 Geometrical design of FZ impeller used in this study.

Parameter	Value
Height of tank	0.245 m
Height of liquid surface	0.20145 m
Depth of tank	0.005 m
Diameter of tank	0.118 m
Lower end position of shaft	0.003 m
Diameter of shaft	0.008 m
Clearance between lower blade and upper blade	0.012 m
Height of lower blade	0.06 m
Outer diameter of lower blade	0.06 m
Inner diameter of lower blade	0.012 m
Clearance between lower and upper blade	0.017 m
Height of upper blade	0.06 m
Outer diameter of upper blade	0.055 m
Inner diameter of upper blade	0.04 m
Angle between lower and upper blade	45 º
Pitch angle of lower blade	45 º

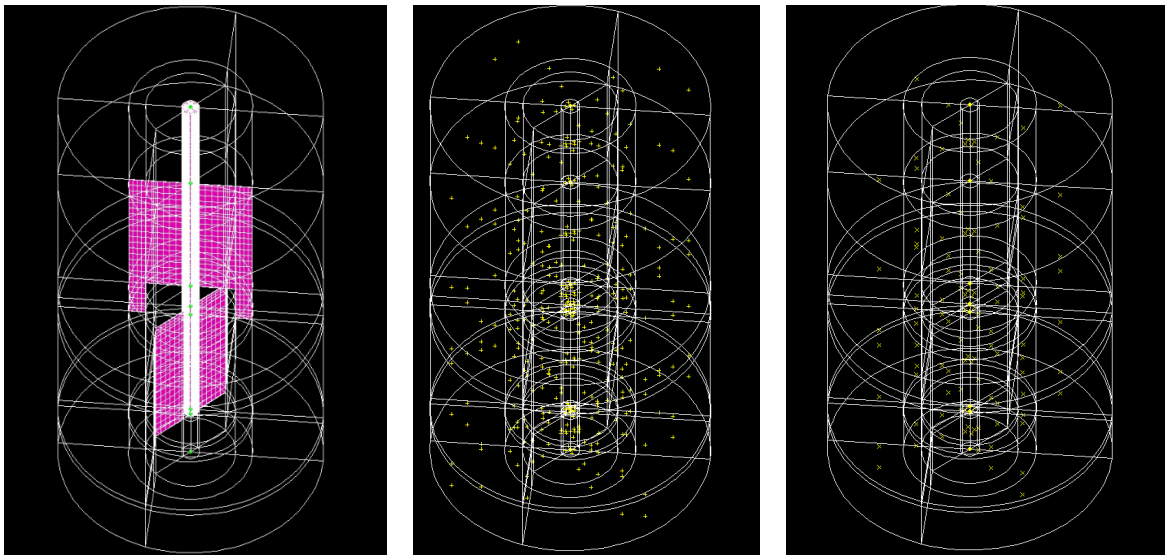


Fig. 19 Mesh generation and design of FZ impeller.

(d) Details of input data for SwingStir® generation used in simulation

Number of impeller mesh; 40320

Number of tank mesh; 204480

Table 6 Geometrical design of SwingStir® impeller used in this study.

Parameter	Value
Depth of Tank	0.005 m
Diameter of Tank	0.118 m
Radius of Tank Bottom	0 m
Lower-End Position of Shaft	-
Diameter of Shaft	0.008 m
Clearance between Blade & Tank Bottom	-
Height of Blade	0.045 m
Outer Diameter of Blade	0.003 m
Inner Diameter of Blade	0.0015 m
Diameter of Blade Inner Edge at Bottom	-
Height of Blade Inner Edge at Bottom	-
Pitch Angle of Blade(Degree)	5 º
Number of Rotation(rpm)	85/ 247 rpm

