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MATHEMATICAL MODELING FOR THE ESTIMATION OF LIPASE ACTIVITY BY AGAR DIFFUSION METHOD

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Abstract – A mathematical model was developed to estimate the lipase activities from the experimentally obtained physical dimension of halo, formed by a diffusing lipase through agar media. A diffusion equation based upon the mass balance was derived in cylindrical coordinates, the integration of which was carried out by using finite difference method. A regressional analysis of the experimental data using the proposed model has brought the values for the hindered diffusivity of lipase through agar media and the minimum effective lipase concentration of 0.817×10^{-6} cm^2/s and 2.86 unit/cm^3, respectively. The procedures, delineated in this work are considered to be useful for the quantitative analysis of halo-forming enzymes at predetermined standard conditions.

Key words: *Lipase, Activity, Halo, Agar, Modeling*

INTRODUCTION

Biotechnology has made an enormous contribution to the human society for the last score or so, including the production of valuable biochemicals using various biological expression systems [Fransman et al., 1995]. Among the many commercial bioproducts, enzymes, a form of protein, are considered to be one of the most valuable products, as a biological catalyst. Lipase is a leading enzyme in the selling quantity, spanning the industries of detergents, lipid hydrolysis, food additives, pharmaceuticals (digestives, diagnostic enzymes, *etc.*) and fine chemicals. The production efficiency is directly related to the economics of lipase production, and the development of a simple and precise assay method to monitor the production is an important matter.

Several assay methods are used for the analysis of lipase, the titration method, the cupric acetate method, the plate agar diffusion method, the SDS PAGE, to cite a few [Lee and Rhee, 1994; Kouker and Jaeger, 1987]. The first two methods are applicable for the quantitative measurement of enzymes. The agar diffusing method is commonly used for the qualitative evaluation of lipase. In this study, a mathematical modeling was carried out to see the applicability of the agar diffusion method to the quantitative analysis of halo-forming enzymes. A mathematical model, based upon the diffusion equation was derived in cylindrical coordinates to express physically the spreading behaviors of enzyme by diffusion from the well in the shallow agar plate. The diffusivity of lipase through agar media and the minimum effective (threshold) lipase concentration were obtained by a regressional analysis which compared the experimentally obtained halo sizes with the calculated values from the proposed model.

THEORETICAL CONSIDERATIONS AND MATHEMATICAL FORMULATION

Lipase, supplied to the agar plate in an aqueous solution, diffuses through the media by molecular diffusion, and decomposes the tributyrin in the media, resulting in the formation of circular halo (clear zone) (Fig. 1). The size (diameter) of halo becomes larger with the proceeding diffusion front as time passes. A mathematical model was formulated based upon the consideration that the dimension of the developing halo will be re-

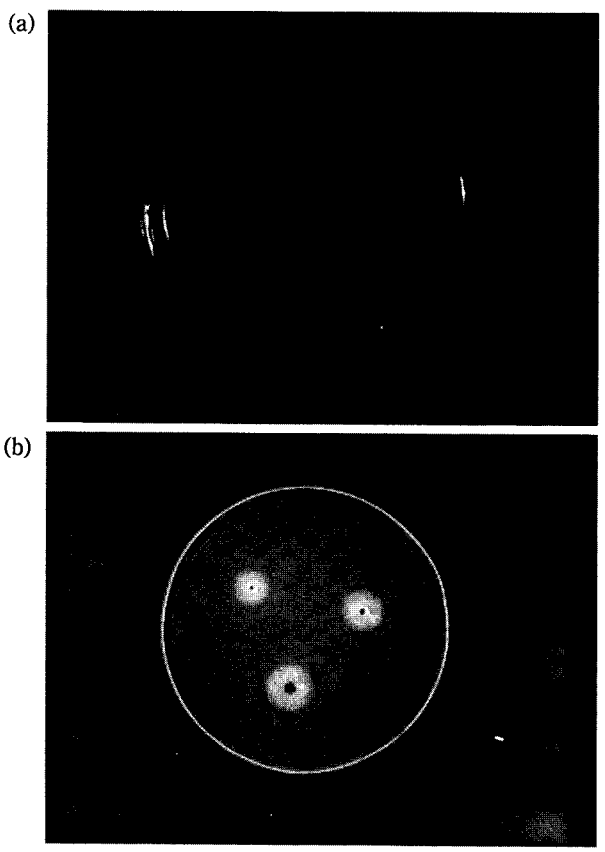

Fig. 1. Halo formations with different well size (2, 3, 4 mm) in (a) LAT media and (b) rhodamine B media.

