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# On-line Conversion Estimation for Solvent-free Enzymatic Esterification Systems with Water Activity Control

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**Abstract** On-line conversion estimation of enzymatic esterification reactions in solvent-free media was investigated. In principle, conversion to ester can be determined from the amount of water produced by the reaction, because water is formed as a by-product in a stoichiometric manner. In this study, we estimated the water production rate only from some measurements of relative humidity and water balances without using any analytical methods. In order to test the performance of the on-line conversion estimation, the lipase-catalyzed esterification of *n*-capric acid and *n*-decyl alcohol in solvent-free media was performed whilst controlling water activity at various values. The reaction conversions estimated on-line were similar to those determined by off-line gas chromatographic analysis. However, when the water activity was controlled at higher values, discrepancies between the estimated conversion values and the measured values became significant. The deviation was found to be due to the inaccurate measurement of the water content in the reaction medium during the initial stages of the reaction. Using a digital filter, we were able to improve the accuracy of the on-line conversion estimation method considerably. Despite the simplicity of this method, the on-line estimated conversions were in good agreement with the off-line measured values.

*Keywords*: lipase-catalyzed esterification, on-line conversion estimation, solvent-free media, water activity control

## INTRODUCTION

Biotransformations already represent an effective and sometimes a preferable alternative to chemical synthesis for the production of fine chemicals and optically active compounds. The use of nonaqueous media for biocatalytic reactions has proved to be an extremely useful approach to expanding the range and efficiency of the practical applications of biocatalysis. The advantages of using nonaqueous media include increased solubility of hydrophobic substrates and favorable shift in reaction equilibria. It is not surprising, therefore, that nonaqueous enzymatic reactions are now being investigated and exploited in numerous academic and industrial laboratories [1,2].

In most cases, enzymatic reactions in nonaqueous media have been performed in batch reactors and conversion has been usually determined by conventional off-line methods (GC, HPLC, *etc*). However, off-line analysis is costly, time-consuming, and suffers from a considerable information lag. Therefore, if possible, on-line monitoring of enzyme-catalyzed biotransformations is a more desirable way of understanding, controlling and optimizing enzymatic processes than off-line sampling and subsequent analysis. In response to this need, real-time instruments capable of measuring enzymatic reaction conversions, such as enzyme thermistors, have been examined as interesting monitoring tools of biotransformations involving enzymes in organic solvents [3-5]. Recently, a nuclear magnetic resonance spectroscopic method has been developed to monitor on-line lipase-catalyzed esterification reactions without the need to sample the reaction medium [6-8]. However, the aforementioned techniques for on-line monitoring of enzyme reactions in nonaqueous systems require instruments for the direct analysis of substrates or products, the availability and reliability of which are still very limited due to their high price, low stability, and high level of complexity. Thus, people have been searching for methods of providing the necessary information from other variables, which are relatively simple and easily measured. As a result, indirect estimation techniques, which make it possible to monitor key variables on-line and in situ without using expensive and complicated analytical instruments, have been developed. However, applications of on-line estimation methods using macroscopic balancing have been limited to fermentation and cell culture systems [9-13]. As far as we are aware, on-line conversion monitoring for enzymatic reactions based on indirect on-line estimations has not yet been reported in the literature.

We previously developed a water activity control sys-

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tem for lipase-catalyzed esterification reactions in solvent-free media. In the case of enzymatic solvent-free ester synthesis, which is one of the new high productivity technologies in the field of nonaqueous enzymology, it is difficult to control water activity because a great amount of water is produced. Although several water activity control systems have been developed, the amount of substrates used was very low, so that the maximum amount of water produced by the reaction was no more than 0.6-2.9 mmol [14-16]. In contrast, we previously succeeded in controlling water activity ($a_w$) in a solvent-free ester synthesis, which involved 320 mmol of substrate [17]. As far as esterifications are concerned, reaction conversion can be estimated by determining the amount of water produced by the reaction, because water is formed as a by-product in the same molar ratio with respect to the product. In other words, the conversion of an ester synthesis reaction can be determined if the amount of water produced by the reaction can be measured. The $a_w$ control system we designed previously enabled us to measure the water content in the gas phase easily using a RH sensor interfaced to a computer. The determination was based on the principle that the water content in the air entering and leaving the reaction medium would allow the amount of water produced by the reaction to be calculated. Therefore, during the course of this work, we have estimated on-line conversions of lipase-catalyzed esterifications from measurements of the relative humidities and the water material balances without using analytical techniques such as gas chromatography. Real-time estimation of reaction conversions makes it possible to follow the progress of the reaction and to control the reaction progress efficiently without the need for expensive analytical equipment. We describe here for the first time the on-line conversion monitoring of enzymatic reactions using indirect estimation techniques.

## MATERIALS AND METHODS

### Materials

Lipozyme IM, *Rhizomucor miehei* lipase immobilized on anion-exchange resin, was a generous gift from Novo Nordisk (Bagsvaerd, Denmark) and used as received. *n*-Capric acid, *n*-decyl alcohol (Sigma, St. Louis, MO, USA) and silica gel (particle size: 3-6 mm; Shinyo Osaka, Japan) were used without further purification. The enzyme powder was stored in a refrigerator prior to use.