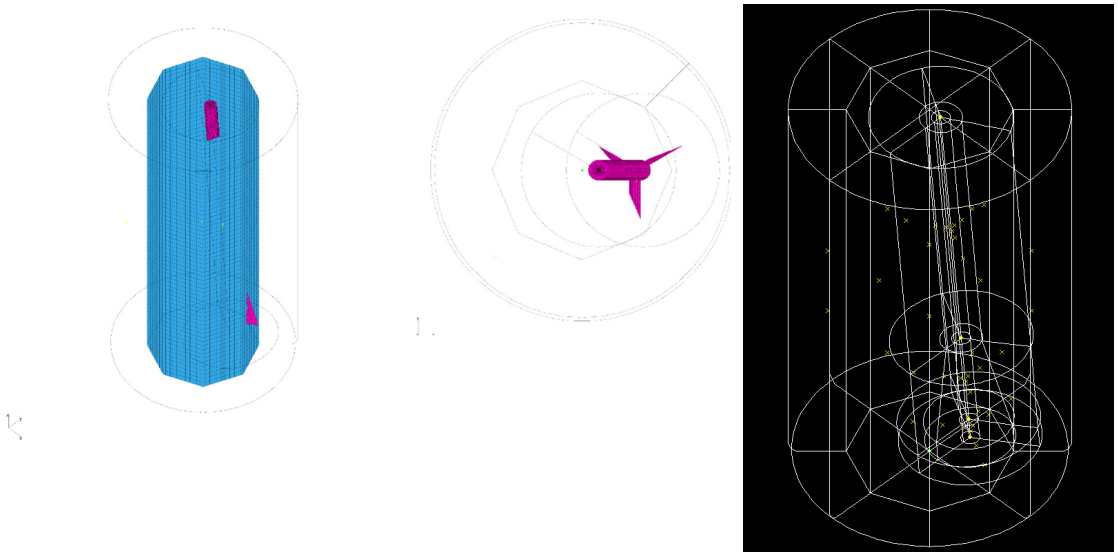


Fig. 20 Mesh generation and design of Swingstir®.

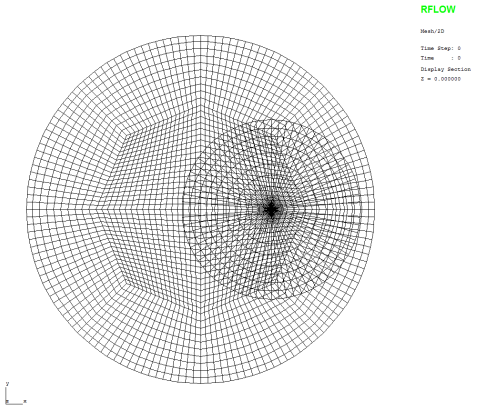


Fig. 18 Mesh generation at liquid surface of fermenter during agitation by Swingstir®.

Boundary conditions

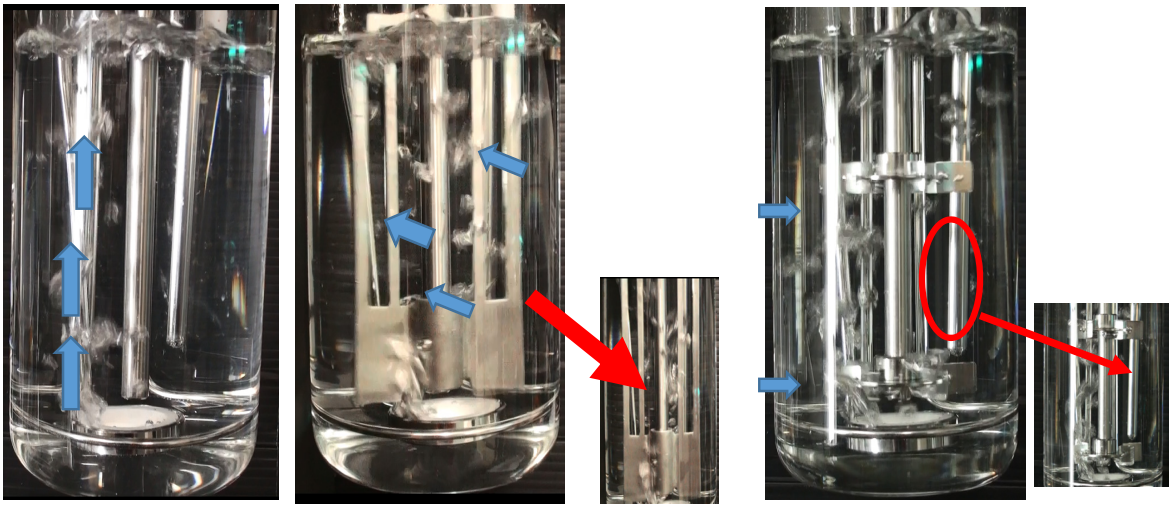
Boundary conditions of simulation were set as follows;

