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Application of Oxygen Uptake Rate Measured by a Dynamic Method for Analysis of Related Fermentation Parameters in Cyclosporin A Fermentation: Suspended and Immobilized Cell Cultures

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Abstract Experimental data for the on-line estimation of cell concentration and growth rate are presented. For this purpose, we utilized the on-line calculation of the oxygen uptake rate (*OUR*), which was derived from a liquid phase dynamic mass balance for the oxygen during the active growth phase in cyclosporin A (CyA) fermentation. The cell yield coefficient, based on the oxygen ($Y_{x/o}$) for both suspended and immobilized cells of *Tolypocladium inflatum*, was estimated as 1.9 g DCW/g O_2 from a very good linear correlation between the cell mass produced and the total oxygen consumed. The calculated yield showed a good agreement with the value of $Y_{x/o}$ generated from the correlation between the cell growth rate and the oxygen uptake rate. In addition, further experimental data are given, which were also applied to determine the specific oxygen uptake rate of *T. inflatum* cells during the exponential phase of CyA fermentation. A theoretical basis for the analysis of these fermentation parameters is also provided.

Key words: Cyclosporin A, fermentation, immobilized cells, *Tolypocladium inflatum*, oxygen uptake rate

For most secondary metabolite fermentations, it is important to measure the current process state and compare it with the desired state in order to apply timely corrective actions for enhancing the productivity of the bioprocesses [1, 17]. In particular, the quantitative characterization of the cell concentration and growth rate can provide a valuable diagnostic tool for optimizing the performance of the bioprocesses. Therefore, real-time information on the cell concentration and growth rate is crucial in the development of such bioprocess optimization practices.

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Normally, the determination of the cell concentration is made based on direct methods, such as determination of cellular dry cell weight, rough estimation of cell concentration by packed cell volume, or turbidity measurement of culture broth [17]. However, such methods have proved to be impractical in industrial fermentations, for a variety of reasons. For example, a dry cell measurement becomes inaccurate if complex insoluble medium ingredients, such as molasses solids, cellulose, or corn steep liquor, are present [6, 19]. Similarly, a turbidity measurement requires a medium that is essentially particle free to avoid the interference from the medium ingredients. Furthermore, calibration curves relating the optical density to the dry cell weight become nonlinear at high values of optical density, requiring an off-line optimum dilution for which a complex liquid dosing apparatus is necessary [17].

As an alternative, indirect methods have been proposed based on measuring the substrate consumption and/or product formation during the course of cell growth. A chemical analysis of the intracellular components, such as the RNA, DNA, and protein, has been applied as an indirect measure for cell growth, yet this also requires fairly sophisticated experimental procedures and becomes too laborious intensive [17, 18]. Furthermore, many industrial complex media contain these components as substrates, thereby limiting the usefulness of these approaches.

The uptake of oxygen is also a viable alternative for monitoring and quantifying cellular growth, especially in the case of fungal cell fermentation. An analysis of the oxygen mass balances can be performed based on the gas phase through measuring the exit gas, particularly with the use of a mass spectrometer [18, 23]. Recent advances in building reliable instruments and computer applications have made this approach very attractive due to its highly specific, rapid, and accurate capabilities. Yet the main limitation to an exit-gas analysis is that the apparatus used

to measure the gas phase concentration and mass flow is too expensive, and thus not often available in a laboratory. Accordingly, the current study proposes that in a fungal cell fermentation, the following mass balance for liquid phase oxygen can be used as a potential method for the on-line estimation of fermentation parameters, such as the cell concentration, cellular growth rate, and oxygen yield coefficient ($Y_{x/o}$):

$$\frac{dC_L}{dt} = k_L a(C^* - C_L) - q_{O_2}X \quad (1)$$

where k_L is the liquid film oxygen transfer coefficient (cm/h), a is the gas-liquid interfacial area per unit volume of liquid (cm^2/cm^3), and C^* and C_L are the saturation and local dissolved oxygen concentration in the liquid medium (mmol/l), respectively [7, 22], while, q_{O_2} is the specific oxygen uptake rate (mmol/g cell/h) and $q_{O_2}X$ is the volumetric oxygen uptake rate (mmol/l/h). Here, the measurement of change in the dissolved oxygen (DO) concentration, during a transient period of no aeration, is utilized as the basis for determining the oxygen uptake rate by the cells.