### Water Adsorption and Desorption Isotherms

The dried enzyme, the substrate solution (molar ratio of *n*-capric acid to *n*-decyl alcohol = 1:2) and the reaction mixtures, composed of substrates and product with four different compositions (representing 30, 48, 75 and 96% conversion), were equilibrated separately with a variety of saturated salt solutions at 30°C for 18 days. When determining the water desorption isotherm of Lipozyme, enzyme particles were fully hydrated with water-saturated air for about 18 days, and then equilibrated with the saturated salt solutions. The saturated salt solutions used were as follows: LiBr ($a_w$=0.06), LiCl (0.11), $CH_3COOK$ (0.22), $MgCl_2$ (0.32), $K_2CO_3$ (0.43), $Mg(NO_3)_2$ (0.51), NaBr (0.56), $CuCl_2$ (0.67), NaCl (0.75), KCl (0.84), $KNO_3$ (0.92), and $Na_2HPO_4$ (0.96). Equilibration was achieved through the vapor phase in sealed vials. After being allowed to equilibrate, the water content in the liquid mixture was measured by the Karl-Fischer method using a Mettler DL 35 KF Coulometer (Mettler, Switzerland) at room temperature. Water bound to the enzyme was calculated by weight difference before and after heating the sample in an oven for 2 weeks.

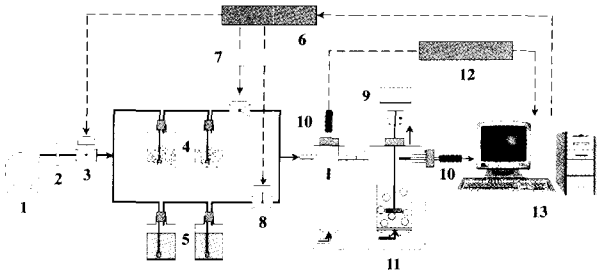


**Fig. 1.** Schematic diagram of the computer system for water activity control and on-line conversion estimation. (1) air pump; (2) air filter; (3) mass flow controller; (4) silica gel; (5) distilled water; (6) digital to analog converter; (7) solid state relay; (8) solenoid valve; (9) stirrer; (10) relative humidity sensor; (11) enzyme reactor; (12) analog to digital converter; (13) computer.

### Water Activity Control

A schematic diagram of the water activity control system for on-line conversion estimation is shown in Fig. 1. Except for the fact that another relative humidity sensor has been added, the control system used in this study is identical to the one we described previously [17]. For $a_w$ measurement in the inlet air stream, another $a_w$ sensor with a transmitter (Novasina Hygrodat 20, Axair AG, Pfäffikon, Switzerland) was added to the system developed in our previous work. The relative humidity probe has an electrolytic measuring cell with a range from 6 to 100% relative humidity (RH) and an accuracy of ±1.0% RH. RH readings were forwarded to a PC via a 16 channel analog-to-digital (A/D) converter (Analog Design Labs, Seoul, Korea).

As described in previous work, an IBM® compatible computer (CPU: Intel® Pentium® II processor) was used as the digital feedback controller. The controller program was developed in Visual C++® 6.0 (Microsoft Corp., Redmond, WA, USA), and the controller output was generated using a digital PI control algorithm. The PI controller parameters $K_c$ and $\tau_I$ were set at 1,000 sccm/% and 2,000 min, respectively. The data-sampling interval was fixed at 1 min.

## Esterification Reaction in Solvent-free Media

The esterification reaction was carried out in a water-jacketed, stirred-tank type of enzyme reactor. A cylindrical glass water-jacketed column of dimensions of 5 × 19 cm was used as the reactor. A sintered glass disc was located at the bottom of the reactor to retain enzyme particles and to allow air to enter. One gram of the enzyme powder was placed in 100 mL of *n*-decyl alcohol in the reactor for 30 min. The reactions were started by adding substrate mixture (22 mL of *n*-decyl alcohol and 62 mL of *n*-capric acid) to the enzyme reactor. Therefore, the reaction medium is a mixture of 320 mmol of *n*-capric acid and 640 mmol of *n*-decyl alcohol without any solvent. All the reactions were performed at 30°C and the reaction medium was stirred vigorously at 500 rpm using a paddle impeller driven by a motor to enable the rapid equilibration of water in the different phases in the reaction system.

The substrate and product concentrations were analyzed on a HP 5890 Series II gas chromatograph (Hewlett-Packard Co., Wilmington, DE, USA) equipped with a flame ionization detector and a HP-INNOWax capillary column (internal diameter: 0.32 mm, length: 30 m). Approximately 0.5 mL of the suspension was taken from the reaction medium, centrifuged to separate the enzyme particles, and 50 μL of the supernatant so obtained was diluted with 750 μL of cyclohexane and analyzed. The column temperature was increased from 160°C to 240°C at 20°C/min and then maintained at 240°C for 3 min. The injector and detector temperatures were 250°C and 260°C, respectively. Helium was used as the carrier gas and supplied to the column at 3.8 mL/min.