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lated to the hindered diffusion rate and the lipase concentration.

The diffusing lipase through agar media is assumed to obey the Fick's law. Additional assumptions to formulate the model are as follows.

- Agar plate is homogeneous, having lipase diffuse to all the circumferential directions at the same speed.
- Tributyrin in the turbid LAT media decomposes quickly to form a clear zone when the concentration of lipase increases to pass the threshold value (Fig. 2).
- Lipase sustains a constant activity in the media throughout the experimental period.

The lipase solution, applied to the well in agar plate in the beginning, is assumed to be instantaneously absorbed into the surrounding media, forming a ring which contains the solution having the same concentration with the feed, as shown in Fig. 3.

The Fick's second law of molecular diffusion, expressed in the cylindrical coordinates, forms a parabolic partial differential equation with the initial conditions, shown in Fig. 3.

$$\frac{\partial C}{\partial t} = D\Delta^2 C \tag{1}$$

$$= D\left(\frac{1}{r}\frac{\partial C}{\partial r} + \frac{\partial^2 C}{\partial r^2}\right) \tag{2}$$

I.C.: $C = C_0$ for $R_w < r < R_r$

$C = 0$ $r < R_w$ & $R_r < r$

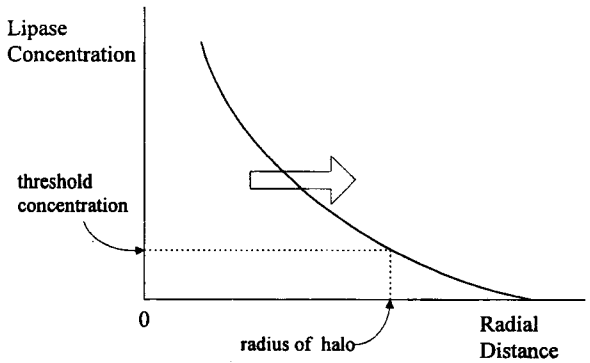

Fig. 2. Halo formation at the threshold concentration of lipase.

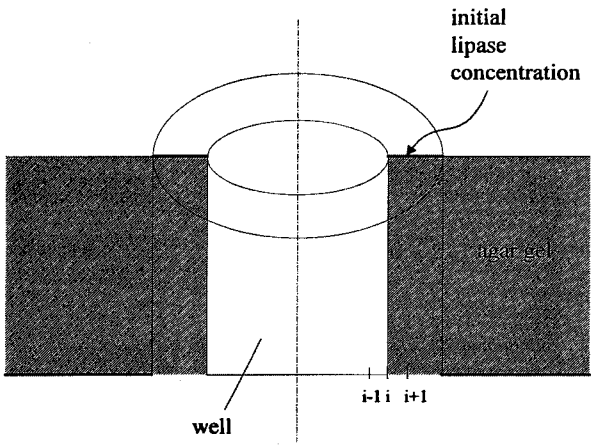

Fig. 3. Initial lipase concentration at the absorption ring surrounding the well.

where D_e is the effective hindered diffusion coefficient, C_0 is the feed enzyme concentration, R_w and R_r are the radius of well and the outer radius of absorption ring, respectively. The lipase concentration as a function of time and radial distance can be solved numerically by using the finite difference method [Constantinides, 1987].

$$\frac{1}{\Delta t}(C_{i,j+1} - C_{i,j}) = \left[\frac{1}{i\Delta r}\frac{D}{2\Delta r}(C_{i+1,j} - C_{i-1,j}) + \frac{D}{\Delta r^2}(C_{i+1,j} - 2C_{i,j} + C_{i-1,j})\right] \tag{3}$$

$$\frac{1}{\Delta t}C_{i,j+1} = \frac{1}{\Delta t}C_{i,j} - \frac{2D}{\Delta r^2}C_{i,j} + \frac{D}{2i\Delta r^2}(C_{i+1,j} - C_{i-1,j}) + \frac{D}{\Delta r^2}(C_{i+1,j} + C_{i-1,j}) \tag{4}$$

$$C_{i,j+1} = \left(1 - \frac{2D\Delta t}{\Delta r^2}\right)C_{i,j} + \frac{D\Delta t}{\Delta r^2}\left[\left(\frac{1}{2i} + 1\right)C_{i+1,j} - \left(\frac{1}{2i} - 1\right)C_{i-1,j}\right] \tag{5}$$

where $C_{i,j}$ is the concentration at the radial grid position i and the time grid of j, Δr and Δt are the grid sizes for radial distance and time, respectively. The hindered diffusion coefficient, D, is an unknown parameter yet to be determined. A regressional analysis with the hindered diffusion coefficient and the threshold value of lipase concentration as parameters was carried out, comparing the model predictions with the experimental data. For the regressional analysis to get the minimum of a function by a quasi-Newton method, the subroutine ZXMIN in IMSL was used in Fortran language [IMSL, 1984].