The fermentations described in this paper are related to the production of cyclosporin A (CyA) in both immobilized and freely suspended cell cultures. CyA is a cyclic undecapeptide comprised of 11 amino acids, and produced by strains of the filamentous fungus *Tolypocladium inflatum* [2, 11, 13, 20, 26] as a secondary metabolite. CyA has been reported to be very effective in preventing foreign tissue rejection and is currently used as a key agent in the immunotherapy of bone marrow and organ transplants [3].

MATERIALS AND METHODS

Microorganism and Inoculum Development

The *Tolypocladium inflatum* ATCC 34921, indicated as *Beauveria nivea* in the Catalog of Fungi/Yeasts of American Type Culture Collection (Rockland, U.S.A.), was initially obtained from the ATCC and subsequently adapted to growth on glucose [5, 14]. The seed cultures for the suspended cell fermentations were prepared exactly in the same manner as described in a previous paper [5], and inoculated into 2 l of a sterilized synthetic medium contained in a fermenter. The methodology of the inoculum development for the immobilized cell system was essentially the same as previously depicted by Chun and Agathos [5]. For the immobilization of the filamentous fungus, *T. inflatum*, celite was selected as a suitable bead matrix. The immobilization procedure, developed by Gbewonyo and Wang [9, 10] using *P. chrysogenum* cells, was adapted to the current mycelial cells with a number of modifications, as described in a previous paper [5]. The matrix used for immobilizing the conidiospores was celite grade 560 (Manville Corp. Lompoc, U.S.A.), made up of beads ranging from 150 μm to 207 μm.

Media, Growth Conditions, and Biomass

The batch fermentations were carried out in a 2.5-l mechanically stirred bioreactor (New Brunswick Scientific, Edison, U.S.A.) with a working volume of 2 l. As the production medium, a modified SM medium was utilized, which had the same composition as the previously formulated SM medium [5], except that 8 g/l of L-valine was used as the sole nitrogen source instead of ammonium sulfate (10 g/l). The pH was controlled at around 5.7 using H_2SO_4 (2 N) as the acid and KOH (2 N) as the base, and the temperature was maintained at 27°C. The air flow rate was set at 2.0 l/min (1 vvm). The foam was controlled by automatic additions of antifoam SAG 471 (Schering Plough, Inc., U.S.A.) *via* an antifoam controller in the fermenter. The cell concentration in the fermentation broth was determined for both the freely suspended and immobilized cultures using the dry cell weight technique, as described in detail in a previous paper [5].

Oxygen Uptake Rate (*OUR*)

The dissolved oxygen (DO) concentration was adjusted to 100% prior to the inoculation using air as the inlet gas at the fermenter temperature and pressure. An electric zero was used for calibration at 0% saturation. This electric zero calibration was confirmed by exchanging air with a pure nitrogen feed. A polarographic DO probe (Mettler-Toledo, Urdorf, Germany) with a replaceable membrane was used, connected to the DO Analyzer, which was included in the fermenter. The DO concentration was continuously recorded throughout the whole period of fermentation on a Fisher Recordall series 500 recorder (Fisher Scientific, Springfield, U.S.A.). The oxygen uptake rate was measured using a dynamic method, as described in detail in the textbooks [7, 22].

RESULTS AND DISCUSSION

Correlations between *OUR* and Growth Rate in Suspended and Immobilized Cell Cultures

In this experiment, two different agitation rates of 400 rpm and 450 rpm were used for the free-cell fermentations. For the immobilized-cell culture, a single batch run was carried out at an agitation speed of 400 rpm, and then compared with the parallel free-cell systems. During the whole fermentation periods, the dissolved oxygen concentration for both systems was maintained above 60% of the saturation level due to good oxygen supply, thereby indicating that the cultures were not limited by oxygen (data not shown).