## Water Balance

Water content changes in the system depend on the mass flow rate of the water vapor entering and leaving the reactor and the rate of water produced by the enzyme action. Since the amount of water in the reaction system is equal to the sum of water in the solid enzyme and in the liquid medium, a water balance in the reaction system yields:

$$\frac{d(V_r C_{w,m} + m_e C_{w,e})}{dt} = F_{w,in} - F_{w,out} + m_e r_w \quad (1)$$

where $C_{w,m}$ = the water concentration in the reaction medium (g/L); $C_{w,e}$ = the water concentration bound to the enzyme particles (g/g of dry enzyme); $V_r$ = the volume of the reaction medium (L); $m_e$ = the amount of enzyme (g); $F_{w,in}$ = the mass flow rate of water vapor entering the reaction system (g/min); $F_{w,out}$ = the mass flow rate of water vapor leaving the reaction system (g/min); $r_w$ = the rate of water production by the reaction (g/(min · g of enzyme)).

Since $V_r$ and $m_e$ remain nearly unchanged during the reaction, Equation (1) becomes

$$\frac{V_r dC_{w,m}}{dt} + \frac{m_e dC_{w,e}}{dt} = F_{w,in} - F_{w,out} + m_e r_w \quad (2)$$

If the water vapor is assumed to behave as an ideal gas, Equation (2) becomes

$$\frac{V_r dC_{w,m}}{dt} + \frac{m_e dC_{w,e}}{dt} = M_w \frac{q_{in} P_{w,in}}{RT_{in}} - M_w \frac{q_{out} P_{w,out}}{RT_{out}} + m_e r_w \quad (3)$$

where $P_{w,in}$ = the partial pressure of water in the inlet air gas; $P_{w,out}$ = the partial pressure of water in the outlet air gas; $q_{in}$ = the volumetric flow rate of the air gas entering the reactor; $q_{out}$ = the volumetric flow rate of the air gas leaving the reactor; $R$ = the gas constant; $T_{in}$ = the temperature of the inlet air; $T_{out}$ = the temperature of the outlet air; $M_w$ = molecular weight of water. Usually $T_{in}$ and $T_{out}$ are maintained at a constant level (*i.e.*, $T_{in} = T_{out} = T$), and therefore Equation (3) can be expressed as

$$\frac{V_r dC_{w,m}}{dt} + \frac{m_e dC_{w,e}}{dt} = \frac{M_w}{RT}(q_{in} P_{w,in} - q_{out} P_{w,out}) + m_e r_w \quad (4)$$

The water activity ($a_w$) in any mixture is defined as the ratio of the fugacity of water in the mixture ($f_w$) to that of pure water at the same temperature ($f_w^o$). Since water behaves nearly ideally, fugacities are commonly replaced by vapor pressures over the entire system ($P_w$) and above pure water ($P_w^o$), respectively:

$$a_w = \frac{f_w}{f_w^o} = \frac{P_w}{P_w^o} \quad (5)$$

Hence, Equation (4) can be rearranged to

$$\frac{V_r dC_{w,m}}{dt} + \frac{m_e dC_{w,e}}{dt} = \frac{M_w P_w^o}{RT}(q_{in} a_{w,in} - q_{out} a_{w,out}) + m_e r_w \quad (6)$$

where $a_{w,in}$ = the water activity in the inlet air, and $a_{w,out}$ = the water activity in the outlet air.

For computer-aided prediction, the discrete-time version of Equation (6) is required. The application of the forward Euler method yields

$$V_r[C_{w,m}(k+1) - C_{w,m}(k)] + m_e[C_{w,e}(k+1) - C_{w,e}(k)] = \frac{M_w P_w^o}{RT}[q_{in}(k) a_{w,in}(k) - q_{out}(k) a_{w,out}(k)]\Delta t + m_e r_w(k)\Delta t \quad (7)$$

In addition, the water production rate can also be expressed in terms of reaction conversion

$$m_e r_w(k) = M_w\left(\frac{S_0}{100}\right)\frac{dX}{dt} = M_w\left(\frac{S_0}{100}\right)\frac{[X(k+1) - X(k)]}{\Delta t} \quad (8)$$

where $X(k)$ = the reaction conversion (%) at the $k$th

sampling; $S_0$ = the initial amount of the substrate, *n*-capric acid (mol). Eliminating $m_e r_w(k)\Delta t$ from Equation (7) using Equation (8) leaves

$$X(k+1) = X(k) + \left(\frac{100}{M_w S_0}\right)\{V_r[C_{w,m}(k+1) - C_{w,m}(k)] + m_e[C_{w,e}(k+1) - C_{w,e}(k)]\} + \left(\frac{100P_w^0}{S_0 RT}\right)[q_{out}(k)a_{w,out}(k) - q_{in}(k)a_{w,in}(k)]\Delta t \quad (9)$$

All parameters in Equation (9) can be determined by experiments except $C_{w,m}$ and $C_{w,e}$. The values of $C_{w,m}$ and $C_{w,e}$ depend on the water activity in the reaction system, and furthermore $C_{w,m}$ is also a function of reaction conversion ($X$)

$$C_{w,m}(k) = f[X(k), a_{w,m}(k)] \quad (10)$$

$$C_{w,e}(k) = g[a_{w,e}(k)] \quad (11)$$

where $a_{w,m}$ is the water activity in the liquid medium phase, and $a_{w,e}$ is the water activity in the solid enzyme phase. A previous study showed that there is a near-equilibrium between the liquid mixture and the exit gas phase during air bubbling: $a_{w,m} \cong a_{w,out}$ [17]. Water in the liquid medium is rapidly distributed to the enzyme particles in the reaction medium, such that the water in the liquid mixture is also nearly equilibrated with water on the biocatalyst: $a_{w,m} \cong a_{w,e}$ (our unpublished experimental data). Consequently, we assumed here that the water activity is at near-equili-brium in all phases in our $a_w$ control system ($a_{w,m} \cong a_{w,e} \cong a_{w,out}$).

