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INVESTIGATION ON THE SCALE-UP CRITERIA FOR FUNGAL FERMENTATION IN SHAKE FLASK

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ABSTRACT

To perform scale-up of fermentation in a shake flask, which the operation efficiency is equivalent to the small-scale bioreactor, many experiments are required to investigate the desired operational conditions. It was needed considerable amounts of material and time. Therefore, in this study, during fermentation of *Aspergillus oryzae* using in shake flasks with different sizes the efficient hydrodynamic parameter for changing the scale by keeping the productivity has been obtained. Finally, results show the same shaking frequency is the most appropriate scale-up criteria that is resulted in a high enzyme activity and biomass by controlling the cell morphology parameters.

KEYWORDS: fermentation, fungi, scale-up, shake flask

I. INTRODUCTION

Basically, 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 in a submerged culture (as a complex three-phase biological fluid) because most of these parameters cannot be predicted through computational fluid dynamic. Down-scale bioreactors have mostly been used for initial stage of research and traditionally have been used for preliminary experiments [1,2]. To scale-up a fermenter which is the fermentation characteristics and enzyme activity of cells equivalent to the small-scale bioreactor, many experiments are required to investigate the desired culture component and operational conditions. It was needed a significant cost, considerable amounts of material and extensive time. A scalable fermenter would require fewer cells and less labor than that of the large-scale systems. Determining optimum operating condition at production scale is expensive and time consuming therefore it is important to know whether a particular process will work correctly before it is employed in full size. In this study, fermentation of *Aspergillus oryzae* in shake flasks with different scale were used to investigate which hydrodynamic parameter could be efficient for changing the scale by keeping the productivity. The alpha-amylase activity, dry cell weight, and macro/micro-morphology of fungal cells at various operational shaking conditions have been compared with small scale results. Finally, among different criteria the best one for scale-up has been decided. Results show fermentation at the same shaking frequency is the most appropriate scale-up criteria that would be resulted in the highest enzyme activity and biomass.

II. MATERIALS AND METHODS

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 ^{o}C. 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} spore 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.

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Fermentation experiments and fermenter configuration

Here a 100 mL shaking flask with a filling volume of V_L = 20 mL was used on an orbital shaker (Labshaker, Kühner AG, Switzerland) with a shaking diameter of d_o = 5 cm and a shaking frequency of n = 200 l/min at 30 ˚C. After 4 days the main cultures were inoculated 1% from the main pre-culture. For the main culture, temperature (T = 30 ˚C), were kept constant, the other parameters in each flask were shown in Table 1.

The maximum local energy dissipation rate (ε_{max}) is generally considered as a good parameter for quantifying hydro-mechanical stress [1]. The experimentally determined ratio of maximum to average energy dissipation rate in shake flasks ranges from 1 to 7 and is therefore more than one order of magnitude lower compared to the range for stirred tank reactors (30–100) [1]. As was shown in Table 1, a constant local energy dissipation rate can be used at the scale-up criterion for stress-sensitive systems such as filamentous microorganism.

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.

Table 1. Properties of each fermentation experiment study.

Flask number	Liquid volume (L)	Re_{in}	P/V (W/m^3)	N (rpm)	Condition	Ne'	P (W)	Maximum energy dissipation (ε_{max}) (W/kg)
1	100 (small-scale)	5434	313	200	-	0.422	0.0313	0.020
2	500	11823	402.0	200	Same *N*	0.330	0.201	0.131
3	500	5434	49.9	92	Same *Re*	0.422	0.025	0.016
4	500	25233	318	190	Same *P/V*	0.302	0.159	0.104

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.

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.

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. 1-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. 1.

$$D_p = \frac{1}{n}\sum d_i \quad (1)$$

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

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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. 1-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.