Figure 1 shows a close relationship between the *OUR* and the cell growth rate for both the immobilized and the free-cell cultures during the active growth phase, despite a slight time shift between the maximum growth rate and the *OUR*. The growth rate was calculated by differentiating the nonlinear regressed values for the growth kinetics, as

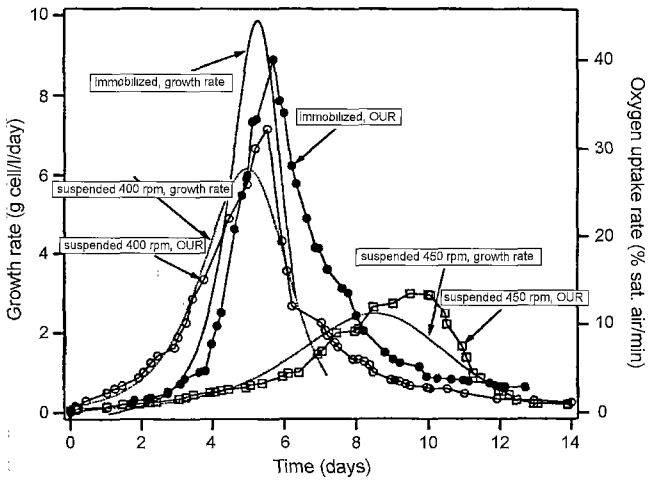

Fig. 1. Comparison of oxygen uptake rate and growth rate as a function of fermentation time between immobilized and free-cell systems in 2.5-l stirred tank bioreactor.
Two-liter working volume at 27°C, 1 vvm, 8 g/l of L-valine as sole N-source in SM; immobilized system: 50% colonized beads (v/v); free-cell system: 5% mycelial inoculum.

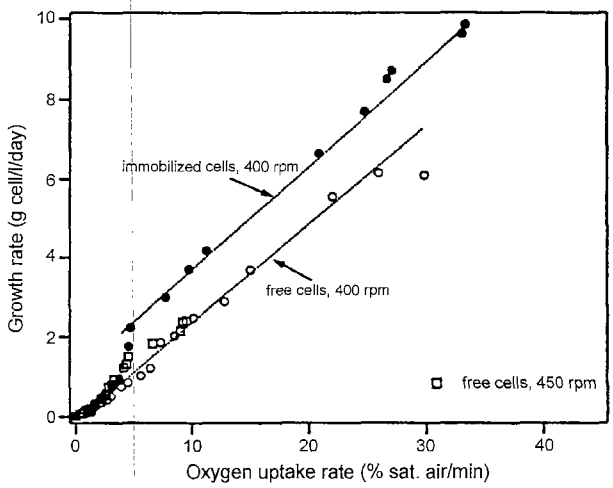

Fig. 3. Correlation between growth rate and oxygen uptake rate between immobilized and free-cell systems in 2.5-l stirred tank bioreactor.
Two-liter working volume at 27°C, 1 vvm, 8 g/l of L-valine as sole N-source in SM; immobilized system: 50% colonized beads (v/v); free-cell system: 5% mycelial inoculum.

described in Fig. 2. In Fig. 3, the significant linear correlation between the *OUR* and the cell growth rate was re-emphasized by plotting the growth rate as a function of the *OUR*. This result implies a good potential for the on-line estimation of the growth rate for the producer microorganism, *T. inflatum*, when using adequate mathematical models with the on-line measurement of the *OUR*. This is particularly important because it is well known that the growth rate is one of the most significant parameters characterizing the fundamental bioprocess in secondary metabolite fermentations [12, 16].