- In this study impeller was rotating and tank wall has been kept stationary.
- At the vessel wall and tank bottom: $V = 0$
- At the free surface $V_z = 0$
- On the impeller: $V = 50/100/300/500$ rpm

Appendix 5; Mass transfer

Effect of impeller as a secondary gas dispersion object

Visualization results of aeration (**Fig. 21**) presented that when using MB impeller, the bubbles were broken by grid parts of the impeller and MB impeller disperses the broken bubble in entire parts of the tank. However, during using DRT impeller most of the bubbles were collected near the turbine blades and there were not any bubbles in the region between the turbine impellers.



$P_v = 0$ W/m^3- bubble dispersion just along the shaft

$P_v = 406$ W/m^3- broken and uniform bubble distribution by agitation with MB impeller

$P_v = 406$ W/m^3- breaking the bubbles near the blades- no bubbles exist between the impeller and tank wall

Fig. 21 Illustration of bubble motion during agitation with impellers and aeration without impeller (aeration; 1 v.v.m, at 30 ºC, water used as a working fluid).

Superficial gas velocity measurement

Superficial gas velocity, v_g, in this study was calculated using Eq. 13. where, Q_g is gas flow rate and D is the diameter of stirred fermenter.

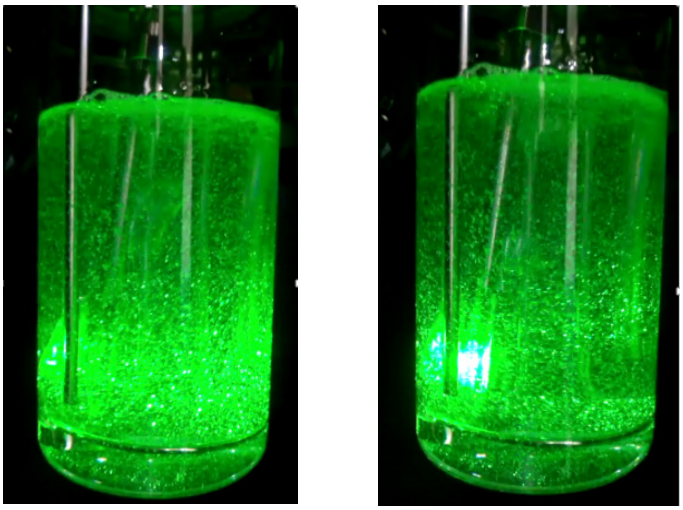
$$v_g = 4Q_g/3.14D^2 \quad (13)$$

The energy dissipation rate was calculated using Eq. (14). P, V_t, and ρ_f are power consumption, total liquid volume, and density of fermentation liquid.