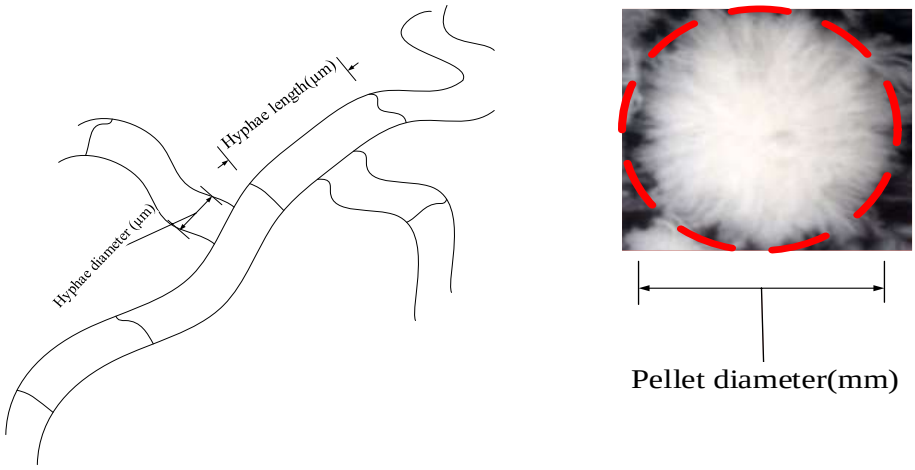

Fig. 1. Illustration of (a) micro- and (b) macro-morphology parameters measured in this study.

Measurement the hydrodynamic properties of shaking fermentation

The *Re* for shake flask is defined as;

$$Re_{\text{in}} = \frac{\rho n d^2}{\mu} \tag{2}$$

Where, ρ , n, and μ are the fluid density, shaking frequency, shaking diameter and viscosity of fluid in the flask. The dimensionless power of shake flasks and power consumption was measured according to Eqs. (3) and (4) [1,5,6].

$$Ne' = 70Re^{-1} + 25Re^{-0.6} + 1.5Re^{-0.2} \tag{3}$$

$$Ne' = \frac{P}{\rho n^3 d^4 V_L^{1/3}} \tag{4}$$

Definition of flow regime in shaken flask

A critical *Re* of *Re* = 60 000 was proposed for the transition from laminar to turbulent flow in un-baffled shake flask and the following method for calculating maximum energy dissipation ε_{max} was described [1]. In this study, all four fermentation conditions were done at laminar condition (*Re* < 60000).

$Re < 60000$ $\quad \varepsilon_{max} = P/(V.\rho)$ (5)

$Re > 60000$ $\quad \varepsilon_{max} = 0.1\,(\pi . n . d)^3/h$ (6)

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).

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 HAAKETM 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 [7]. In the present study cell adherence and some problems

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with dense pellet cells caused unwanted fluctuations while working with the rheometer (HAAKETM 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 model having high regression coefficients. 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.

III. RESULTS AND DISCUSSIONS

During cell growth in fermentation process the DCW were measured when four different constant scale-up criteria employed during fermentation and the results were presented in Fig. 2. Results of DCW indicates that the biomass production at the same *Re* condition was relatively same as the shaking at the same shake frequency. In this case, it is important to investigate the glucose consumption at the same DCW to confirmed which criteria is the most appropriate parameter for scale-up.

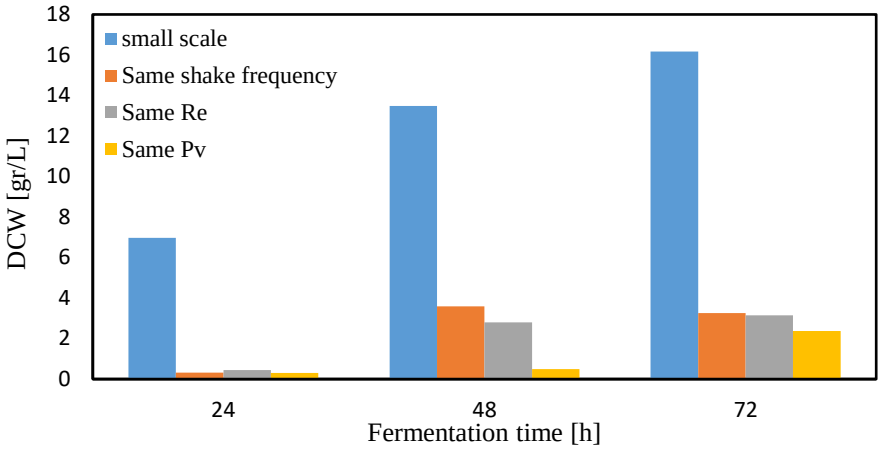

Fig. 2. Effect of different scale-up criteria on the dry cell weight (DCW), during fermentation of A.oryzae in shake flasks (The average error for the calculated values of DCW was up to 5%).

Due to this reason, the glucose concentration in fermentation culture after each sampling have been measured. The results were presented in Fig. 3. The glucose concentration at the same shake frequency condition was lower than that of the same P_v and same *Re* in most of the sampling times and this finding was in agreement with high enzyme activity in the condition with the same shake frequency (Fig. 4). In addition, the results show that fermentation at the same shake frequency by consuming less sugar than the condition of same *Re*, the DCW production could be performed in convenient condition.

