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Cyclosporin A 생산을 위한 액체배양과 고정화배양의 생물반응기에서의 산소전달 비교 연구

전 계 택
강원대학교 자연과학대학 미생물학과

Comparative Bioreactor Studies in Terms of Oxygen Transfer between Suspended and Immobilized Fungal Systems for Cyclosporin A Fermentation

G. T. Chun
Department of Microbiology, College of Natural Sciences,
Kangwon National University, Chunchon 200-701, Korea

ABSTRACT

In fermentations with a 4-liter stirred tank bioreactor, a better than two-fold enhancement of the gas-liquid mass transfer coefficient (k_La) in the celite-immobilized fungal cultures of *Tolypocladium inflatum* over the parallel conventional free-cell was observed at identical biomass concentrations, despite the higher specific oxygen uptake rate of the immobilized fungi during exponential growth. As a result, oxygen sufficient conditions, i. e., dissolve oxygen (D. O.) concentrations exceeding 75% air saturation, could be maintained throughout exponential growth period of the immobilized culture, in contrast to the suspended fungal culture, whose D. O. levels fell below 50% air saturation. A linear monotonic dependence of k_La upon impeller agitaion rate was found for both immobilized and conventional cultivation modes over a range of 250 to 550rpm, the slope being a function of biomass concentration for the free but not for the immobilized cell system. In contrast, oxygen transfer rate was a much weaker function of aeration rate up to about 2.5 vvm for both culture configurations. Above this level, aeration rate had no further effect on the mass transfer. In addition, the immobilized cultures sustained good morphological and physiological states, leading to almost two times higher cyclosporin A (CyA) productivity over the parallel free cell system. These experiments suggest that the celite-immobilized fungal system in a stirred tank reactor has considerable promise for scaling up cyclosporin A production in terms of high-density cultivation.

INTRODUCTION

Cyclosporins are cyclic peptide antibiotics composed of eleven amino acids produced as secondary metabolites by strains of the filamentous fungus, *Tolypocladium inflatum* (1, 2). Due to its remarkable selective immunosuppressive effects, cyclosporin A (also spelled cyclosporine, ciclosporin and abbreviated as CyA in this article) has been the focus of great interest in recent

years. CyA is very effective in organ transplants as a key agent used to prevent foreign tissue rejection, and has considerable promise in the control of autoimmune and parasitic diseases (3). The non-ribosomal synthesis of CyA *via* an enzyme thiotemplate mechanism was proposed (4), and this hypothesis was subsequently supported experimentally by synthesizing CyA and other analogs *in vitro* (5, 6, 7). The directed biosynthesis of CyA and analogs *in vivo* has also been demonstrated (2, 8, 9) and, like its *in vitro* counterpart (7), it is compatible with the low substrate specificity of peptide antibiotic biosynthetic enzymes (synthetases) (10, 11). Very recently, detailed characterization of cyclosporin synthetase, a single multifunctional enzyme, has been described by purifying the enzyme to near-homogeneity, and it was confirmed that cyclosporin synthetase follows a thiotemplate mechanism (12). This has also been observed previously for the biosynthesis of other peptide antibiotics (13).

In the cultures of mycelial organisms such as fungi, an immobilized cell system has several additional advantages compared with a conventional suspended culture system. A key feature of the growth of these mycelial organisms lies in the characteristic highly branched hyphal filaments. This pattern of cell growth results in high viscosities, thus leading to decreased mass transfer capacity in conventional suspended culture. The whole fungal culture fluid may be induced to assume a rheology similar to that of a mycelial pellet system by confining the mycelial cells into porous biosupport materials, such as celite (14, 15), thus allowing a switch from extended mycelial growth to particulate growth. This can be used to control cell morphology and broth rheology, thus leading to increased mass transfer capacity primarily arising from lower viscosity of the immobilized cell broth.

Despite the numerous clinical and basic immunological studies of CyA and its analogs (3, 16, 17, 18), there is still little published information on the basic biology and process engineering of this secondary metabolic bioprocess (1, 2, 5, 7, 12). In our laboratory, CyA has been sucessfully produced using immobilized cells of *T. inflatum* entrapped into celite beads which have been found to be very compatible for physical entrapment of the mycelial cells, and their considerable potential for optimal production of CyA has been demonstrated (19, 20). Because of the complexities of secondary metabolic pathways and largely unknown cellular response of immobilized cells, from their confined microenvironments, it was suggested that the intrinsic properties of immobilized biocatalysts should be carefully characterized separately from the corresponding free cell system (21).

In this study we compared bioreactor kinetics and oxygen transfer in parallel suspended and immobilized fungal systems for CyA production. Special emphasis was placed on the analysis of oxygen uptake and the gas-liquid mass transfer coefficient (k_La) assessed during these comparative fermentations. The dependency of the mass transfer coefficient on cell growth and morphology was illustrated in order to characterize the detrimental effects of filamentous mycelial morphology on the gas-liquid mass transfer capabilities.