EXPERIMENTAL

1. Lipase Solution

Lipase (MW, 50,000) is an esterase, produced by *Candida rugosa*. Lipase solution was prepared by solving 1 g of lipase in the form of powder reagent (*Candida rugosa*, 360,000 unit/g) and 0.03 g of sodium azide in 100 ml of sterile 0.1 M potassium buffer solution (pH 7.0). Addition of sodium azide is for the prevention of microbial growth. The diluted lipase solutions by two and five times were used in addition to the original solution. The activity of original lipase solution was 10,800 unit/cm^3.

2. Agar Plate Preparation

The LB agar media containing trybutyrin (LAT media), and the rhodamine B media were used as solid media for the analysis of lipase activity. The LAT media was prepared by addition of 0.5 % (w/v) tributyrin into 100 ml LB media with the subsequent sonication. The solution was sterilized after the addition of 1.5 % (w/v) agar, and poured into the petri dish at between 40 °C and 50 °C to make gel plate with the thickness of 0.5 cm. The rhodamine B media was made of 0.3 g beef extract, 0.5 g Bacto-tryptone, 0.4 g NaCl, 1.5 g agar and 2.5 g olive oil in 100 ml distilled water, with pH adjusted at 7.0. At 60 °C, after sterilization, the solution was added by 1 ml rhodamine B solution of 0.001 % (w/v) and sonicated for 1 min. Removing air bubbles by settling, the solution was poured into the petri dish to make gel plate with the thickness of 0.5 cm.

The holes to hold the lipase solutions were made using gel punchers with diameters of 2, 3 and 4 mm. The solutions with different concentrations (undiluted, twofold and fivefold diluted) were added to the holes of each sizes in triplicate. The volumes of feed solution were as much as to fill the holes to the top, and were roughly 4, 9 and 16 μl for the holes with diameters of 2, 3, 4 mm, respectively. The size of halos was measured by a ruler with the constant time interval in 9 LAT media plates, incubated at 37°C. The halos in LAT and rhodamine B media were observed without and with the aid of UV light, respectively.

RESULTS AND DISCUSSION

1. Halo Formation

Esterase is a hydrolysing enzyme, which attacks an ester, splitting it into 2 groups, one of which is an acid. As a common practice of detecting esterases, agar plate containing LAT media was used. At the same time, agar plate containing rhodamine B media was used for specific detection of lipase, which belongs to a group of esterase. Halo formations in these plate were shown in Fig. 1. The applied solution was observed to disappear and absorb quickly into the surrounding gel. As expected, the diameter of halos increased with time in both media. The size of halos were observed to increase with increasing well diameter at a given time. In both experimented media of LAT and rhodamine B, halo sizes were found to be almost same at a given time. The time course of halo formations in the LAT media were measured (Table 1), and employed for the analysis in this study. The average values were taken from the triplicated experiments.

2. Numerical Integrations and Regressional Analysis

The numerical calculations were carried out by using the finite difference method with the aid of a personnal computer (CPU, Pentium). The outer radius of the absorption ring, Rr, was set to the radius of the well, multiplied by $\sqrt{2}$ [$\pi \times R_w^2 \times l = \pi \times (R_r^2 - R_w^2) \times l$, l: thickness of agar plate]. For the reliable convergence, the lipase concentration at the point (C_{i-1}), just inside the radius of the well was put to be same with the concentration at the inner radius of the absorption ring (C_i). The convergences were successful with the grid size of 0.01 cm and 1 sec for radial distance and time, respectively. The next condition is known to be crucial to the convergence of this method [Constantinides, 1987].

$$\frac{D\,\Delta t}{\Delta r^2} \leq \frac{1}{2} \tag{6}$$

Fig. 4 presents model predictions of the case with the well diameter of 2 mm, containing undiluted lipase solution. As expected, concentration profiles became lower and spreaded to the radial direction with time.