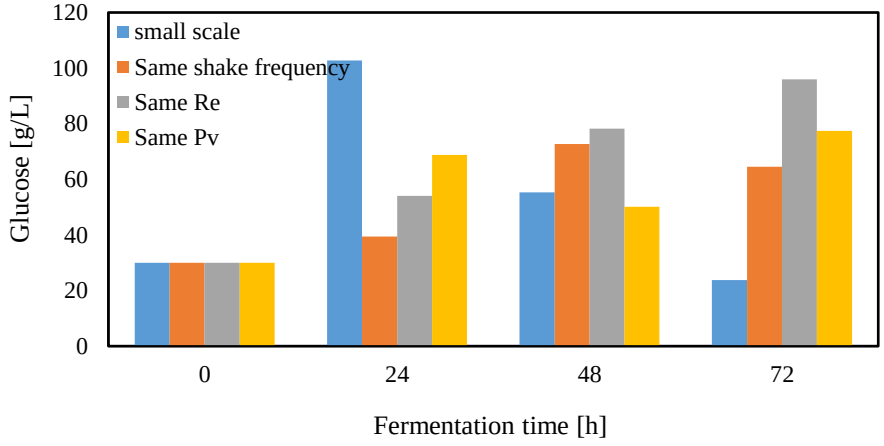

Fig. 3. Effect of different scale-up criteria on the glucose consumption during fermentation of A.oryzae in shake flasks.

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Results of enzyme activity in the condition with the same shake frequency indicate the importance of increasing the oxygen supply by increasing the shake frequency comparing with the shake frequency in the fermentation by the two other constant criteria.

According to the literature [8,9], 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 weather 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 complicated.

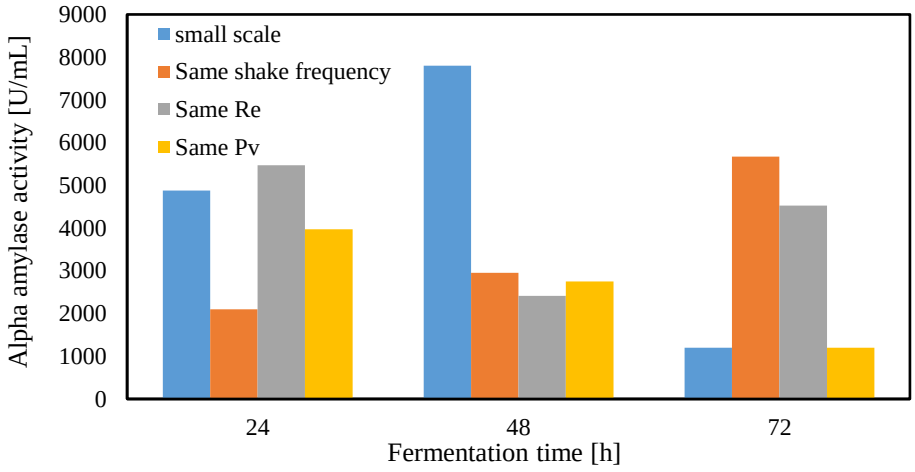

Fig. 4. Effect of different scale-up criteria on the alpha amylase activity, during fermentation of A.oryzae in shake flask (The average error for the calculated values of DCW was up to 35%).

In this research, results of pellet diameter measurement (Fig. 5) showed that except the fermentation at the constant Re, fermentation experiments at different scale-up criteria did not have any significant effect on the macro-morphology (pellet diameter) of fungal cells. By increasing the pellet diameter at this condition (Fig. 6), the oxygen penetration into the hairy and core zones of pellets are very difficult.