MATERIALS AND METHODS

Microorganism and Inoculum Development

Tolypocladium inflatum ATCC 34921, indicated as *Beauveria nivea* in the American Type Culture Collection Catalog of Fungi/Yeasts (22) was initially obtained from ATCC and subsequently adapted to growth on glucose (23). The seed cultures for suspended cell fermentations were prepared exactly in the same manner, as described in the previous paper (20), and inoculated into 3 liters of sterilized synthetic medium contained in the fermentor. The methodology of inoculum development for the immobilized cell system was essentially the same as previously depicted by Chun and Agathos (20).

Cell Immobilization Methodology

Celite was selected as a suitable bead matrix

for immobilization of CyA-producing *T. inflatum*. The immobilization procedure developed by Gbewonyo and Wang (14) with cells of *P. chrysogenum* was adapted to our mycelial cells with a number of modifications. The matrix used for immobilization of conidiospores was celite grade 560 (Manville Corporation) made up of beads ranging from 150 μm to 207 μm. The celite beads were pretreated by washing with distilled water several times and heated in a furnace overnight at 600℃ to remove volatile materials. The particles were then steamautoclaved for one hour and allowed to dry at 121℃ for 30minutes. The prepared spore suspension was added by use of a peristaltic pump to the dry celite beads in twice its volume of packed celite (50% v/v). The inoculated spores were adsorbed and immobilized into celite beads at 27℃ under the agitation rate of 300rpm and aeration rate of 1vvm. After two hours of incubation, the supernatant was decanted and the celite beads were washed with sterile distilled water to remove unentrapped spores. Finally the modified synthetic medium (see below) that had been autoclaved at 121℃ for 30minutes was added aseptically to the immobilized celite beads contained in the reactor system and the inoculated beads were incubated at 27℃ inside the reactor for growth and CyA production. The pH of the production medium was initially adjusted to 5.5.

Media and Growth Conditions

A modified SM medium was utilized, which has the same composition as the previously formulated SM medium (23) except that 8g/ℓ of L-valine was used as a sole nitrogen source instead of ammonium sulfate (10g/ℓ). Approximately 7.5times intensified SM medium was supplemented at hour 139 of fermentation to each of the two systems, which were operated batchwise until the pulse time. The added concentrated SM medium (200ml) was composed of: glucose 300g/ℓ, L-valine 45g/ℓ, $MgSO_4$ 3.75g/ℓ, $CaCl_2$ 0.75g/ℓ, KH_2PO_4 5.625g/ℓ and 0.75% v/v trace metal solution (23).

The batch fermentations were carried out in a 4-liter mechanically stirred bioreactor (Model MF-200 SPL, New Brunswick Scientific, Edison, NJ) for both immobilized and freely suspended cells. The working volume was 3 liters. The main characteristics of the fermentor are as follows: type of impeller, turbine; number of impellers, 2; number of blades, 6; liquid height, 21cm; tank diameter, 14cm; impeller diameter, 7.5cm; type of sparger, *ca* 0.5mm single hole; number of baffles, 4 of 1.8cm width. Agitation rate of 300rpm was utilized for the two systems. It was found that 300rpm was the minimum agitation rate for preventing a dead zone due to accumulation of the biosupport particles at the bottom of the fermentor by employing two 6-blade turbine impellers. The pH was controlled at around 5.5 using H_2SO_4 (2N) and KOH (2N). The air flow rate was set at 3.0L/min (1vvm). The temperature of each fermentation was controlled at 27℃ ± 1℃. The foaming problem was solved by automatic addition of antifoam SAG 471 (which was kindly provided by Schering Plough, Inc., Union, NJ) *via* the fermentor's antifoam controller.

Analytical Methods

Analysis of cyclosporin A was carried out by use of HPLC as previously described by Chun and Agathos (20).

Biomass

The cell concentration in the fermentation broth was determined by the dry cell weight technique for both freely suspended and immobilized cultures.

For the free cell cultures, approximately 30ml of whole broth sample was obtained every sampling time from the fermentor, and homogenized. From the homogenized fermentation broth, one or two 10ml of sample(s) were taken for cell mass determination (the other 10ml of the homogenized sample was used for CyA analysis). The samples were centrifuged for 5minutes at 15000rpm three times and dried at 85℃ for 24hours.

For the immobilized cell cultures, cell homogenization was not necessary. 10ml samples composed of culture fluid and solids (cells and beads) were taken. After each of five consecutive centrifugations for 5minutes at 15000rpm, the samples were washed with distilled water to completely remove the residual nutrients from the deep inside of the celite particles. The washed solids were dried at 85℃ for 24hours to obtain the dry weight of dry cells plus celite particles. After heating the solids in a furnace at 600℃ for 5hours, the weight of celite particles alone was obtained. The dry cell concentration of each sample was calculated by deducting the weight of the celite particles from the weight of the 85℃-dried solids. Finally the amount of cell growth obtained in the immobilized cultures was determined by averaging the measured dry cell mass of the samples.

Oxygen Uptake Rate (*OUR*) and Gas-liquid Oxygen Transfer Rate Coefficient(k_La)

The oxygen uptake rate and gas-liquid mass transfer coefficient, k_La, were measured by use of a dynamic method as described in detail in the textbook (24).