$$\varepsilon = P/(V_t . \rho_f) \quad (14)$$

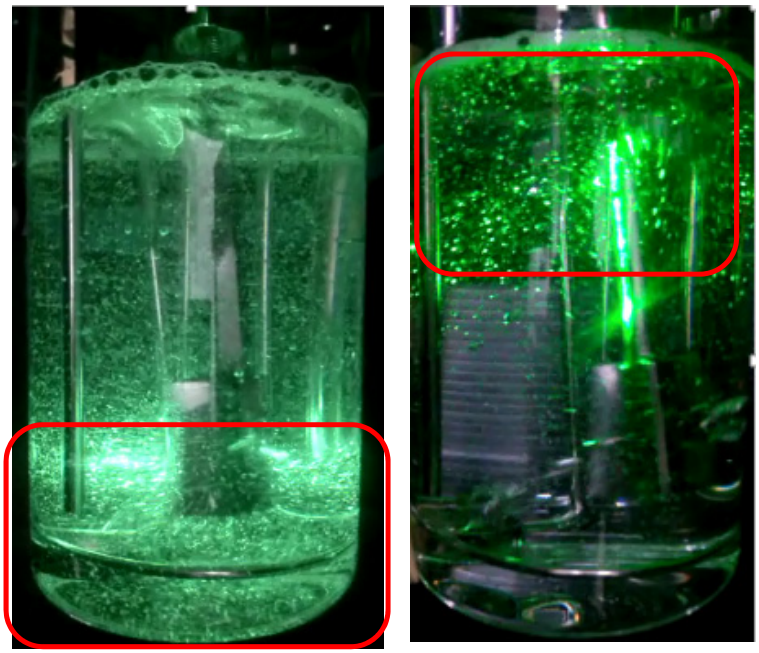
Flow pattern of gas-liquid mixing using glycerol (Newtonian fluid using Swingstir®, impeller by laser sheet method

Before measuring the mixing time for a given process, it is necessary to perform a number of flow visualization studies. The simplest technique for examining flow patterns in a mixing system is light sheet visualization. A narrow light sheet is shown through the mixing vessel, illuminating reflective tracer particles in the bulk fluid. Streak photography can be used in any transparent laboratory mixing vessel, and the light sheet can be either vertical, to provide axial or radial flow information. Here, the visualization was done at the volumetric power consumption same as fermentation condition to investigate how is the flow pattern of gas-liquid flow during the first 20~24 h from the fermentation when the cell concentration was near zero and liquid medium was a Newtonian fluid (glycerol). Investigation on the pattern of bubble dispersion showed (**Fig. 22**) bubble dispersion at the top, middle and bottom of stirred tank using Swingstir® were relatively homogenous in both low and moderate P_vs.



P_v = 150 Wm^{-3}, N =1.83s^{-1} P_v = 690 Wm^{-3}, N =3.83s^{-1}

Fig. 22 Gas- liquid pattern of mixing using Newtonian fluid agitated by Swingstir®.

