고정상 *Tolypocladium inflatum*균의 세포성장 지속성과 Cyclosporin A 생산성 향상

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Sustained Cell Growth and Improved Cyclosporin A Production Capablity of Immobilized *Tolypocladium Inflatum* Cells

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ABSTRACT

In batch bioreactor fermentations for cyclosporin A (CyA) production, good potential for bioprocess improvement was demonstrated in the immobilized cell system, providing appreciably better utilization of the catalytic activity of the biomass than the freely suspended cells, especially during the exponential phase. When concentrated nutrient medium was added pulsely during the exponential phase of cell growth (at hour 139 of fermentation), reactivation and regermination in both immobilized and suspended cell cultrues were observed to contribute to the longevity of CyA production, maintaining maximum CyA titre until 250 hours of fermentation. Contrarily, simple batch fermentations without any supplement of medium in both systems showed repid decrease in CyA concentrations during the late stationary phase. Notably, the CyA yield coefficient($Y_{p/x}$) for the immobilized cell system was maintained quite high even after the pulse addition of the concentrated full medium, reaching almost 80% of the level attained during the exponential phase. This is in sharp contrast when compared with the corresponding value of 58% in the case of parallel suspended cells. This pattern of CyA production resulted in considerably enhanced CyA production in the immobilized cell system, reaching almost 2 times higher maximum CyA production in comparison with the free cell system.

INTRODUCTION

The cyclosporins are a group of oligopeptides produced as secondary metabolites by the fungus *Tolypocladium inflatum*. Cyclosporin A(CyA) is the main component of this family of eleven-membered cyclic peptides, which is very effective in organ transplantation as a key agent because of its powerful immunosuppressive properties to prevent foreign tissue rejection reactions (1). The remarkably selective immunosuppressive effects of CyA are characterized by a preferential action against proliferating T cells and by low myelot-oxity (2). Very recently it was reported

that cyclosporin synthetase consists of single polypetide chain harbouring all catalytic activities necessary for peptide formation (3). The non-ribosomal synthesis of CyA via an enzyme thio-template mechanism has also been observed previously for the biosynthesis of other peptide antibiotics (4).

In fermentation bioprocesses for production of secondary metabolite using filamentous microorganisms, special benifits can be realized by employing immobilized cell technology such as: good broth rheology leading to enhanced mass transfer and mixing (5); decoupling and optimization of growth phase and production phase relatively easily during the continuous fermentation process, which is specifically important in the synthesis of non-growth associated secondary metabolites (6); easy control of overgrowth problems, making reactor operation comfortable (7); development of good reactor operating schemes for the reduction in the loss of chemically unstable products, by applying continuous or semi-continuous mode of operation. This last advantage appeared to be particularly important for the production of thienamycin, a potent broad spectrum antibiotic, since the antibiotic is extremely susceptible to degradation by medium components and metabolites produced during the fermentation (8). However, there is still a serious lack of fundamental understanding of the different physiological metabolic aspects which are associated with the biocatalyst stability and enhanced intrinsic rates claimed for such immobilized cell systems. Hence, some limitations still exist for the development of sophisticated biopr-ocesses which could outcompete the traditional free cell fermentations for the production of secondary metabolites.

In our laboratory, several prior investigations have been accomplished through an analysis of bioprocess implications arising from major differences in metabolic functions between parallel immobilized and suspended cultures(9-11). The immobilized cell system was observed to provide practical operating advantages such as greater shear stability and significantly higher gas-liquid

mass transfer rate in comparison with the free cell system. In this study, the very active metabolic capabilities of the immobilized cells for both primary and secondary metabolism will be demonstrated in batch bioreactor fermentations. Special emphasis will be placed on the effects of cellular growth stimulation on the longevity of CyA production when concentrated full medium was pulsely supplemented during the exponential phase in both immobilized and freely suspended cultures.

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(12) was initially obtained from ATCC and subsequently adapted to growth on glucose(13). The seed cultures for suspended cell fermentations were prepared exactly in the same manner, as described in the previous papers(10), 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(10).

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 by use of a peristaltic pump to the imobilized celite beads contained in the reactor system and the inoculated beads were incubated at 27℃ inside the reactor for growth and CyA production.

Media and Growth Conditions

The batch fermentations were carried out in a 4-liter mechanically stirred bioreactor(Model MF -200 SPL, New Brunswick Scientific, Edison, NJ). 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 with.