### On-line Conversion Estimation

Using the equations described above, the reaction conversion was indirectly estimated from the RH and the mass flow rates of the inlet and outlet air. A flowchart for the conversion estimation procedure is shown in Fig. 2. First, $a_{w,in}(k)$, $a_{w,out}(k)$, $q_{in}(k)$, $q_{out}(k)$, and $a_{w,out}(k+1)$, measured by experiment were used as input values with a known value of $X(k)$. Then $C_{w,m}(k)$ and $C_{w,e}(k)$, and $C_{w,e}(k+1)$ were calculated from $a_{w,out}(k)$ and $a_{w,out}(k+1)$, respectively, using correlations between $a_w$ and the water content, which were obtained from separate experiments (see Equations (12) and (13)). Since $C_{w,m}$ is also a function of $X$, $C_{w,m}(k+1)$ was estimated using an iterative technique: $C_{w,m}(k+1)$ was calculated using an assumed reaction conversion value, $\hat{X}(k+1)$, and then it was determined whether the resultant $C_{w,m}(k+1)$ satisfied Equation (9). The iteration process was repeated until the difference between $\hat{X}(k+1)$ and $X(k+1)$ calculated from Equation (9) was less than 0.001% conversion. This iteration procedure yields the estimated values of $X(k+1)$ and $C_{w,m}(k+1)$ simultaneously.

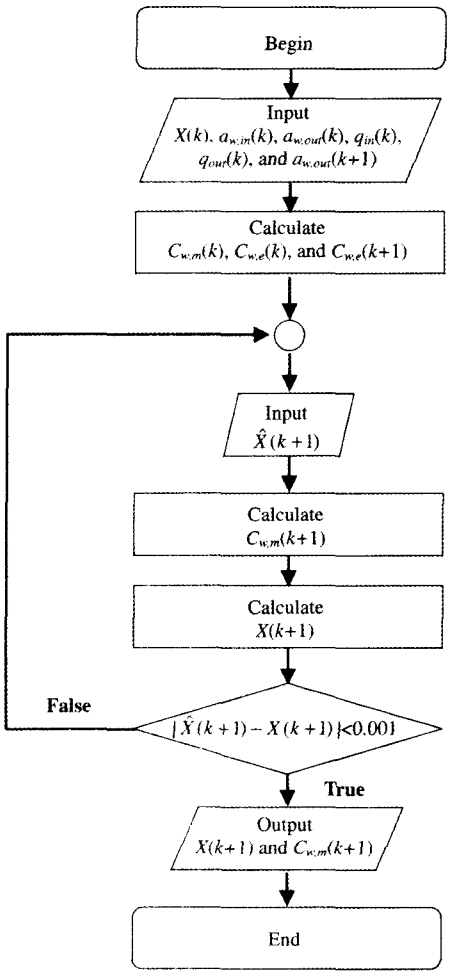


**Fig. 2.** A flowchart for calculation of the reaction conversion.

## RESULTS AND DISCUSSION

### Determination of the Correlation between $a_w$ and Water Content

In order to calculate the conversion $X(k+1)$ starting from a known initial condition $X(0)$ using Equation (9), the correlation between $a_w$ and the water content in the reaction medium (Equation (10)) and in the enzyme particles (Equation (11)) should be determined. The dependencies of the water contents in the liquid medium and in the enzyme particles on $a_w$ are shown in Fig. 3(a) and 3(b), respectively. Fig. 3(a) represents the water solubility curves in the reaction mixture at five different compositions (0, 30, 48, 75, and 96% reaction conversion) at a reaction temperature of 30°C. A reduction in $C_{w,m}$ increasing conversion to the ester is expected, since the relatively polar fatty acid and alcohol are being replaced by the nonpolar ester. The experimental data (closed symbols) fitted well ($r^2$ = 0.997) with the polynomial equation (solid lines) obtained by non-linear regression analysis:

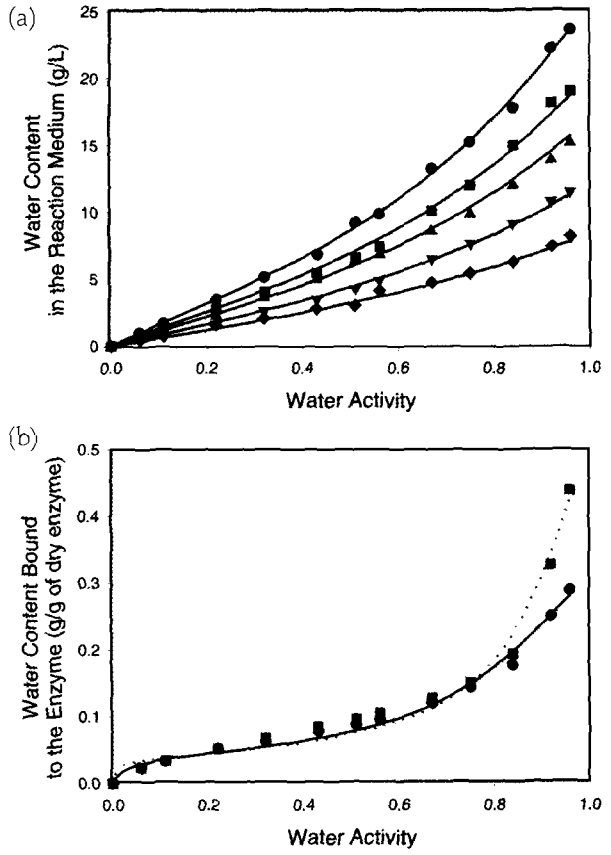


**Fig. 3.** (a) Water solubility curves in the reaction medium at five different levels of conversion at 30°C. Symbols: (●) 0%; (■) 30%; (▲) 48%; (▼) 75%; (◆) 96%. The solid lines represent nonlinear regressions of the experimental data. (b) Water adsorption and desorption isotherms of Lipozyme IM in air at 30°C. Symbols: (●) adsorption; (■) desorption. The solid line and dotted line denote the data fitting of the adsorption and desorption with the generalized BET equation, respectively.

$$C_{w,m} = (-0.1123a_w + 0.06534a_w^2 - 0.1330a_w^3)X + (16.85a_w - 7.054a_w^2 + 15.80a_w^3) \quad (12)$$

As can be seen from Equation (12), the $C_{w,m}$ was found to be linearly dependent upon the reaction conversion.

Fig. 3(b) shows the water adsorption isotherm of Lipozyme in air at 30°C (closed circle). The result is similar to that reported in the literature [18,19]. Several thermodynamic models have been proposed to fit water adsorption isotherms for nearly anhydrous proteins hydrated from the vapor phase. A linear combination of Langmuir sorption and Henry's law was used to predict water sorption by proteins suspended in nearly anhydrous organic solvents [20]. Recently, the adsorption of water by alcohol dehydrogenase from baker's yeast was measured in a continuous flow gas reactor at varying temperatures, and in this case, Huttig's isotherm was found to fit the experimental data extremely well [21]. However, none of the aforementioned models worked well in our systems. Hence, we utilized the generalized BET equation with three parameters [22]:

$$C_{w,e} = \frac{mca_w}{(1-a_w)}\left[\frac{1-(n+1)a_w^n + na_w^{n+1}}{1+(c-1)a_w - ca_w^{n+1}}\right] \quad (13)$$

where $m$ = the content of water required to form a complete monolayer; $c$= the affinity of water molecules; $n$ = the maximum number of adsorbed layers. As shown in Fig. 3(b), the experimental data fit the generalized BET model well (solid line) ($r^2$=0.990). The model parameters, $m$, $c$, and $n$ were found to be 0.04 g/g 35.2, and 14, respectively. The water desorption isotherm is also shown in Fig. 3(b) (closed rectangle). Although hysteresis was observed, as reported by others [20,21], the desorption isotherm was very similar to the adsorption isotherm up to a water activity of 0.8. Desorption data were also fitted using the generalized BET model (dashed line) and the parameters, $m$, $c$, and $n$ were 0.04 g/g, 100.6, and 26, respectively ($r^2$=0.983).

For the real-time estimation of reaction conversion, we used Equation (13) and assumed that the water sorption in organic solvents is identical to that in air. It has been reported that the difference in the water adsorption isotherms of enzymes in air and in organic solvents is insignificant for most enzymes [23]. For example, the water adsorption isotherms of immobilized lipase from *Candida antarctica* were very similar in air and in isooctane up to very high values of $a_w$ [24]. In another work on the free lipase of *Candida rugosa* [25], good agreement was obtained between the adsorption isotherms determined either in air or in organic solvents including isooctane, cyclohexane and isobutyl methyl ketone. Since water was removed for $a_w$ control throughout the present work, the parameter values obtained from the water desorption isotherm were used for the on-line conversion estimation.

## Performance of the On-line Conversion Estimation

In order to test the performance of the on-line conversion estimation, a lipase-catalyzed esterification reaction in solvent-free media was conducted with water activity controlled. Ester synthesis with $a_w$ control was carried out under the conditions described in Materials and Methods. Fig. 4(a) shows the time-course profiles of the water activity during the reaction with $a_w$ control at 0.2. Initially humidified air was supplied to increase $a_w$ to the 0.2 setpoint, subsequently dried air was bubbled into the reaction vessel to remove the water produced at the air flow rate determined by the PI controller. This explains the change of $a_w$ in the inlet air gas (closed circle). The minor oscillations of $a_w$ in the dried air might result from fluctuations in the air flow rate, and variations in the relative humidity of the atmospheric air entering the silica gel bottles. The relative humidity measured in the reactor headspace (closed rectangle) was successfully held at a constant level in