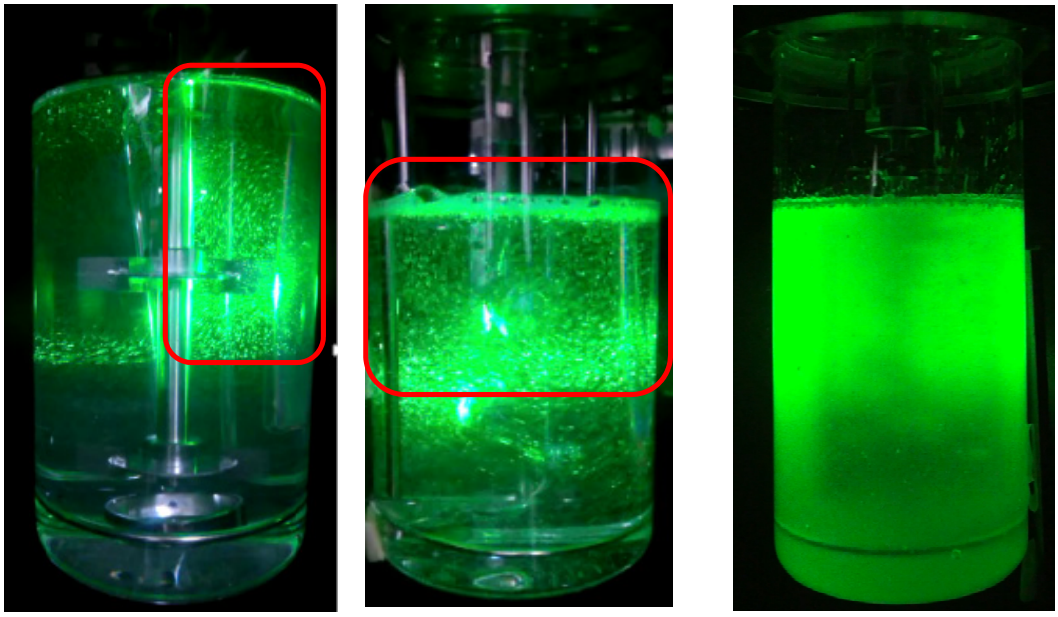


P_v = 690 Wm^{-3}, N =3.83s^{-1} P_v = 690 Wm^{-3}, N =3.83s^{-1}

Fig. 23 Gas- liquid pattern of mixing in bottom and upper side of stirred fermenter using Newtonian fluid agitated by Swingstir®.

Results of **Fig. 23** and **24** indicate the bubble dispersion using Swingstir® at the top, middle, and bottom of the stirred tank were uniform. However, when using DRT the bubble

accumulation and dispersion around the upper turbine blade was locally more than that of the other region of tank. Investigation on the results of **Fig. 25** showed bubble dispersion during mixing by MB and FZ (at the same P_v/Re) were relatively uniform in whole tank.



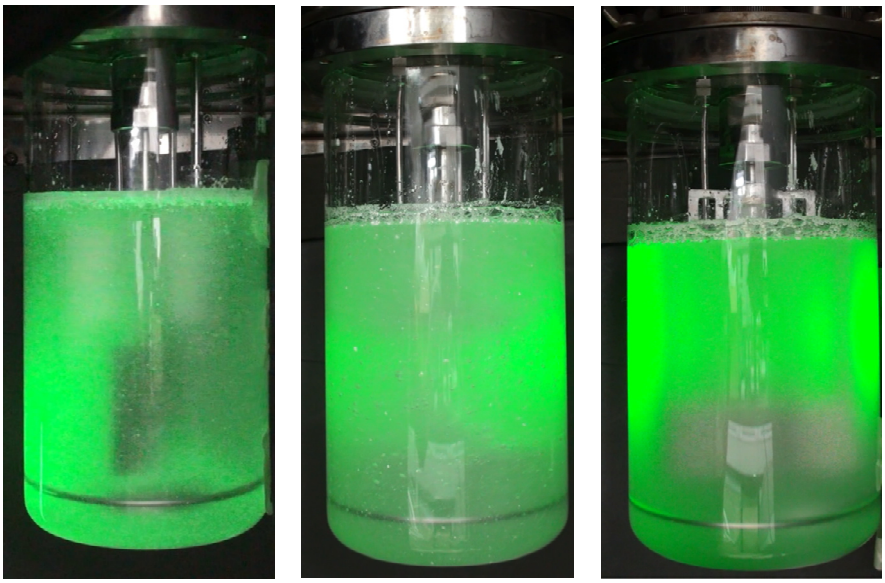
DRT, $P_v = 690\ Wm^{-3}$, $N = 3.08 s^{-1}$

Swingstir®, $P_v = 690\ Wm^{-3}$, $N = 3.83 s^{-1}$

MB, $P_v = 690\ Wm^{-3}$, $N = 2.83 s^{-1}$

Fig. 24 Gas- liquid pattern of mixing at upper side of stirred fermenter using Newtonian fluid agitated by DRT, Swingstir® and MB impellers.

Based on the experimental investigation on the flow pattern of gas- liquid mixing in the regime flow similar to fermentation liquid (at the same P_v and relatively same Re). The local high oxygen and nutrient dispersion in the vicinity of DRT impeller (upper turbine blade) was resulted in growing and accumulation of cells near the upper blade and finally, production of rigid zone at the center of fermenter (**Fig. 26**).