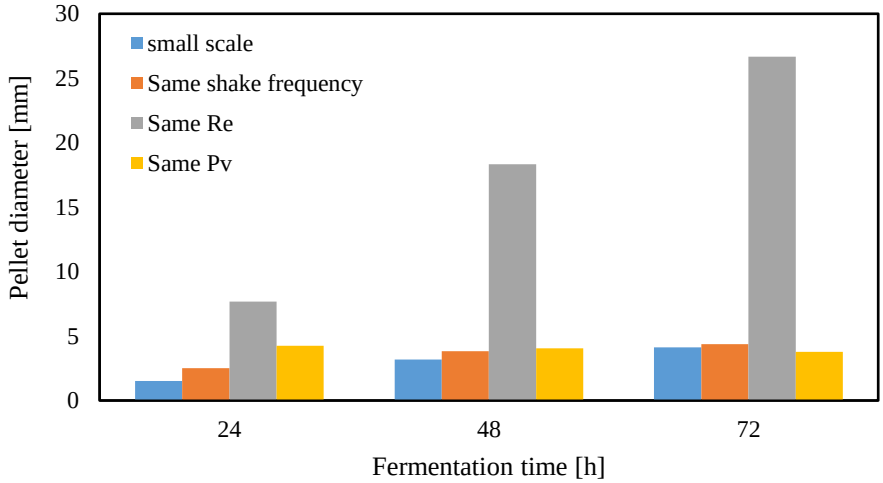

Fig. 5. Effect of different scale-up criteria on the pellet diameter (macro-morphology), during fermentation of A.oryzae in shake flasks (The average error for the calculated values of DCW was up to 3%).

Results of micro-morphology presented (Fig. 7) that by increasing the scale of shake flask hyphae diameter in the whole of the scale-up criteria have been increased. Therefore, it could be concluded that during the fermentation of fungal cells micro-morphology is a dependent parameter upon the scale. However, investigation on the results

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of hyphae length showed that hyphal length (Fig. 8) did not have a clear dependency on the scale of flask or scale-up criteria.

It is mentioned that some information about the morphology characterization could not be achieved using the down scale experiment. The other issue which should be investigated in future for choosing the best scale-up parameter is shear stress exposed to the cells. Due to this reason, results of current research combining with future study on the effect of each scale-up criteria on formation of shear stress in fermentation culture could be useful for scale-up of fermentation using shaking method.

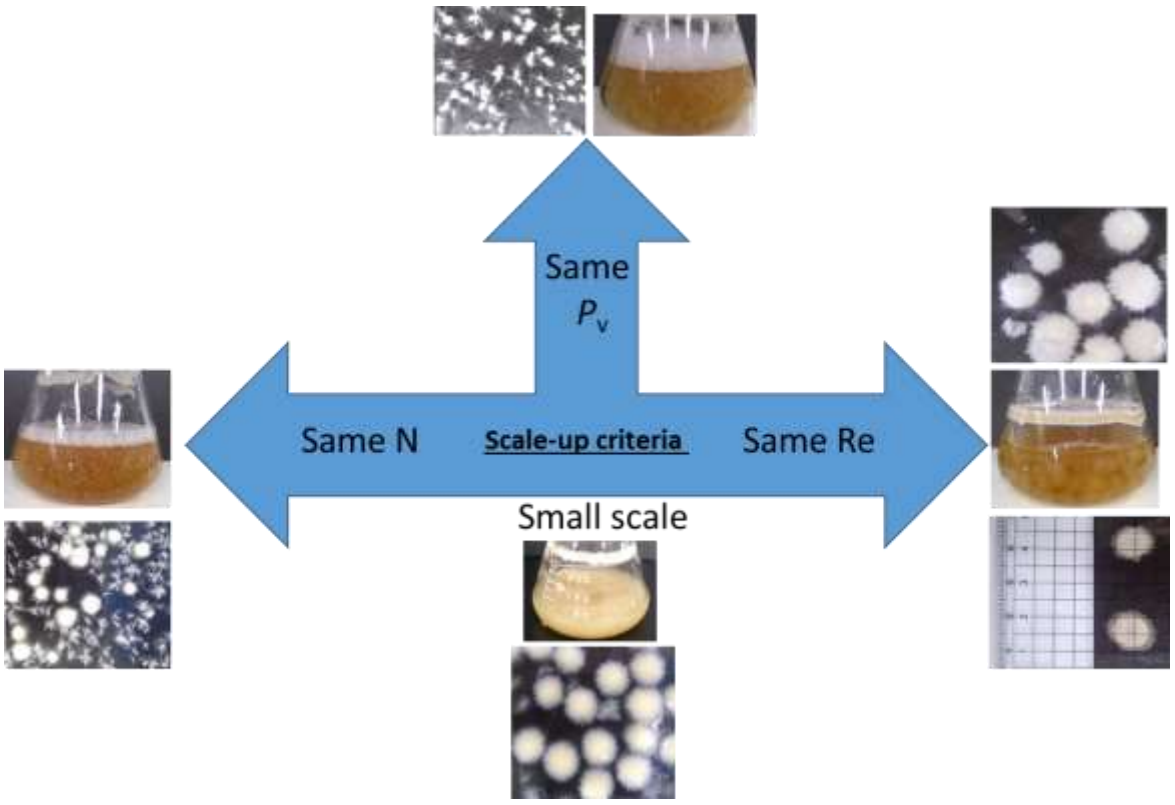

Fig. 6. Visualization of pellet morphology during fermentation at different scale-up criteria.