The amount of lipase in the plate at a given time was calculated by integrating the concentration profile of model prediction (Fig. 4), and compared with those at different times.

$$A = \int_{R_w}^{R_h} 2\pi r l \; C(r)\, dr \tag{7}$$

where A is the total amount of lipase in terms of enzyme activity, R_w and R_h are the radius of well and halo, respectively, C(r) is the concentration at the radial distance of r, and l is the thickness of agar plate. Numerical integration for Eq. (7) was carried out by using the Simpson's formula with the aid of a personal computer. The total amount of lipase in the plate each time were equal to each other within the error bound of 2.5 %. This confirms the appropriateness of the numerical calculations, employed in this study. The resulting errors between them are ascribed to the negligence of the lipase concentration lower than the threshold value, which corresponds to the outskirts of the expanding halo.

The most probable diffusivity and threshold enzyme concentration to give the best fitting between the model predictions and the experimental data were sought by computer calculations. A regressional analysis was carried out to locate the local minima of the squared discrepancy between model predictions and observations by using subroutine ZXMIN in IMSL. The 5 most probable minima are listed in Table 2 for 9 cases, which are 3 dilutions for each 3 well sizes. Among them, the set of the hindered diffusion coefficient and the threshold concentration of lipase of 0.817×10^{-6} cm^2/s and 2.86 unit/cm^3, respectively (the values with asterisk in Table 2), gave the most frequent appearances (6 out of 9 cases) as a local minimum. This set of values is presumed to present the global minimum, along with the con-

Table 1. Radius of halos (cm) with well diameter and dilution as parameters

Well diameter / Time (hr)	0.2 cm			0.3 cm			0.4 cm		
	×1	×2	×5	×1	×2	×5	×1	×2	×5
1.0	0.45	0.30	0.30	0.42	0.38	0.35	0.50	0.47	0.40
2.5	0.47	0.45	0.40	0.55	0.47	0.45	0.63	0.58	0.50
4.0	0.65	0.63	0.58	0.63	0.60	0.55	0.70	0.65	0.60
5.5	0.77	0.70	0.68	0.83	0.79	0.75	0.90	0.88	0.83
7.0	0.80	0.77	0.72	0.88	0.85	0.80	0.97	0.90	0.85
8.5	0.90	0.82	0.77	0.95	0.90	0.85	1.02	1.00	0.92
10.0	0.96	0.87	0.82	1.03	0.95	0.90	1.10	1.05	1.00
11.5	0.98	0.91	0.85	1.06	1.00	0.95	1.11	1.09	1.00

*Data triplicated

**×1: no dilution, ×2: twofold dilution, ×5: fivefold dilution

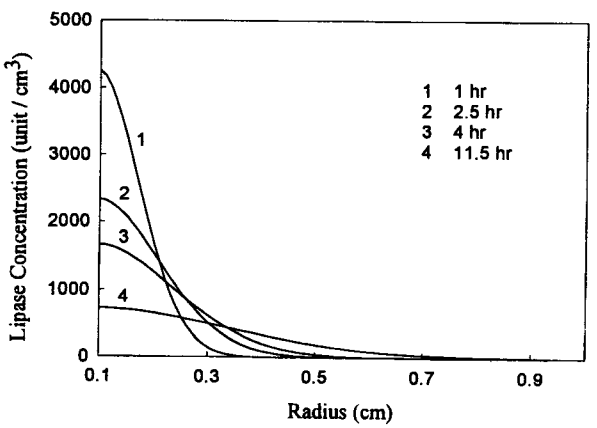

Fig. 4. Calculated lipase concentration profiles with time as a parameter.

Table 2. Regressional results with hindered diffusivity and threshold concentration as parameters