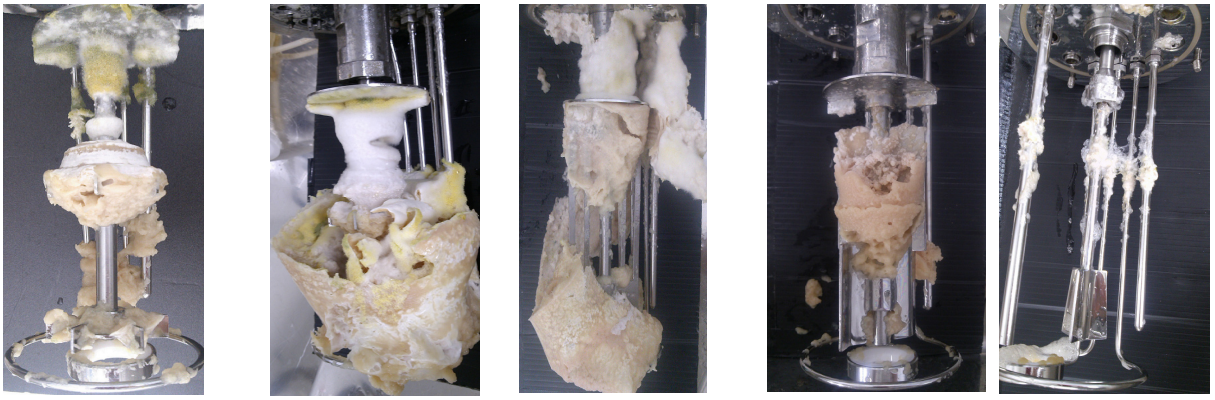


FZ, P_v = 690 Wm^{-3} , N =2.67s^{-1}

DRT, P_v = 690 Wm^{-3} , N =3.08s^{-1}

MB, P_v = 150 Wm^{-3} , N =0.95s^{-1}

Fig. 25 Gas- liquid pattern of mixing at upper side of stirred fermenter using glycerol agitated by FZ, DRT, and MB impellers.



a *DRT,* P_v = 1487 Wm^{-3}, N = 8.33 s^{-1}

b DRT, P_v = 690 Wm^{3}, N = 5.00s^{-1}

c *MB,* P_v = 690 Wm^{-3}, N = 5.00s^{-1}

d *FZ,* P_v = 690 Wm^{-3}, N = 5.00 s^{-1}

e *Swingstir®,* P_v = 690 Wm^{-3}, N = 4.11s^{-1}

Fig. 26 Effect of localized oxygen dispersion on localized stagnant zone preparation around the **(a)** and **(b)** DRT, **(c)** MB and **(d)** FZ impeller; no stagnant zone and localized cell growth using **(e)** Swingstir® impeller (all the experiments were done at the same P_v).

Accumulation of cells near the MB blade (**Fig. 26**) has two reasons; first; breaking the air bubble by grid part of blade would be resulted in increasing the surface area of bubble and finally mass transfer between bubble-liquid, and the second; is the surface area of blade. The

blade with high surface area (such as MB) has a potential of cell adherence to the blade and finally, leaded to unwanted growing the cells on the impeller blade. However unwanted cell adherence when using Swingstir® was not seen. Besides, in the case of using FZ the wasted biomass was lower than that of mixing using DRT and MB. It might be due to the low oxygen and nutrient gradient during agitation with FZ impeller.

Appendix 6; Shear rate

Combining the equations of P-N_p, energy dissipation rate versus power consumption, and $\dot{\gamma}_{ave}$ versus k_s, and by considering to the relation between liquid volume, tank diameter and height of liquid the dependency of $\dot{\gamma}_{ave}$ on energy dissipation rate and non-dimensional power number has been shown in **Table 7**. From the results of **Table 7**, it could be concluded that at relatively same ε and N_p the $\dot{\gamma}_{ave}$ of MB was higher than that of the DRT.

Table 7 Dependency of $\dot{\gamma}_{ave}$ to energy dissipation rate and non-dimensional power number using DRT and MB impellers.

Impeller	$\dot{\gamma}_{ave} = f(\varepsilon, N_p)$
DRT	$\dot{\gamma}_{ave} = 173.19\epsilon^{0.33}N_p^{-.33}$
MB	$\dot{\gamma}_{ave} = 261.60\epsilon^{0.33}N_p^{-.33}$

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