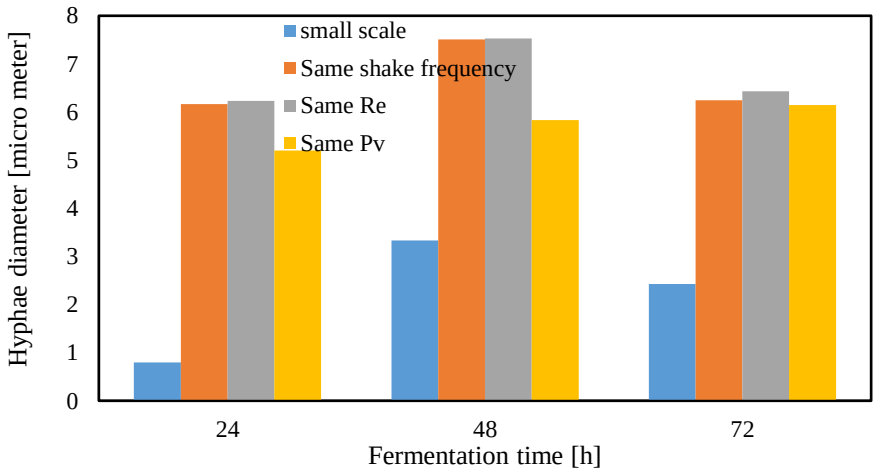

Fig. 7. Effect of different scale-up criteria on the hyphae diameter (micro-morphology), during fermentation of A.oryzae in shake flask (The average error for the calculated values of DCW was up to 2%).

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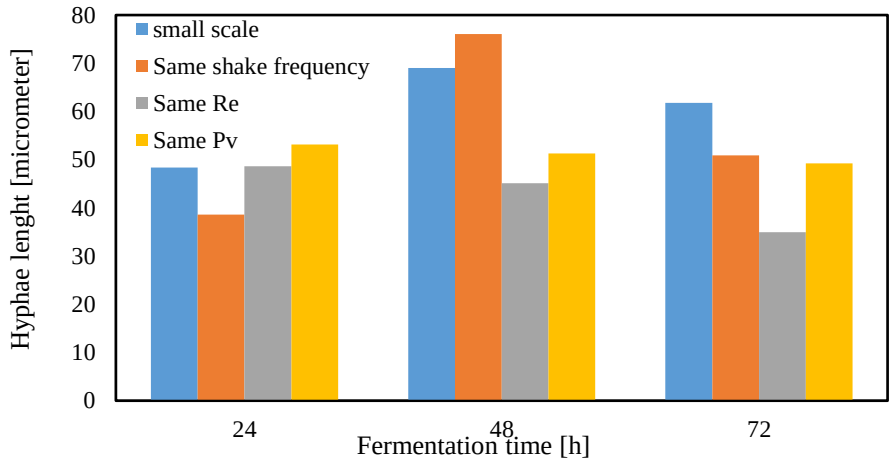

Fig. 8. Effect of different scale-up criteria on the hyphae length (micro-morphology), during fermentation of A.oryzae in shaken flask (The average error for the calculated values of DCW was up to 3.2%).

IV. CONCLUSION

In this paper, scale-up of fermentation of *A.oryzae* using Erlenmeyer flask was resulted in the highest enzyme activity when performing the fermentation at the same shaking frequency. Among the three scale-up criteria conditions the highest dry cell biomass was belonged to the same shaking frequency condition. Pellet diameter of fermentation condition at the same *Re* was the largest, and it is a big challenge for substrate diffusion in cells. At the same shaking frequency, the hyphae elongation has been increased by increasing the fermentation time. At the same P_v condition hyphae elongation was constant during the fermentation. By increasing the maximum energy dissipation during the scale-up of fermentation at the same shaking frequency condition nutrient mass transfer, enzyme activity and biomass production were enhanced. At the same P_v condition using large flask, the remained glucose concentration was higher than that of the small scale. Final enzyme activity of small and large flask at the same P_v was the same.

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