RESULTS AND DISCUSSION

Enhanced Oxygen Mass Transfer in Immobilized Cell Cultures

It is well known that the solubility of oxygen in aqueous solution under 1atm of air is extremely low, of the order of 10parts per million (ppm) near ambient temperature, resulting in only a small reservoir in solution available for the cells. In particular, gas-liquid oxygen mass transfer rates have been shown to become significantly diminished during mycelial growth in submerged cultures, due to the three-dimensional structure of the mycelial organisms imparting very high non-Newtonian viscosities to the fermentation broth. Among all the different factors that affect the rate of oxygen mass transfer, temperature, pressure and fluid rheology are the most important as physical factors. In this paper, we emphasized particularly the effects of rheological properties of the fermentation broth on the oxygenation.

In order to accurately investigate the effects of *OUR* (oxygen uptake rate) and *OTR* (oxygen transfer rate) on the CyA fermentation, operating conditions such as agitation rate and aeration rate were chosen carefully so that the dissolved oxygen level of the fermentation broth remains above the critical D. O. level, and thus oxygen does not act as a growth-limiting nutrient. These conditions were necessary because it has been previously reported that the measured *OUR* is significantly influenced by the D. O. level of the fermentation broth, especially when the D. O. level is around critical D. O. level (15, 24). Actually, it was found that the rate of oxygen uptake by the cells of *Penicillium chrysogenum* was strongly limited by low D. O. concentration (15). In our own experimental system, the agitation rate of 300rpm at an aeration rate of 1vvm was found by trial and error to be suitable to this end (*i. e.*, no oxygen-limitation during the whole period of fermentation in both immobilized- and free-cell systems). Notably, in our system, by setting the agitation speed and aeration rate at the respective value above, we were able to obtain almost same profiles of cell growth (Fig. 1) and essential carbon-source consumption (data not shown) in the immobilized and free cell systems, thus indicating that *OUR* and *OTR* can be compared more accurately between the two systems under these physiologically similar conditions.

The most interesting result in this pulse experiment is the higher D. O. level observed in the immobilized cell system in comparison with the free cell cultures performed under the identical operational (300rpm and 1vvm) and nutritional conditions during the whole period of exponential growth phase (Fig. 2). The concentration of dissolved oxygen in the free cell system fell down almost to 50% of air saturation level during the course of fermentation, whereas in the immobilized cell cultures the minimum value of D. O.

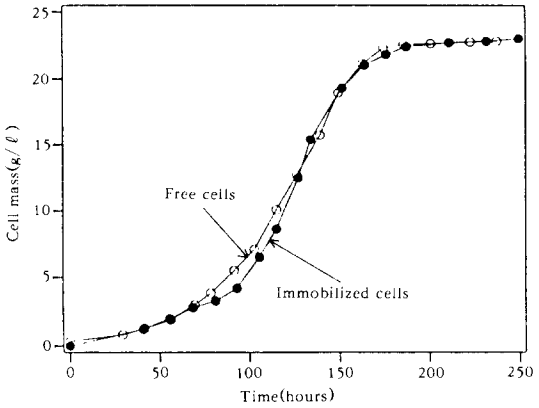

Fig 1. Comparison of cell growth as a function of fermentation time between immobilized and free cell systems in a 4ℓ stirred tank bioreactor. (27℃, 300rpm, 3ℓ working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM). Immobilized system(●):50% colonized beads (v/v), additional pulse feeding at 135hr; Free cell system (○):5% mycelial inoculum, additional pulse feeding at 139hr.

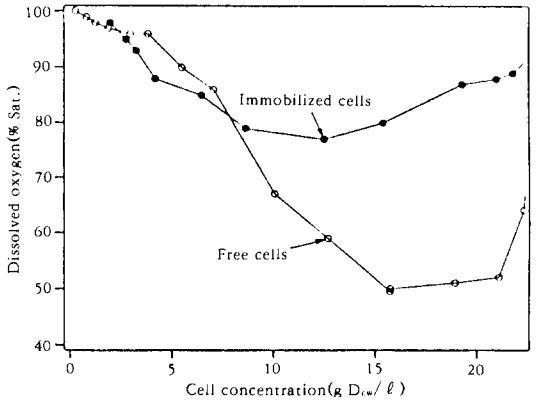

Fig 2. Comparison of dissolved oxygen profile as a function of cell concentration between immobilized and free cell systems in a 4ℓ stirred tank bioreactor. (27℃, 300rpm, 3ℓ working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM). Immobilized system (●):50% colonized beads (v/v), additional pulse feeding at 135 hr; Free cell system (○):5% mycelial inoculum, additional pulse feeding at 139hr.