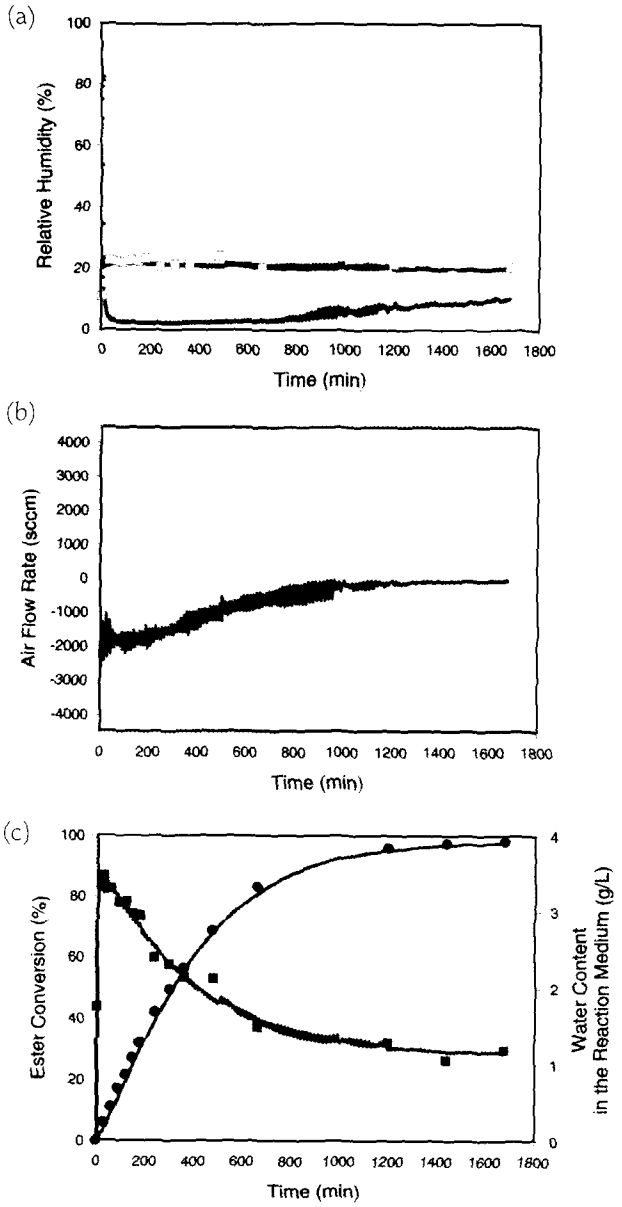


**Fig. 4.** (a) Time-course profiles of the relative humidity in the lipase-catalyzed esterification at a water activity control set point of 0.2. Symbols: (●) water activity in the air entering the reaction medium; (■) water activity in the air leaving the reaction medium; (□) water activity in the reaction medium. (b) The flow rate of humid air (positive value) and dry air (negative value) supplied for water activity control. (c) Time-course profiles of the products. (●) ester conversion; (■) water content in the reaction medium. The solid line denotes the on-line estimated conversions and water contents.

spite of the massive production of water after reaching the desired point. Variations in the air flow rate are shown in Fig. 4(b), where positive and negative values represent the flow rates of humid air and dry air, respectively. As the water production rate declined, the flow rate of dry air decreased. Fig. 4(c) shows the concentrations of ester (closed circle) and water (closed rectangle) as monitored by gas chromatography (GC) and Karl Fischer titration throughout the reaction. Even though water was produced at the same molar ratio as the ester, the water content in the reaction medium was gradually decreased by water activity control. The on-line estimated values (solid lines) were in good agreement with the measured values. As described in our previous study [17], we also compared the water activity measured in the gas phase (closed rectangle) with that calculated from the reaction conversion and the water content data (open rectangle) in Fig. 4(a). It was reconfirmed that the water was at a near-equilibrium when $a_w$ was controlled in our system.

As depicted in the above case during which $a_w$ was controlled at 0.2, Lipozyme-catalyzed esterification reactions were also carried out with $a_w$ control at different values (0.4, 0.6, and 0.8). During these reactions, the reaction conversions were estimated on-line by using Equations (9), (12), and (13). Ester conversions measured off-line by GC and on-line by computation were compared during the reaction. For esterification at $a_w$ value of 0.4, the on-line conversion estimation was as successful as it was at 0.2. However, when $a_w$ was controlled at 0.6 and 0.8, the estimated conversion did not agree with the measured values (data not shown).

## Conversion Estimation Using a Digital Filter

In order to investigate the reason for the discrepancy between the estimated and actual ester conversion with $a_w$ control at higher values, each term in the right side of Equation (9) was analyzed versus reaction time at the four $a_w$ control levels of 0.2, 0.4, 0.6 and 0.8. As a result, the $(100V_r/M_wS_0)[C_{w,m}(k+1) - C_{w,m}(k)]$ term was found to fluctuate more so under these conditions at the beginning of the reaction, particularly before $a_w$ had reached the desired set point. Fig. 5(a) and 5(b) show the variation of the value of $(100V_r/M_wS_0)\ \Delta C_{w,m}$ for reactions with $a_w$ control values of 0.2 and 0.6, respectively. The dotted lines denote the times to reach the set points. $C_{w,m}$ was initially increased, and then decreased due to the supply of dried air during $a_w$ control. Therefore, $\Delta C_{w,m}$ should be positive before $a_w$ reaches the desired set point, and thereafter it should be negative. However, as shown in Fig. 5, fluctuations of $\Delta C_{w,m}$ were observed, and these were particularly more severe for reactions with $a_w$ control at 0.6 (Fig. 5(b)). This is because $\Delta C_{w,m}$, which is sensitive to $a_w$ changes, tends to be inaccurate at the beginning where $a_w$ in the medium is increased rapidly to reach the required set point by bubbling humid air. As the set point is increased, the time required for $a_w$ to reach the set point also increases, which explains the more severe fluctuations in $\Delta C_{w,m}$ at a set point of 0.6.