In these batch fermentations, a modified SM medium was utilized, which has the same composition as the previously formulated SM medium (13) except that 8g/ℓ of L-valine was used as a sole nitrogen source instead of ammonium sulfate (10g/ℓ). 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) and blowing into the fermentor through the ca. 0.5cm single hole sparger. The temperature of each fermentation was controlled at 27℃±1℃. A waterbath (Haake-Buchler Instruments, Inc., Saddle Brook, NJ) equipped with both a cooling unit and a heater was used for temperature control by circulating water through water-pipe inside the fermentor. During the exponential phase of cell growth, significant foaming problem was encountered and this was solved by automatic additions

of antifoam SAG 471(which was kindly provided by Schering Plough, Inc., Union, NJ) via the fermentor's antifoam controller.

Analytical Methods

Analytical methodology for CyA and glucose was essentially the same as previously described by Chun and Agathos(10).

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). Homogenization of the fermentation broth was necessary, because this strain was found to form a lot of pellets during the fermentation. Otherwise it was very difficult to obtain a representative sample. The samples were centrifuged for 5minutes at 15000rpm, and then the supernatant was decanted and kept in the freezer at −20℃ for further analysis. The compacted sample was washed with 10ml of distilled water three times to completely remove the residual nutrients. After completion of this operation the centrifuged cell mass was transferred to a pre-weighed aluminum dish and dried at 85℃ for 24hours to obtain constant dry cell weight. Finally the aluminum dish was weighed and from the average difference in weight the dry cell concentration was determined in gDCW/L of fermentation broth. The centrifuge employed in this analysis was an RC5C from Sorval Instruments(Chadds Ford, PA).

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 sam-

ples were washed with distilled water to completely re-move the residual nutrients from the deep inside of the celite particles. The original supernatant was decanted and kept in the freezer at $-20\,°C$ for further analysis. The washed solids were dried at $85\,°C$ for 24hours to obtain the dry weight of dry cells plus celite particles. These solids were heated further in a furnace at $600\,°C$ for 5 hours to get the weight of celite particles alone. The dry cell concentration of each sample was calculated by deducting the weight of the celite particles from the weight of the $85\,°C$-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.

RESULTS AND DISCUSSION

Simple Batch Fermentation without Further Supplement of Medium

The comparison of growth kinetics and growth rate calculated by differentiating non-linear regressed values is shown in Fig. 1(A) and (B). The two different agitation rates of 400rpm and 450rpm were used for the free cell fermentations, while a single batch run at the agitation speed of 400rpm was carried out for the immobilized cell culture. The dissolved oxygen concentration was maintained at above 60% of saturation in each experiment, thus the cultures were not limited by oxygen(data not shown). The active primary metabolism of the immobilized cells during the exponential phase of cell growth is well illustrated in this figure by its high growth rate, although almost the same levels of maximum cell density were obtained in both systems under the same agitation rate(400rpm). CyA production showed similar trends as the cell growth with a slightly higher maximum CyA titre in the immobilized cell system(Fig. 2). The specific CyA production capability of the immobilized cells(6.3mg CyA/g cell) during the exponential growth phase was observed to be almost the same as that of the free cells under the same agitation speed(400rpm). The rapid decline of CyA concentration should be
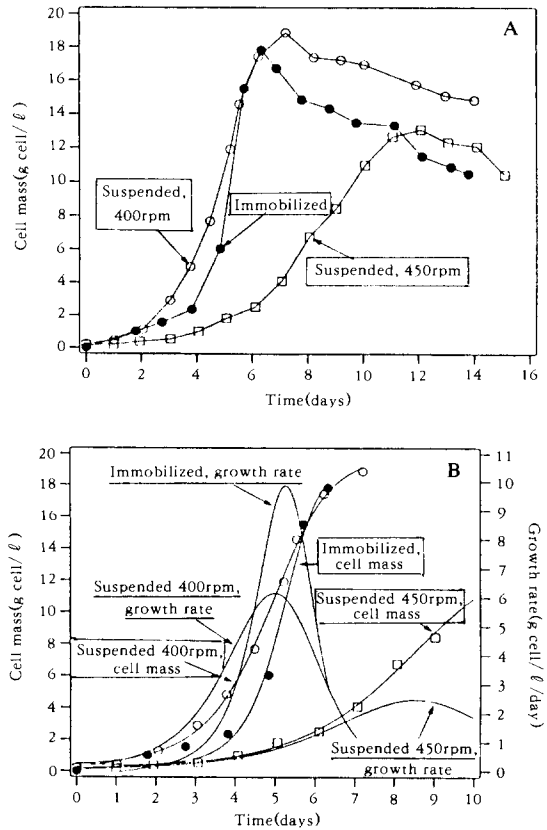