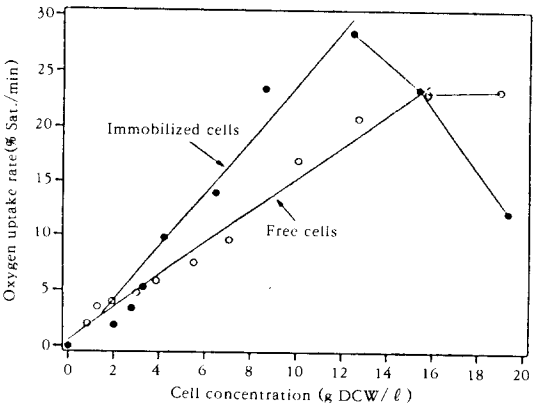

Fig 3. Comparison of oxygen uptake rate as a function of cell growth between immobilized and free cell systems in a 4ℓ stirred tank bioreactor (27℃, 300rpm, 3ℓ working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM). Immobilized system (●):50 % colonized beads (v/v), additional pulse feeding at 135hr; Free cell system (○):5% mycelial inoculum, additional pulse feeding at 139hr.

reached was as high as 80% of air saturation level. This result is quite interesting because the oxygen uptake rates of the immobilized cells were maintained higher than those of the parallel free cells during this period (Fig. 3). In Fig. 3, it should be noted that the slope of the fairly linear line denotes the average specific oxygen uptake rates under these operating conditions, demonstrating more active cellular metabolism of the immobilized cells in comparison with the freely suspended cells (approximately 66% higher specific *OUR* than that of the free cells during the exponential phase of cell growth). The possible cause for the deviation of the data from linearity can be explained on the following basis, *i. e.*, the observed increase in dry weight during the late exponential and stagnant phases is frequently due to the cellular production and accumulation of various polymeric substances, occasionally in large amounts. The restricted, stressing microenvironment of compressed growth into the inter-

stitial space of the celite beads may account for the high *OUR* of the immobilized cells. On the other hand, one other possible explanation for the observed active respiration rates in the immobilized cells of *T. inflatum* during the exponential phase is that the immobilized cells may be composed of actively growing young cells of almost the same generation. This can be inferred by the fact that only spores were employed as an inoculum for the immobilized cell culture. In the case of the free cell system, however, the inoculum was composed of a mixed population of mycelial cells. Hence, such behavior as active synchronous germination of the cells observed in the immobilized cell culture could not occur in the free cell system. It is worthwhile to mention here that when a pure spore inoculum was used in the free cell culture, the lag period was so long, possibly due to high shear-producing environment for the spores, that it resulted in an uneconomical bioprocess for CyA production. Based on these results, the protective environment provided by the immobilization appeared to be another cause for the active primary metabolism of the immobilized cells, which is frequently mentioned as a beneficial advantage for immobilized plant cell and mammalian cell cultures (25, 26). In addition, a few reports were published revealing that cells grew well and stably when they were immobilized, due to a strong affinity of the cells for surfaces of either organic or inorganic materials (27). Indeed it was mentioned that the adherence properties and surface colonization are important features of natural ecology in the case of filamentous fungi (28).

The decline of D. O. down to 50% of air saturation in the free cell cultivation did not appear to affect the primary metabolism of the suspended fungi as demonstrated by the almost same growth rate and final cell density as those obtained in the immobilized cell culture (Fig. 1). This can be explained by the fact that the D. O. level of 50% of air saturation was not gorwth-limiting to the freely suspended cell cultures. However, as shown in Fig. 4, different secondary

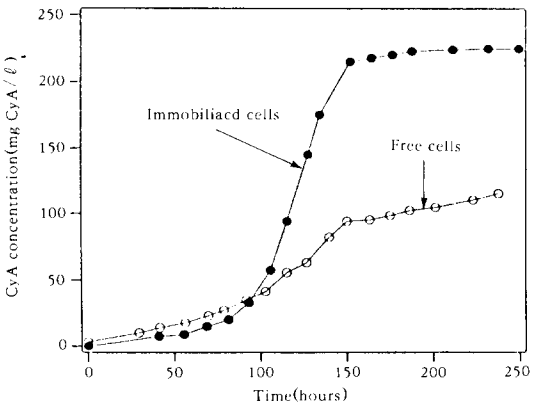

Fig. 4. Comparison of CyA production as a function of fermentation time between immobilized and free cell systems in a 4ℓ stirred tank bioreactor. (27℃, 300rpm, 3ℓ working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM). Immobilized system (●): 50 % colonized beads (v/v), additional pulse feeding at 135hr; Free cell system (○): 5$ mycelial inoculum, additional pulse feeding at 139hr.

metabolic activity was quite clear in the case of immobilized cell cultures, reaching almost two times higher maximum CyA production in comparision with the parallel free cell cultures.