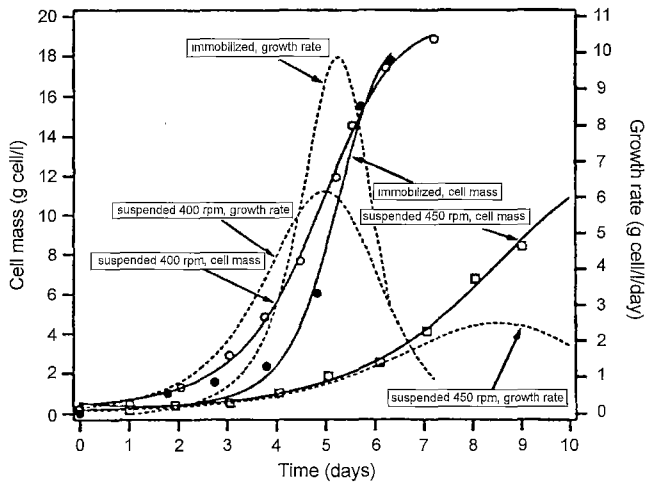

Fig. 2. Comparison of growth kinetics and growth rate between immobilized and free-cell systems in 2.5-l stirred tank bioreactor.
Two-liter working volume at 27°C, 1 vvm, 8 g/l of L-valine as sole N-source in SM; immobilized system: 50% colonized beads (v/v); free-cell system: 5% mycelial inoculum.

In fact, several important fermentation parameters can be evaluated using an on-line measured *OUR*. From a growth model based on the cell yield ($Y_{x/o}$) term and maintenance coefficient term (m) adopted by Zabriskie *et al.* [25], the *OUR* can be represented by:

$$Q_{O_2}=\frac{\mu X}{Y_{x/o}}+mX \cong \frac{\mu X}{Y_{x/o}} \quad (m=0) \tag{2}$$

In the above expression, it is assumed that, during the active growth phase, all the oxygen consumed is directed into cell mass formation. Therefore, when the above equation was applied to Fig. 3, the slope became the cell yielded based on the oxygen ($Y_{x/o}$). Hence, the good linearity in Fig. 3 implies that $Y_{x/o}$ in both the immobilized and the free-cell cultures of *T. inflatum* remained nearly constant, especially during the exponentially proliferating phase.

The CyA production showed a similar trend to the cell growth kinetics in all the fermentations (maximum production of 102.8 mg CyA/l for free cells and 112.6 mg CyA/l for immobilized cells) and the specific CyA production capability of the immobilized cells (6.3 mg CyA/g cell) was observed to be almost identical to that of the free cells (400 rpm and 450 rpm) (data not shown).

Estimation of Cell Concentration and Cell Yield Coefficient ($Y_{x/o}$) from Measurement of Total Oxygen Amount Consumed

Theoretically, when using the experimentally obtained yield factor ($Y_{x/o}$), the on-line estimation of the cell concentration (X) is as follows:

$$X=Y_{x/o}\int_0^t (OUR)dt \tag{3}$$

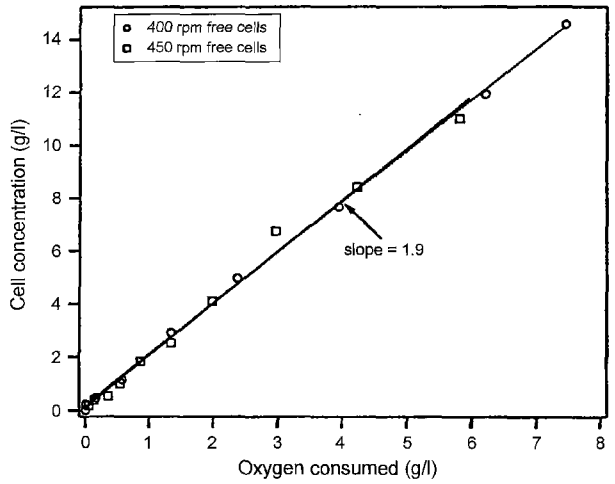

Fig. 4. Cell yield of suspended cells based on oxygen in 2.5-l stirred tank bioreactor.
Two-liter working volume at 27°C, 1 vvm, 8 g/l of L-valine as sole N-source in SM; immobilized system: 50% colonized beads (v/v); free-cell system: 5% mycelial inoculum.