Fig. 1. Comparison of (A) growth kinetics and (B) growth rate between immobilized and free cell systems in a 4 liter stirred tank bioreactor(27°C, 3liter working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM; immobilized system: 50% colonized beads(v/v); free cell system: 5% mycelial inoculum).

noted after reaching the maximum CyA titre in both systems(Fig. 2). One possible cause for the loss of CyA during the stationary phase appears to be peptidase-dependent degradation of CyA molecules, perhaps partly due to cell lysis following the glucose depletion in the medium(Fig. 3).

Fig. 3 also demonstrates that the immobilization of the fungal cell accelerates the rate of glucose uptake, especially during the exponential phase of cell growth. But the average value of
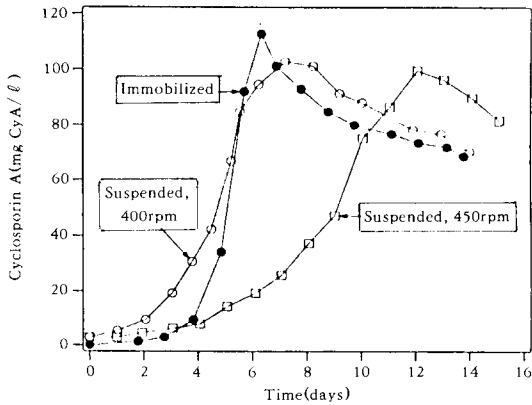
Fig. 2. Comparison of CyA production as a func-
tion of fermentation time between immo-
bilized and free cell systems in a 4 liter
stirred tank bioreactor(27℃, 3 liter work-
ing volume, 1vvm, 8g/ℓ of L-valine as sole
N-source in SM; immobilized system: 50%
colonized beads(v/v); free cell system: 5%
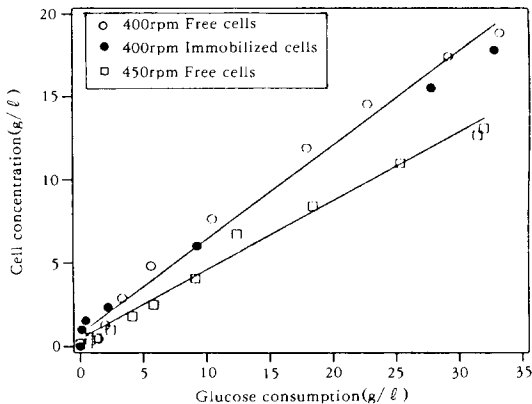mycelial inoculum).

Fig. 3. Comparison of glucose consumption be-
tween immobilized and free cell systems in
a 4 liter stirred tank bioreator(27℃, 3 liter
working volume, 1vvm, 8g/ℓ of L-valine
as sole N-source in SM; immobilized
system: 50% colonized beads(v/v); free cell
system: 5% mycelial inoculum).

the immobilized cell yield based on glucose($Y_{x/s}$),
which can be estimated from the slope of Fig. 4,
remained approximately at the same level(0.61g
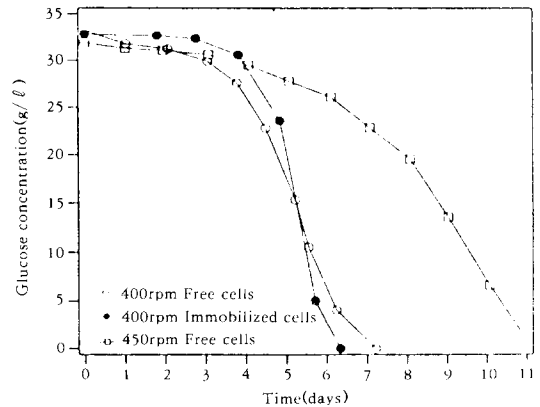

Fig. 4. Comparison of cell yield based on glucose
between immobilized and free cell systems
in a 4 liter stirred tank bioreactor(27℃, 3
liter working volume, 1vvm, 8g/ℓ of L-
valine as sole N-source in SM; immobi-
lized system: 50% colonized beads(v/v);
free cell system: 5% mycelial inoculum).