Fig. 5 demonstrates that the rheology of free mycelial fermentation is strongly dependent on the development of the microorganism. During the initial period of fermentation (approximately up to 3g/ℓ of cell density), nearly Newtonian behavior of the free cell fermentation broth was visually observed, showing relatively small variation in the k_La value (ranging from 0.9/min to 1.0/min). However, during the second fermentation period when the cell density increased from 4g/ℓ to 21g/ℓ, a steep change in the rheological behavior of the fermentation broth was observed, as demonstrated by a significant reduction in k_La value from 0.9/min to less than 0.6/min in the free cell culture. In contrast, for the case of the immobilized cell culture, remarkably higher k_La values were sustained over the full range of cell

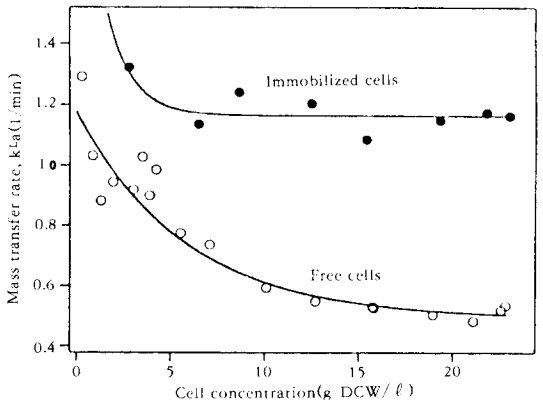

Fig 5. Comparison of gas-liquid mass transfer rate as a function of cell concentration between immobilized and free cell systems in a 4ℓ stirred tank bioreactor. (27℃, 300 rpm, 3ℓ working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM). Immobilized system (●): 50% colonized beads (v/v), additional pulse feeding at 135hr; Free cell system (○): 5% mycelial inoculum, additional pulse feeding at 139hr.

concentrations in comparison with the free cell system. Much slower decline in the k_La value was observed during the early phase, and this value leveled off to 1.2/min from around 10g/ℓ of cell concentration. This was about 2.5times higher than the value obtained at the corresponding free cell concentration. It is noteworthy that even at the highest immobilized cell concentration of 22g/ℓ, the k_La value still remained as high as 1.18/min. At the corresponding free cell concentration, the k_La value was found to be only 0.5/min (42.4% of the value for the immobilized cells). Consequently it can be concluded that these significantly higher k_La values in the immobilized cell system accounted for the observed higher D. O. levels sustained, despite more active oxygen uptake rates (Fig. 2 and Fig. 3).

It is well known that in batch growth of filamentous fungi, the mycelial suspension culture is usually fraught with many problems, due to changes in the rheology of the mycelial broth during the course of fermentation. High apparent viscosity and non-Newtonian behavior of these filamentous culture broths have long been noted as causing inefficient mixing and suboptimal oxygen mass transfer rates (29). Although the apparent viscosities of the two cultures were not measured during the progress of fermentation, significantly different flow patterns of the fermentation broths were visually observed. Thick viscous mycelial suspensions were observed in the free cell culture system, while still apparent Newtonian flow properties of the immobilized cell broth were manifested even at the relatively high cell density of 23g/ℓ. Although some released free cells were present in the broth of the confined cell culture, the culture appeared to still remain Newtonian since these released-free cells were mainly composed of shortly broken mycelial fragments or round-shaped spores as observed under the microscope. In the case of the free cell fermentation, more notably non-Newtonian behavior was observed, especially when the cell concentration was greater than 22g/ℓ. The significantly thick viscosity at this high cell density caused a stagnant zone (dead volume) at the bottom of the stirred bioreactor, potentially decreasing the productivity of the suspended microorganism. However, in the immobilized cell cultures, there were no stagnant zones even at higher cell concentrations under the same bioreactor operation conditions, clearly due to the low viscosity resulting from the confinement of the mycelial cells into the porous biosupport particles. This fact is particularly important for product enhancement since it is reported that stagnant zones in the bioreactor can easily decrease the productivity of the microorganism and cause the production of undesirable metabolites (30). On the other hand, it was found that the presence of cell-free bead particles did not affect the k_La values obtained under these conditions, as those reached in the bead-free (cell-free) liquid media at several different agitation rates (300rpm, 400rpm and 500rpm at 1vvm). This indicated that the apparently reduced viscosity of the im-

mobilized cell system appeared to be a major part of the reason for the increased k_La values (data not shown). This experiment was done by varying (cell-free) celite bead concentration in the media (*i. e.*, 10% v/v, 30% v/v and 50% v/v), and the obtained k_La values were compared with those values attained in the bead-free (cell-free) liquid media under the identical conditions.

So far, several reports have been published, classifying the culture broths of filamentous mycelial fermentations as Bingham plastic or pseudoplastic fluids (31). With regard to the detrimental effect of non-Newtonian viscosity on the mass transfer rate, Ryu and Humphrey (32) suggested the following correlation for the mass transfer capacity, by considering the effect of viscosity together with operational and process control parameters:

$$k_La \propto (P/V)^{\alpha} (V_S)^{\beta} (\eta_{app})^{\gamma}$$

where P/V is power input per volume, V_s is the superficial air velocity, η_{app} is the apparent viscosity of the medium, and α, β and γ are experimental exponent factors. These workers provided the following relationship, demonstrating logarithmic reduction in mass transfer coefficient with respect to the rise in mycelial broth viscosities:

$$k_La \propto (\eta_{app})^{-0.86}$$

For practical purposes, the potential detrimental effect of intraparticle diffusional limitation on *OUR* in the immobilized cell cultures should be mentioned at this moment. As will be theoretically explained in the subsequent paper, however, diffusional resistance did not appear to have a significant effect on *OUR* in the immobilized cell system. This can be further supported by comparing our results against the data reported by Gbewonyo and Wang (15). In their low shear-producing bubble column reactor, they observed negligible diffusion limitation in the compact mycelial microenvironment formed in the immobilized cells of *Penicillium chrysogenum* when a diameter of celite beads ranging from 300 μm to 500 μm was utilized, with a final loaded biomass of 0.3g per g celite. They identified the trivial diffusion resistance in their system by showing that the specific oxygen uptake rates for the confined cell cultures were closely identical with the values obtained with the three cell cultures when *OUR* was measured under fully oxygen-saturated medium conditions. In comparing very similar *in vitro OUR* for the free and confined cells, Wang *et al.* (33) also demonstrated that there was probably no limitation of oxygen transfer from the liquid to the mycelia on the surface or the inside of the celite beads. In their experiments, a celite particle diameter of 180-500 μm was employed and the maximum cell density obtained was more than 0.35g cells/g biosupport. By comparing our own data against these results, it could be inferred that there existed no intraparticle diffusion limitation within the beads in our highly shear-producing stirred tank reactor, since the celite beads used in our experiments were composed of small sized beads, ranging from 150 μm to 207 μm in diameter. Furthermore, the loaded maximum cell density was not as compact, but rather approximately 0.2g cell/g biocatalyst. It should be also noted that the maximum *OUR* in the immobilized cell system exhibited a 21% higher value than that of the free cell system during exponential phase, even though the *OUR* was measured and compared at different D. O. levels for each system.

Comparison of Oxygen Mass Transfer Rate as a Function of Agitation Speed, Aeration Rate and Cell Concentration in Immobilized and Free Cell Systems

The influence of physical and biological factors on k_La has been examined by investigating experimentally the effects of agitation rates and aeration rates at different cell concentrations in both systems. This research has been undertaken in order to further analyze systematically the enhanced gas-liquid mass transfer rate of oxygen in the immobilized cell system. Fig. 6 shows the influence of agitation rates on the oxygen mass transfer rates at various cell concentrations for both immobilized and suspended cell systems. The k_La values of the free cell system were seen to be

affected by cell densities, as a result of the detrimental effect of non-Newtonian rheology on the mass transfer of oxygen caused by the changes in the physical properties of the filamentous broth during the free cell fermentation. It was observed that the lower the cell density, the more sensitive became the effect of agitation rates on the oxygen gas-liquid mass transfer rate, as explained by the value of the slope for each linear curve obtained (Fig. 6). At 10.1g/ℓ of cell concentration, k_La values were increased from 0.38min^{-1} to 2.23min^{-1} for agitation rates ranging from 250rpm to 550rpm at an aeration of 1vvm, with the highest slope value (100%) among all three selected free cell samples. When the cell concentration was 15.8g/ℓ, the k_La values were observed to increase relatively slowly with agitation rates. In this case, the slope was determined to be 89% for the linear curve obtained from the plot of k_La vs. agitation rate. The slowest increase in k_La with agitation rates within the same range was revealed when the cell concentration reached 22.6g/ℓ, implying a lower efficiency of agitation in increasing the gas-liquid mass transfer rate at the higher cell densities. This was indicated by the lowest value of the slope, approximately 75%, among the linear correlated values of k_La vs. rpm for the free cell fermentations.

In contrast, in the immobilized cell system, the extent of the increase in k_La was not significantly affected by the immobilized biomass concentration, thus indicating the homogeneous Newtonian viscosities of the immobilized cell fermentation broth. At the immobilized cell concentrations between 15.4g/ℓ and 21.8g/ℓ, the k_La values were found to overlap with each other, and, on average, an increase from 0.58min^{-1} to 3.77min^{-1} was observed as the agitation rate was changed from 250rpm to 500rpm. The calculated linear curve for the plot of k_La vs. rpm, using the linear regression method, is also shown in Fig. 6 with the greatest slope value (203%). This result indicates that the influence of increased agitation rate on the enhancement in k_La value becomes more significant at high cell concentrations in comparison with the free cell system.

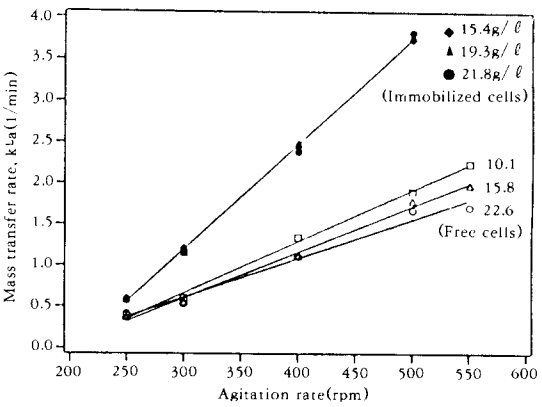

Fig 6. Comparison of gas-liquid mass transfer rate as a function of agitation rate between immobilized and free cell systems in a 4ℓ stirred tank bioreactor. (27℃, 300rpm, 3ℓ working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM). Immobilized system (closed symbols): 50% colonized beads (v/v), additional pulse feeding at 135hr; Free cell system (open symbols): 5% mycelial inoculum, additional pulse feeding at 139hr.