where the integral denotes the total oxygen consumed, which can be calculated by an on-line numerical summation of the *OUR* with respect to the fermentation time using a computer. In the freely suspended cell cultures (400 rpm and 450 rpm), a very good linear correlation was observed between the cell mass produced and the oxygen amount consumed (Fig. 4). This result shows further potential for the on-line estimation of the cell concentration by measuring the total oxygen consumed *via* the oxygen mass balance in the liquid phase. In this experiment, the total oxygen amount consumed was calculated by integrating the slope of the oxygen transient curve relative to the time (based on the liquid-phase dynamic balance of oxygen during a sequence of "non-aeration" transients), using a trapezoidal rule approximation. The solubility of oxygen at 27 and 1 atm was taken to be 0.257 mmol/l [7].

In the above expression, it should be noted that the yield factor ($Y_{x/o}$) can also be derived from the slope of a plot for the cell concentration vs. the total oxygen consumed. For both fermentations, the calculated yield (1.9 g DCW/g O_2) agreed well with the value of $Y_{x/o}$ obtained from the data analysis of the cell growth rate (dX/dt) and the *OUR* (dC/dt), as revealed in Fig. 3. In Fig. 4, the fermentation results for the immobilized cells are not shown, because of a lack of available immobilized cell mass data, especially during the exponential phase (in fact, the growth rate data for the immobilized cells in Fig. 3 was obtained by simulating a nonlinear regressed growth curve for the immobilized cell system). The oxygen yield obtained (1.9 g DCW/g O_2) appeared to be somewhat higher when compared with previously quoted values for a bacterial culture on a carbohydrate carbon source (1.5 g DCW/g O_2) [1] and for suspended *P. chrysogenum* cells (1.56 g DCW/g O_2) [15]. Yet, in the case of *Saccharomyces cerevisiae* cells, Zabriskie and Humphery [25] also obtained relatively high $Y_{x/o}$ values, ranging from 2.11 to 2.70 g DCW/g O_2, when performing a material balance for the oxygen and employing a kinetic model for the molecular oxygen utilization. It was noted that almost the same level of $Y_{x/o}$ was observed for both free-cell fermentations (450 rpm and 400 rpm), when considered together with the lower level of $Y_{x/s}$ in the 450 rpm fermentation (0.61 g DCW/g glucose and 0.44 g DCW/g glucose for 400 rpm and 450 rpm fermentations, respectively) (data not shown). This reflects that a greater portion of the carbon source was utilized for cell maintenance, thereby leading to a lower growth rate at 450 rpm.

The specific growth rate (μ) during the exponential phase can also be estimated on-line using the *OUR* and calculated total oxygen consumed, as follows:

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{OUR}{\int_0^t (OUR)dt} \quad (4)$$

The linearity between the *OUR* and the growth rate observed in this experiment (Fig. 3) implies that μ and X, which are both very valuable fermentation parameters, can also be estimated directly *in situ* during filamentous fungal cell fermentations, simply by using an oxygen electrode (through the method based on a liquid-phase dynamic mass balance for the oxygen) together with an on-line computer analysis system.

Determination of Specific Oxygen Uptake Rate

The average specific *OUR* during the exponential phase was estimated from the slope of the linear portions in Fig.