DCW/g glucose) as that of the free cells cultured
under the identical conditions.

In Fig. 1, it is important to note that the
growth rate of the free cells was very retarded at
the agitation speed of 450rpm. This slow growth
rate also resulted in low CyA productivity and
low glucose uptake rate as shown in Fig. 2 and
Fig. 3. The specific oxygen uptake rate was also
depressed significantly(approximately 50%) as
compared with that of that free cells cultured at
400rpm(data not shown). The lower growth rate
and CyA productivity at the higher agitation rate
(450rpm) might be attributable to the detrimen-
tal shear forces in the fermentor and the resul-
tant morphological changes of the suspended
cells. The cell yield based on glucose($Y_{x/s}$) in the
450rpm free-cell fermentation was only 70% the
400rpm-free cell fermentation(0.44vs. 0.61g
DCW/g glucose). This indicates that a greater
portion of the carbon source was diverted to the
cells' maintenance activities under the high shear
-producing conditions(Fig. 4). When observed
under a micoscope, the cultures grown at higher
agitation speed revealed shorter mycelia and

smaller pellet size. This implies that the stressed mycelia with short hyphal length is related to the declined rates of cell growth and CyA production. It has been reported that the high agitation rate can directly damage the morphology and physiology of filamentous fungi(15, 16). This becomes more complicated by the occurrence of a natural process of vacuolation of the older cells in hyphae which may lead eventually to autolysis(17). In a very recent investigation, Smith *et al.* (18) found that at high agitation speeds(1000 and 1200rpm) in 10-L fermentor the rate of decrease in the mean effective hyphal length was faster and the rate of penicillin production was lower than at 800rpm.

In contrast, these detrimental shear effects appear to be less significant in the immobilized cell system because the interconnected bead matrix can provide the favorable environment for the cells to endure high shear forces and elongate hyphae within the bead matrix. These differences in the culture morphology between the immobilized and free cells could be one factor for the higher growth rate of the immobilized cell system at 400rpm in comparison with the free cells cultivated at 400rpm and 450rpm.

It is also noteworthy that the increased shear stress at the surface of the bioparticles is important if the beads have strong mechanical stability to overcome such high stress. Especially in the case of oxygen, it is well known that intensive agitation could not only increase the gas-liquid mass transfer coefficient, $k_l a$, but also improve the delivery of oxygen by diminishing mass transfer resistance external to the beads(19). We have already shown the great stability of the immobilized cells against shear forces by demonstrating easy germination of the entrapped spores in the previous article(10). Relatively good mechanical stability of the celite particles were also observed experimentally.

For many cases of immobilized cell cultures, it is well known that usually, due to diffusion limitation, immobilized cell preparations show reduced affinity for their substrates, resulting in an increase in K_m value and a decrease in μ_{max} value. In our own system, however, the intraparticle diffusion limitation appeared to be insignificant in view of the small bioparticle size used, the biomass load(in this case, the loaded biomass of 0.14g DCW/g catalyst is only 28% of the full capacity of the biosupports) and the adopted turbulence-producing operational conditions(note that the dissolved oxygen level was greater than 60% of air saturation at the maximum immobilized cell density).

In conclusion, the batch results obtained in this section suggest a good potential of the immobilized cell system for further process development because, as noted previously, CyA is usually actively produced during the active growth phase in a growth-associated mode(20).

Sustained Cell Growth and CyA Production Capability in Batch Fermentations with Pulse Addition of Concentrated Medium

It has been observed in the previous section that the production of CyA in the simple batch fermentations without any supplement of medium declines very rapidly during the late stationary phase(Fig. 2). This behavior suggests that the enzyme mechanism involved in CyA biosynthesis might be inactivated or the cells might be rapidly lysed leading to CyA biodegradation possibly by peptidase induced under the stressed environment in the absence of essential nutrients.