In summary, the remarkable enhancement in the gas-liquid mass transfer rate in the immobilized cell system can be described well by comparing the k_La values against those of the cells under almost identical cell concentrations, as shown in Table 1. These results, once again, reveal that the immobilized cell cultures provided significantly enhanced mass transfer capacity by entrapping the filamentous mycelial cells into the spherical porous biosupport particles. This was particularly true when dense cell concentration were exposed to higher agitation rates, *i. e.*, 500rpm, as clearly indicated in this table. Hence, the phenomenon observed in the immobilized cell system shows a promising potential for further process improvement, because CyA is produced in an essentially growth-associated mode and operation at higher cell densities is clearly desirable.

In order to further develop operating strategies,

Table 1. Comparison of gas-liquid mass transfer coefficient between immobilized and free cell systems at almost identical cell densities.

rpm	Mass transfer coefficient immobilized cells: 15.4g/ℓ free cells: 15.8g/ℓ			Mass transfer coefficient immobilized cells: 21.8g/ℓ free cells: 22.6g/ℓ		
	Immo. system	Free system	% increase	Immo. system	Free system	% increase
250	0.60	0.55	9.1	0.57	0.52	9.6
300	1.21	1.11	9.0	1.18	1.10	7.3
400	2.44	1.78	37.1	2.38	1.67	42.5
500	3.73	1.97	89.3	3.82	1.70	124.7

Unit of mass transfer coefficient(k_La): 1/min

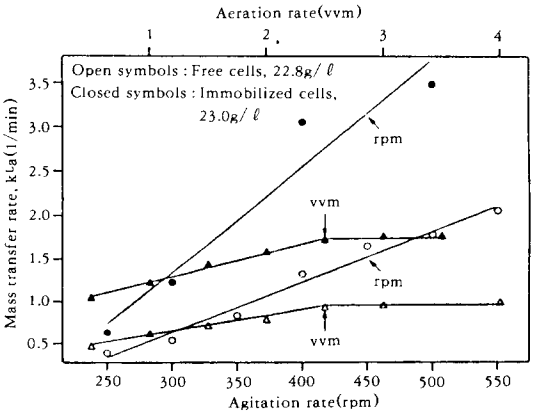

Fig 7. Comparison of gas-liquid mass transfer rate as a function of agitation and aeration rates between immobilized and free cell systems in a 4ℓ stirred tank bioreactor. (27 ℃, 300rpm, 3ℓ working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM). Immobilized system (closed symbols): 50% colonized beads (v/v), additional pulse feeding at 135hr; Free cell system (open symbols): 5% mycelial inoculum, additional pulse feeding at 139hr.

the influences of aeration rates as well as agitation speeds on k_La have been investigated and compared in the two systems, as shown in Fig. 7. The k_La values as a function of the variation of aeration rate were obtained at a fixed agitation rate of 300rpm in both immobilized and freely suspended cell systems. A fixed aeration rate of 1vvm was used in order to examine the effects of agitation rate on k_La value in the two systems. The free cell cultures containing 22.8g/ℓ of cells at the end of fermentation were used as a control to compare against the immobilized cell cultures at almost identical cell density of 23.0g/ℓ and also at almost the same culture age.

The k_La values in both systems were observed to be relatively insensitive to the increase in aeration rate. In addition, almost the same slope, which shows the impact of aeration rate on k_La, was seen in both systems, suggesting that the aeration rate alone is not sufficient to obtain significant enhancement of k_La in the immobilized cell system. However, it should be noted that the immobilized cell system showed almost two-fold higher values of k_La than the freely suspended cell system over the full range of aeration rates. As aeration rate was varied from 0.5vvm to 2.5vvm, the k_La values in the free cell cultures increased from $0.47min^{-1}$ to $0.95min^{-1}$, whereas these values increased from $1.05min^{-1}$ to $1.75min^{-1}$ in the immobilized cell system within the same range of aeration rate. Notably, at aeration rates above 2.5vvm, the measured k_La values remained constant in both systems, *i. e.*, approximately $1.75min^{-1}$ for the immobilized cell cultures and approximately $0.95min^{-1}$ for the free cell cultures. In fact, a change in the gas flow pattern in the fermentation broth was seen as a consequence of increased aeration rate in both immobilized and free cell systems. Hence, the less sensitivity of k_La values to aeration rate at higher aeration rates in both systems appeared to be partly attributable to the formation of large single bubbles and pronounced gas channeling, which might generate low interfacial area and mean residence time. Schügerl (34) explained in his review article that no matter how the air is distributed at the bottom of an air sparged fermentor, in a viscous broth, large spherical bubbles are formed which capture and engulf the smaller bubble swarms that contribute to the interfacial area. In their experiment with a bubble column

reactor, Gbewonyo and Wang (15) observed large single bubbles surging through the center of the column at high aeration rate (2cm/sec) after a certain threshold cell concentration, thus promoting bubble coalescence in the column.