On-line estimation techniques using balancing methods are known to suffer from instrumental inaccuracies [26]. The errors in the primary measurement are often large, and these errors can have profound effects on the accuracy of the estimates obtained. Accumulations of

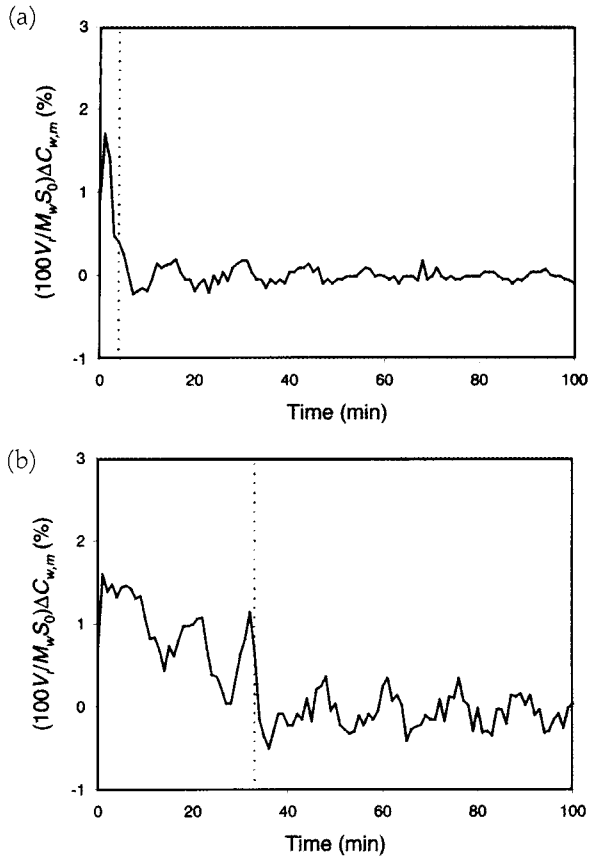


**Fig. 5.** Time-course profiles of $(100V_r/M_wS_0)\Delta C_{w,m}$ during the lipase-catalyzed esterifications with controlling $a_w$ at (a) 0.2 and (b) 0.6.

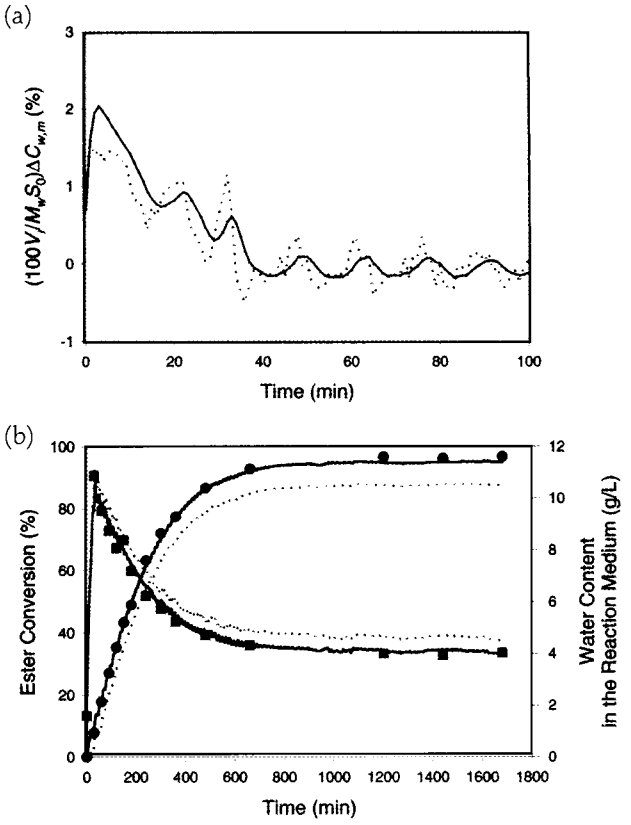


**Fig. 6.** (a) Time-course profiles of $(100V_r/M_wS_0)\Delta C_{w,m}$. (b) The performance of conversion estimation during a reaction with controlling $a_w$ at 0.6 ($\alpha$=0.35). The dotted and solid lines denote unfiltered and filtered values, respectively.

such errors can compound deviations of on-line biomass estimates from the off-line assay values as fermentation progresses [11]. Indeed, in some cases it has been found necessary to evaluate the biomass concentration in the midst of fermentation as the deviation became unacceptably large due to noisy oxygen measurements [9]. Thus, a good noise filtration algorithm should be employed to improve the reliability of the estimated values.

In order to reduce the oscillations of $\Delta C_{w,m}$, as shown in Fig. 5, a double exponential digital filter was introduced. There are several popular digital filters available, such as exponential filters, double exponential filters, moving-average filters, and noise-spike filters. Double exponential filters are known to provide better filtering of high-frequency noise. The filtered measurement is a weighted sum of the current measurement and of the past filtered values [27]

$$y_n = \alpha^2 x_n + 2(1-\alpha)y_{n-1} - (1-\alpha)^2 y_{n-2} \quad (14)$$

where $x_n$ is the measured value of the variable at the $n$th sample; $y_n$ is the filtered value of the variable corresponding to $x_n$. The smoothing constant, $\alpha$, decides the weighting. When $\alpha$ is equal to 1, no data is filtered. The closer to zero the smoothing constant becomes, the more the current measurement is ignored.