In this experiment, we investigate the effects of growth stimulation on the longevity of CyA production in both immobilized and free cell systems. The reactivation and regermination of the cells was studied by the pulse addition of 200-mL of concentrated SM medium. Approximately 7.5 times 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$_4$ 3.75/ℓ, CaCl$_2$ 0.75g/ℓ, KH$_2$PO$_4$ 5.625g/ℓ and 0.75% v/v trace metal solution(13). A single agitation rate of 300rpm was
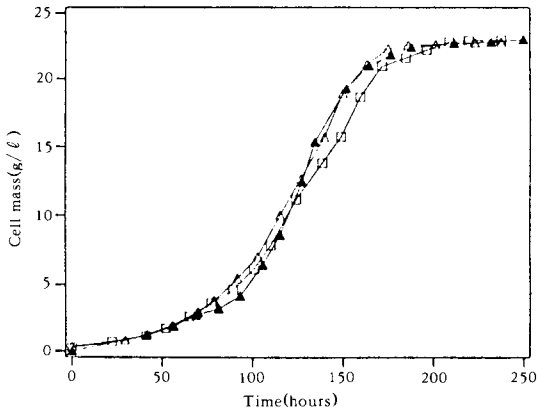
Fig. 5. Comparison of cell growth as a function of fermentation time between immobilized and free cell systems in a 4 liter stirred tank bioreactor(27℃, 3 liter working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM). Immobilized system (▲): 300rpm, 50% colonized beads(v/v), additional pulse feeding at 135hr; Free cell system: 300rpm (△) and 250rpm (□), 5% mycelial inoculum, additional pulse feeding at 139hr.
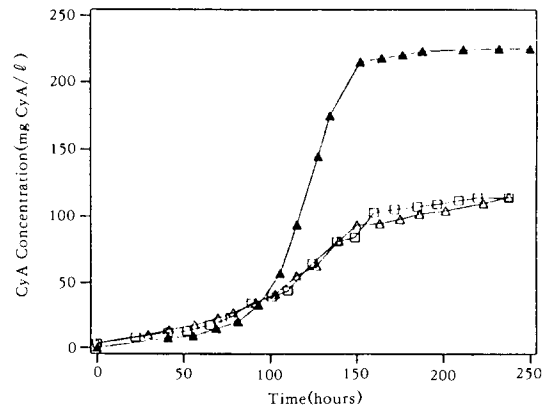
Fig. 6. Comparison of CyA production as a function of fermentation time between immobilized and free cell system in a 4 liter stirred tank bioreactor(27℃, 3 liter working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM). Immobilized system (▲): 300rpm, 50% colonized beads(v/v), additional pulse feeding at 135hr; Free cell system: 300rpm (△) and 250rpm (□), 5% mycelial inoculum, additional pulse feeding at 139hr.

utilized for the immobilized cell cultivation. For the free cell cultures, twe batch runs were carried out at the agitation speeds of 300rpm and 250rpm and compared with the immobilized cell fermentation. 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 when employing two 6-blade turbine impellers.

As shown in Fig. 5, all three fermentations gave almost the same level of maximum cell density of about 23g/ℓ at the end of each fermentation. On the other hand, the difference in the final titre of CyA between the immobilized and the freely suspended cell cultures was remarkably significant. As shown in Fig. 6, further addition of concentrated SM medium during the exponential phase of growth resulted in considerable increase in cellular CyA production with the immobilized cell system, reaching almost 2 times higher maximum CyA production in comparison with

the free cell system. This result is quite interesting since the cellular growth rate of the immobilized cell was almost the same as that of the parallel free cells within the hour 139 to 240 of the incubation(Fig. 5). Different secondary metabolic activiy is quite clear when referring to Fig. 7 which shows the comparison of CyA production as a function of cell concentration between the immobilized and the free cells. In this plot, the slope implies the specific CyA production capability(based on unit cell mass), demonstrating greater than 2 times higher specific production capacity($Y_{p/x}$) of the immobilized-cell as compared with that of the free-cell cultures even before the pulse addition of the intensified SM medium (Table 1). Furthermore, it is quite remarkable that upon a pulse addition of the concentrated full medium, the specific production($Y_{p/x}$) for the immobilized cell system was maintained quite high, almost 80% of that attained during the exponential growth phase. However, a significant
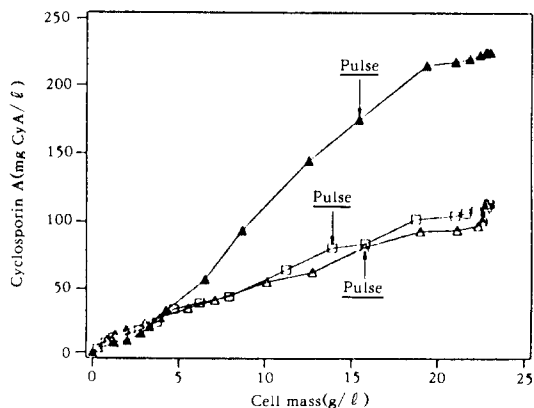
Fig. 7. Comparison of CyA production as a function of cell concentration between immobilized and free cell systems in a 4 liter stirred tank bioreactor(27℃, 3 liter working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM). Immobilized system (▲): 300rpm, 50% colonized beads(v/v), additional pulse feeding at 135hr; Free cell system: 300rpm (△) and 250rpm (□), 5% mycelial inoculum, additional pulse feeding at 139hr.