In contrast, the higher sensitivity of k_La values on agitation rate rather than on aeration rate should be noted in both immobilized and free cell systems, as demonstrated by the steeper slopes in Fig. 7. Indeed, in the immobilized cell culture, much higher increases in k_La values were observed, with values ranging from $0.63min^{-1}$ to $3.49min^{-1}$, when the agitation rates were varied from 250rpm to 500rpm at a constant aeration of 1vvm. It was revealed, however, that the free cell culture was less responsive to the increasing agitation rate, as demonstrated by slower increase in the k_La values from $0.39min^{-1}$ to $1.79min^{-1}$ as a function of agitation rates varying from 250rpm to 500rpm. These quantitative results, once more, demonstrate experimentally the remarkable improvement of k_La in the immobilized cell system. In addition, these data illustrate the importance of the role of power input (rpm) for the enhancement of gas-liquid mass transfer rate. It is well known that only mechanically agitated systems are capable of attaining high values of k_La as is required in industrial antibiotic fermentation processes (35). It has been also recognized that the non-mechanically agitated systems would result in liquid blow-out before reaching high aeration rates for obtaining high values of k_La (34). It should be mentioned, however, that the application of increased power input, *viz*, increased stirring rate for the enhancement of *OTR* has some drawbacks in the case of an immobilized cell system because of the mechanical limitation intrinsic to the biosupport particles utilized.

CONCLUSIONS

As a conclusion, it can be stated from the observed data that the immobilized cell culture offers favorable conditions for cell growth as well as CyA production in the stirred tank bioreactor. Due to the remarkably enhanced gas-liquid oxygen mass transfer rate, adequately high levels of dissolved oxygen were maintained throughout the whole period of the immobilized cell fermentation runs. In contrast, when suspended mycelial cells of *T. inflatum* were employed for the production of CyA, the highly viscous non Newtonian nature of the fermentation broth due to the characteristic filamentous structure of the producer microorganism was found to impose serious problems on the gas-liquid mass transfer rate of oxygen, leading to serious mass transfer limitation in the free -cell culture. As a result, it was possible that the immobilized cultures sustained good morphological and physiological states, enhancing the CyA productivity over the corresponding values of the free cell system. In view of the fact that maintaining appropriate supply of oxygen to the cells becomes particularly serious under high cell density cultivations commonly encountered during industrial fermentations, these results demonstrate that celite-immobilized fungal system in a stirred tank reactor could be successfully applied for scaling up CyA production, providing a promising alternative to conventional free cell fermentation. On the other hand, it should be noted that in the case of dense immobilized cell cultures, additional problems are often imposed due to oxygen diffusion resistance caused by the entrapment of cells inside the biosupport matrix, such as diffusion through the stagnant liquid film surrounding the cell aggregates, and diffusion through the matrix of the aggregate. In this paper, we have also demonstrated that by carefully selecting optimal-sized beads and operating conditions, the diffusional oxygen resistance within the celite bead matrix did not appear to have any significant effect on the respiration rates of the immobilized cells.

요 약

4ℓ 교반식 생물반응기에서, celite담체에 고정화된 *Tolypocladium inflatum* 균주 배양시의 산소전달계수(k_La)가 같은 세포농도 하에서 액상배양시의 값

과 비교할 때, 고정상균주의 높은 비산소흡수율에도 불구하고, 2배 이상 증가되었다. 그 결과 고정상배양의 경우, 용존산소량이 포화상태의 75%를 초과하는 충분한 산소량이 배양기간 내내 유지될 수 있었으나, 액상배양의 경우에는 용존산소량이 포화상태의 50% 이하까지 감소되었다. 임펠러의 교반속도에 따른 k_La의 단순 선형 의존 현상이, 250rpm에서 550rpm 범위에서 고정상배양 및 액상배양 모두에서 관찰되었으며, 그 의존정도는 액상배양의 경우 세포농도와 함수관계인 반면, 고정상배양의 경우에는 세포농도와 무관하였다. 반면에 두 배양시스템 모두에서, 통기율 변화에 따른 산소전달율은 2.5vvm까지는 함수관계를 보였으나 그 의존도는 임펠러 교반속도의 변화에 따른 영향과 비교할 때 훨씬 미미하였으며, 2.5vvm 이상의 통기율에서는 산소전달현상에 별로 영향을 주지 못했다. CyA 생산 면에서 볼 때, 고정상세포는 형태학 또는 생리학적으로 훌륭한 배양상태를 유지할 수 있어서, 동일조건의 액상배양과 비교해서 약 2배 이상 생산성이 증가되었다. 그러므로 교반식 생물반응기를 이용한 celite-고정상배양법은 고농도배양이 가능하다는 측면에서 볼 때, CyA 대량생산 산업화를 위한 대체 공정으로서 훌륭한 전망을 제시해 준다.

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