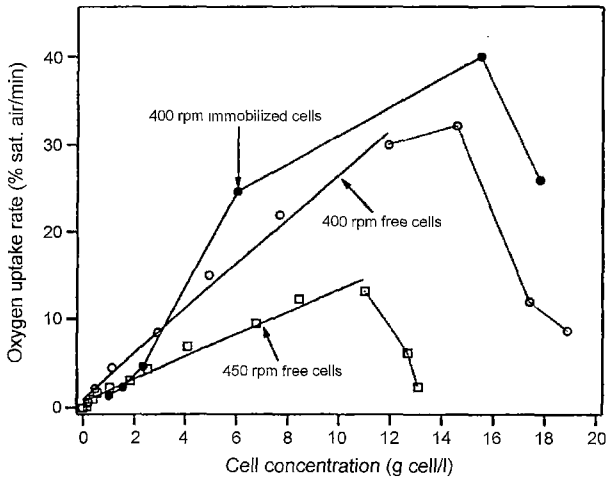

Fig. 5. Comparison of oxygen uptake rate as a function of cell growth between immobilized and free-cell systems in 2.5-l stirred tank bioreactor.
Two-liter working volume at 27°C, 1 vvm, 8 g/l of L-valine as sole N-source in SM; immobilized system: 50% colonized beads (v/v); free-cell system: 5% mycelial inoculum.

5 (approximately 0.41 mM O_2/g cell/h for the free cells at 400 rpm and 0.20 mM O_2/g cell/h for the free cells at 450 rpm). The deviation from the linearity after the active growth phase may have been due to inaccurate estimates of the active biomass concentration during the later stage of fermentation. As pointed out by Calam [4], the estimation of the net dry weight is hampered by the simultaneous existence of living and dead cells during the whole fermentation process, thereby leading to an accumulation of reserve materials in the system. The decrease in the *OUR* during the stagnant phase, reflecting both a slow-down of the cell proliferation and a decline in the cell viability, could have been another possible reason for the deviation.

A notable result was the higher specific respiration rates of the immobilized cells, especially during the exponential phase (Fig. 5). Shortly after the start of the fermentation, the specific oxygen uptake rates of the immobilized cells were slightly lower than those of the free cells, yet these rates increased rapidly with the increasing cell concentrations during the exponential phase. Since the measured values of the oxygen uptake rate (*OUR*) in the immobilized cell system were apparent values without considering the effect of the intraparticle diffusion resistance of the oxygen, the intrinsic oxygen uptake rates may have been actually higher than the values shown in Fig. 5. The possible reason for the enhanced *OUR* of the immobilized cells during the exponential phase could have been due to the simultaneous germination of entrapped spores during the active growth phase, although the lower specific *OUR* during the lag phase of the cell growth suggested a relatively long adaptation period for the immobilized spores related to germination and outgrowth inside the immobilizing matrix environment. Changes in the metabolic activity of immobilized cells after cell confinement into a porous matrix have been frequently observed during the cultivation of microbial as well as mammalian cells. Galazzo and Bailey [8] reported increased rates of glucose uptake or glucose phosphorylation after the immobilization of *Saccharomyces cerevisiae* in alginate beads. Wohlpart *et al.* [24] also demonstrated that the specific respiration rates of alginate-entrapped hybridoma cells were about 3 times higher than those observed for freely suspended cells, regardless of the bead size and cell loading. They proposed that the unusually high specific respiration rates of the entrapped cells may be due to the greater maintenance energy requirements of such cells related to their environment.

CONCLUSIONS

The dynamic mass balance technique for dissolved oxygen would appear to offer a good potential for the on-line estimation of fermentation parameters, such as the cell concentration, cellular growth rate, and oxygen yield coefficient ($Y_{x/o}$), which are often regarded as the key process variables affecting secondary metabolite fermentation bioprocesses. A sequence of "no aeration" transients was utilized to measure the *OUR* in both immobilized and suspended fungal cell fermentations. Very active metabolic capabilities for the immobilized cells were also noted, as documented in terms of the specific oxygen uptake rates.

However, certain technical problems still need to be solved: Due to the possibility of the DO probe being fouled, especially during filamentous fungal cell fermentation, it is important that the probe is inserted in a region with turbulence sufficiently to help maintain a clean probe for extended fermentation periods. In addition, the position-dependent nature of dissolved oxygen in a large fermenter implies that the placement should be considered carefully in order to obtain the most representative DO values.

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