Table 1. Extent of sustenance of specific production capability($Y_{p/x}$) after pulse addition of concentrated media into free and immobilized cell cultures.

$Y_{p/x}$ Cultures(rpm)	$Y_{p/x}$ during simple batch period (mg CyA/g cell)	$Y_{p/x}$ during pulse batch period (mg CyA/g cell)	Sustenance of $Y_{p/x}$ after pulse medium addition(%)
Free cells(250)	5.76	3.36	58.3
Free cells(300)	5.10	2.94	57.6
Immobilized cells(300)	12.73	10.20	80.1

reduction in the specific production of the parallel suspended cell cultures was observed, and the level was only approximately 60% of that obtained before nutrient pulse-addition(Table 1). One possible explanation for these phenomena is that, in the free cell cultures, some energy might be expended(at the later exponential growth phase) for the adapt-ation to the new environment(maintenance). On the other hand, it could be inferred that, upon pulse addition of the concentrated full medium, the enzymes involved in the biosyntheis of CyA antibiotic might be somewhat repressed, possibly due to more ready utilization of the substrates for cell growth rather than for secondary metabolic functions, thus resulting in low levels of specific CyA production in the freely suspended cell system.

In contrast, for the case of the immobilized cell run, the extra nutrients appeared to have stimulated the CyA production, thus resulting in a increase greater than 3 times in the production yield($Y_{p/x}$) in comparison with the free cell system during the rest of the fermentation. Therefore, the high productivity of CyA in the immobilized cell system during the later parts of this run after the pulse addition of the extra nutrients can be considered as being related to the growth of the immobilized cells leading to the continuous synthesis of newly active enzymes as well as the good maintenance of the activity of the orginal enzyme system without degradation of CyA under the relatively good microenvironmental conditions prevailing in the bioparticles. In Fig. 6, it should be noted that the pulse addition of the extra nutrients at hour 139 resulted in the prolonged maintenance of the maximum CyA concentration even during the later stationary period of fermentation in both immobilized and parallel free cell systems. Namely, the intracellular CyA accumulated in the immobilized and the suspended cells was not biodegradaded/ or the cells were not lysed even after the deficiency of the nutrients in the medium. In all of the pulse experiments, the residual glucose concentration at the later stage of fermentation was approximately 9g /ℓ, which appeared to be enough for the cells to maintain their activities without cell lysis(Fig. 8). In contrast to these results, the previous simple batch runs resulted in complete consumption of all the available carbon source, leading to rapid decline of both cell mass and corresponding CyA.
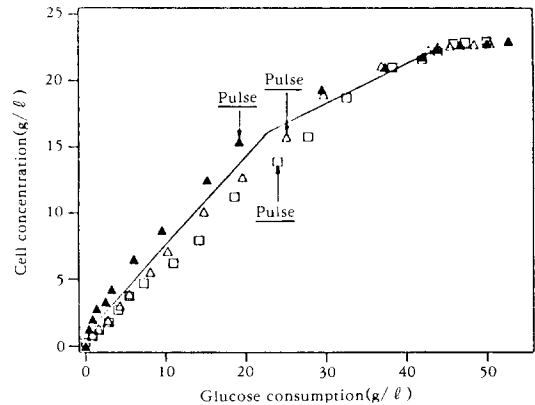
These observations suggest that it is possible to
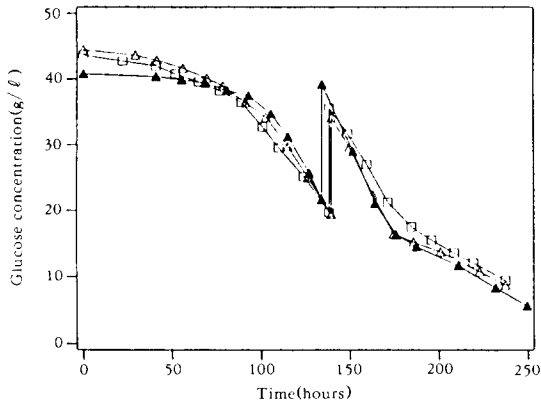
Fig. 8. Comparison of glucose consumption as a function of fermentation time between immobilized and free cell systems in a 4 liter stirred tank bioreactor(27℃, 3 liter working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM). Immobilized system (▲): 300rpm, 50% colonized beads(v/v), additional pulse feeding at 135hr; Free cell system: 300rpm (△) and 250rpm (□), 5% mycelial inoculum, additional pulse feeding at 139hr.