With the filtered values of $\Delta C_{w,m}$, the conversion estimation was repeated for esterifications with $a_w$ control values at the various set points. In cases of reactions with $a_w$ control at 0.2 and 0.4, the digital filtering had little effect on the performance, and the filtering ($\alpha$<0.5) worsened the performance (data not shown). In contrast, when the set point was 0.6, the performance of the conversion estimation was considerably improved by employing the double exponential filter. Fig. 6(a) shows a comparison between the time-course of $(100V_r/M_wS_0)\ \Delta C_{w,m}$ term without (dotted line) and with (solid line) the digital filter with an $\alpha$ value of 0.35 The values shown were smoothed with using the digital filter. In Fig. 6(b), the ester conversion estimate using filtered data is compared to the estimate without the filter. The performance of the conversion estimation was remarkably improved. Similarly lipase-catalyzed esterification at $a_w$ value of 0.8 was also estimated using the filter. An $\alpha$ value of 0.25 resulted in a significant improvement in the conversion estimate (data not shown). Higher set points than 0.8 made the smoothing constant closer to zero. At high values of $\alpha$, reduction in the noise level tends to be poor but the filter tracks real signal changes more easily. When $\alpha$ is small, the double

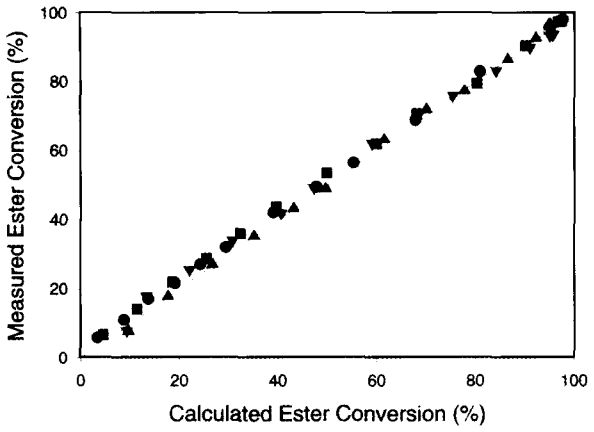


**Fig. 7.** Comparisons between on-line estimated conversion and off-line measured conversion during enzymatic esterifications with $a_w$ control at four different set points. The solid line represents an ideal line. Symbols: (●) 0.2; (■) 0.4; (▲) 0.6; (▼) 0.8.

exponential filter reduces the noise substantially but the filter is sluggish. Therefore, α has an optimum value, which depends on the process dynamics, the noise characteristics and the sampling period [28]. As a summary, comparisons between the on-line estimated and off-line measured conversion of Lipozyme-catalyzed reactions with $a_w$ control values of 0.2, 0.4, 0.6 and 0.8 are shown in Fig. 7. They showed a good agreement within ±2%.

## CONCLUSION

On-line conversion estimation through humidity monitoring was performed for lipase-catalyzed esterification reactions whilst simultaneously controlling the water activity. The measurement of relative humidity and water material balance were found to offer a means of providing real-time estimates of reaction conversion without expensive analytical instrumentation. It was shown that the on-line estimated conversion agreed well with the off-line measured values within ±2% during lipase-catalyzed esterification reactions with water activity control. When the set point $a_w$ was 0.6 or 0.8, the discrepancy between the estimated conversion and the measured value was significant, because of inaccurate measurement of the water content in the reaction medium, while the water activity was increased initially to the desired value by humid air bubbling. Using a double exponential digital filter, we were able to improve the accuracy of the method considerably. Consequently, the computer-aided control system that we developed in this work makes it possible to control water activity for enzymatic reactions in nonaqueous media and at the same time to estimate the on-line reaction conversion.

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## NOMENCLATURE

| | |
|---|---|
| $\alpha$ | Smoothing constant |
| $a_w$ | Water activity |
| $c$ | Affinity of water molecules in the generalized BET equation |
| $C_{w,e}$ | Water concentration bound to the enzyme particles |
| $C_{w,m}$ | Water concentration in the reaction medium |
| $f_w$ | Fugacity of water in the mixture |
| $F_w$ | Mass flow rate of air |
| $k$ | Number of sampling |
| $m$ | Monolayer water content in the generalized BET equation |
| $m_e$ | Amount of enzyme |
| $M_w$ | Molecular weight of water |
| $n$ | Maximum number of the adsorbed layers in the generalized BET equation |
| $P_w$ | Partial pressure of water in the air |
| $q$ | Volumetric flow rate of the air |
| $R$ | Gas constant |
| $r_w$ | Rate of water production by the reaction |
| $S$ | Amount of substrate |
| $t$ | Time |
| $T$ | Temperature |
| $V_r$ | Volume of the reaction medium |
| $X$ | Reaction conversion |
| $x_n$ | Measured value of the variable at the $n$th sample |
| $y_n$ | Filtered value of the variable corresponding to $x_n$ |

### Subscripts

| | |
|---|---|
| $in$ | Inlet |
| $out$ | Outlet |

### Superscript

| | |
|---|---|
| ° | Pure compound |

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