Fig. 9. Comparison of cell yield based on glucose between immobilized and free cell systems in a 4 liter stirred tank bioreactor(27℃, 3 liter working volume, 1vvm, 8g/ℓ of L-valine as sole N-source in SM). Immobilized system (▲): 300rpm, 50% colonized beads(v/v), additional pulse feeding at 135hr; Free cell system: 300rpm (△) and 250rpm (□), 5% mycelial inoculum, additional pulse feeding at 139hr.

prevent unwanted cell lysis and deactivation of the cells during fed-batch or continuous run by supplying extra amount of nutrients during the fermentation process, finally leading to the extension of the CyA production phase. We believe that the sustained increase in CyA biosynthesis during the exponential phase came from the active regermination and outgrowth of the immobilized cells as well as the continuous production of released free cells into the bulk medium rather than an increase in the specific activity of the immobilized cells. In this connection, the data obtained from fed-tatch and continuous runs will be published later and comparisons between immobilized and freely suspended cell systems will be made. This explanation can be supported by Berk et al.(21), who reported that, in a three-phase fluidized bed reactor for the production of patulin with immobilized cells of Penicillium urticae, intermittent pulse addition of nutrients stimulated the slow growth of cells and resulted in an extended

production up to 525hr. They concluded that this long term activity was the result of the formation of new cells rather than the maintenance of the original activity. Likewise, Arcuri et al.(22) found that, in a fermentation using immobilized cells of Streptomyces cattleya with the strategy of total medium change-over(from growth medium to semisynthetic production medium followed by complex production medium) in a novel continuous 2-L bubble column reactor, the higher volumetric rate of thienamycin production obtained was predominantly the result of an increase in cell loading and not an increase in the specific production rate.

On the other hand, no particular differences were observed in the pattern of glucose consumption profiles for each system, as shown in Fig. 8. However, it is noteworthy that the average cell yields based on glucose($Y_{x/s}$), which can be estimated from the slopes of Fig. 9, declines upon the pulse addition of the intensified medium for all the fermentations investigated in this experiment,

Table 2. Comparison of cell yield based on glucose between immobilized and free cell systems($Y_{x/s}$) after pulse addition of concentrated media

$Y_{x/s}$ Cultures	$Y_{x/s}$ during simple batch period (g cell/g glucose)	$Y_{x/s}$ during pulse batch period (g cell/g glucose)
Free cells(250rpm)	0.57	0.49
Free cells(300rpm)	0.62	0.37
Immobilized cells (300rpm)	0.69	0.30

as is clearly demonstrated in Table 2. These results imply that upon the pulse addition of extra nutrients, a greater portion of the supplemented glucose was utilized for cell maintenance rather than biomass accumulation, possibly due to the sudden change in environmental conditions imposed by the increase in substrates for both the immobilized and the free cell systems during the late fermentation period. It could also be reasoned that the growth plus biosynthetic activity of the cultures(both the immobilized and the free cells) might be severely limited by other nutrients rather than glucose during the periods after pulse additions of concentrated medium.

In summary, this study clearly demonstrated that the immobilized cell system had a good potential for (semi-)continuous production of CyA. The significant extension of the exponential CyA production period by the immobilized cells was the key factor to this potential because it was observed that in the traditional freely suspended cell system, a considerable portion of substrate supplemented at the stage of high cell concentration was primarily converted into biomass instead of CyA biosynthesis. In fact, sustained activities of secondary metabolite enzymes after cell immobilization have been observed by several researchers(6, 21, 23, 24). We believe that this phenomenon intrinsic to immobilized cell system is particularly significant since one of the most important factors in a successful continuous process for the production of secondary metabolite like CyA is the longevity of the catalytic activity of the cells.

요 약

Cyclosporin A(CyA) 생산을 위한 회분식 생물반응기 실험에서, 고정상세포를 이용함으로써 액상배양과 비교할 때 생물공정 개선의 가능성이 있음을 제시하였다. 고농도 배지를 생산균주가 지수기 생장 단계인 발효개시 후 139시간에 첨가하였을 때, 고정상배양과 액상배양 모두에서, 균주의 재활성 및 재생장으로 인해 CyA의 생산기간이 연장되어, 발효개시 후 250시간까지 최대 CyA 농도를 유지하였다. 반면에 배지의 첨가가 없는 단순 회분식 배양의 경우, 두 경우 모두 정체생장 단계에서 CyA의 생산성이 빠른 속도로 감소하였다. 주목할 점은 고정상 세포의 경우 CyA수율($Y_{p/x}$)이 고농도 배지를 첨가한 후에도 지수기 때의 수율의 80%에 이르는 높은 값을 계속 유지할 수 있었으나, 이와는 대조적으로 액상 세포는 단지 58%만을 유지할 수 있었다. 그 결과 고정상배양의 최대 CyA생산성이 액상배양과 비교하여 약 2배 정도 증가하였다.

REFERENCES

1. J. F. Borel(1986), *Ciclosporin, Progress in Allergy*, (J. F. Borel, ed), **38**, 9, Karger, Basel.
2. J. F. Borel(1983), *Transplant. Proc.*, **15**(1), 2219.
3. A. Lawen and R. Zocher(1990), *J. Biol. Chem.*, **265**, 11355.
4. H. Kleinkauf and H. V. Döhren(1988), *Criti, Rev. Biotechnol.*, **8**(1), 1.
5. K. Gbewonyo and D. I. C. Wang(1983), *Biotechnol. Bioeng.*, **25**, 2873.
6. Y. M. Deo and G. M. Gaucher(1985), *App. Microbiol. Biotechnol.*, **21**, 220.
7. J. H. Kim, D. K. Oh, Y. K. Park Y. H. Park and D. A. Wallis(1986), *Biotechnol. Bioeng.*, **28**, 1838.
8. E. J. Arcuri, J. R. Nichols, T. S. Brix, V. G. Santamarina, B. C. Buckland and S. W. Drew (1983), *Biotechnol. Bioeng.*, **25**, 2399.
9. G. -T. Chun and S. N. Agathos(1989), *J. Biotechnol.*, **9**, 237.

10. G. -T. Chun and S. N. Agathos(1991), *Biotechnol. Bioeng.*, **37**, 256.

11. G. -T. Chun and S. N. Agathos(1993), *J. Biotechnol*, **27**, 283.

12. American Culture Type Collection(1987), Catalogue of Fungi and Yeasts. 17th ed., Rockland.

13. J. Lee(1989), Ph. D. Thesis, Dept. of Chem. & Biochem. Eng., Rutgers University, New Brunswick.

14. K. Gbewonyo and D. I. C. Wang(1983), *Biotechnol. Bioeng.*, **25**, 967.

15. H. Tanaka, T. Mizzuguchi and K. Ueda (1975), *J. Ferment. Technol.*, **53**, 35.

16. F. Vardar and M. D. Lilly(1982), *Eur. J. Microb. Biotechnol.*, **14**, 203.

17. J. C. van Suijdam and B. Metz(1981), *Biotechnol. Bioeng.*, **23**, 149.

18. J. J. Smith, M. D. Lilly and R. I. Fox(1990), *Biotechnol. Bioeng.*, **35**, 1011.

19. S. O. Enfors and B. Mattiasson(1983), Immobilized Cells and Organelles, (B. Mattiasson, ed), **2**, 41, CRC Press, Boca Raton.

20. S. N. Agathos, C. Madhosingh, J. W. Marshall and J. Lee(1987), *Ann. N. Y. Acad. Sci.*, **506**, 657.

21. D. Berk, L. A. Behie, A. Jones, B. H. Lesser and G. H. Gaucher(1984), *Can. J. Chem. Eng.*, **62**, 112.

22. E. J. Arcuri, G. Slaff and R. Greasham (1986), *Biotechnol. Bioeng.*, **28**, 842.

23. Y. M. Deo and G. M. Gaucher(1983), *Biotechnol. Lett.*, **5**, 125.

24. A. Jones, D. Berk, B. H. Lesser, L. A. Behie and G. M. Gaucher(1983), *Biotechnol. Lett.*, **5**, 785.