Well diameter	Dilutions	Hindered diffusivity [cm^2/s]	Threshold concentration [$unit/cm^3$]	Squared errors
0.2 cm	×1	0.103442E-05	5.70155	0.0127
		0.111808E-05	7.11293	0.0137
		0.103442E-05	6.58781	0.0163
		*0.816898E-06	*2.86495	*0.0189
		0.111587E-05	4.45803	0.0208
	×2	*0.816898E-06	*2.86495	*0.0164
		0.101870E-05	5.92035	0.0182
		0.101870E-05	6.84600	0.0188
		0.109821E-05	7.63357	0.0192
		0.136957E-05	13.5523	0.0236
	×5	0.102770E-05	4.48278	0.0199
		0.104003E-05	4.69827	0.0221
		0.114635E-05	4.95636	0.0216
		0.811086E-06	2.85505	0.0224
		*0.816898E-06	*2.86495	*0.0229
0.3 cm	×1	0.916338E-06	4.93668	0.0191
		*0.816898E-06	*2.86495	*0.0197
		0.984601E-06	6.82713	0.0225
		0.123172E-05	17.7728	0.0236
		0.879379E-06	5.84078	0.0240
	×2	*0.816898E-06	*2.86495	*0.0325
		0.105749E-05	7.87652	0.0361
		0.904792E-06	7.87652	0.0388
		0.111151E-05	7.79283	0.0400
		0.904792E-06	9.14654	0.0445
	×5	*0.816898E-06	*2.86495	*0.0309
		0.921498E-06	4.09174	0.0328
		0.921498E-06	4.68301	0.0332
		0.990993E-06	4.93685	0.0339
		0.884664E-06	4.50253	0.0347
0.4 cm	×1	*0.816898E-06	*2.86495	*0.0177
		0.875078E-06	7.65407	0.0214
		0.127958E-05	19.7995	0.0218
		0.102233E-05	7.65407	0.0222
		0.107601E-05	17.1141	0.0229
	×2	*0.816898E-06	*2.86495	*0.0285
		0.119190E-05	15.6813	0.0320
		0.986070E-06	10.6544	0.0329
		0.119190E-05	18.1723	0.0334
		0.115358E-05	10.6544	0.0335
	×5	*0.816898E-06	*2.86495	*0.0488
		0.103279E-05	10.0421	0.0520
		0.106895E-05	8.10201	0.0521
		0.908203E-06	7.15527	0.0533
		0.914485E-06	8.10201	0.0539

siderations that the sets, representing minima for the other 3 cases, have different values from each other. Noting that the diffusivity of a solute in free solution is in the order of 10^{-5} cm^2/s, the value for the hindered diffusion coefficient of 0.817×10^{-6} cm^2/s is judged to be reasonable. It is also notable that this value for D is not much different from those for the other 3 cases, which are 0.916, 1.034 and 1.027×10^{-6} cm^2/s. The comparisons between the experimental data and the model predictions, using the optimum values for the diffusivity and

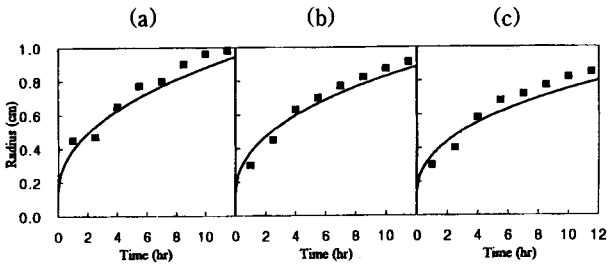

Fig. 5. Comparisons of the halo sizes between the model predictions (—) and the observations (■) with different dilutions, well size: 2 mm, a) no dilution, b) twofold dilution, c) fivefold dilution.

the threshold concentration for the case with the well diameter of 2 mm are shown in Fig. 5. The unpresented graphs for the cases of 3 and 5 mm are of similar patterns with that of 2 mm. This model with the proposed numerical calculations appears to predict the experimental data with a reasonable precision. The discrepances between the experimental values and the predictions are thought to come from the experimental error, mostly in the part of measuring the size of halos. Commercial image analyzers may be used for the accurate measurement of halo size. The assumption of instantaneous ring formation mentioned in the previous section may have provided another source of this discrepancy.

Following the procedures described above, the activity of most halo-forming enzyme solution can be determined by measuring the halo size as a time course, followed by a regressional analysis with the predetermined values of the diffusivity and the threshold concentration. Conventionally, the activities of halo-forming enzymes are decided by the size of halo at a given time from the standard curve of enzyme activity and halo size, as a simple and qualitative way, unless more reliable quantitative assay methods are available.

CONCLUSIONS

A mathematical model to predict the diffusing behaviors of the halo-forming lipase was proposed, based upon the mass balance of enzyme in the agar plate. The model employed several assumptions, such as a quick uniform diffusion of the enzyme solution from the well into the surrounding media at the beginning, an instantaneous reaction between tributyrin and lipase, and a lasting activity of lipase. The resulting partial differential equation, in the form of Fick's second law of molecular diffusion, was solved numerically by using the finite difference method. A regressional analysis, comparing the model predictions with the experimental data, gave the values of 0.817×10^{-6} cm^2/s and 2.86 $unit/cm^3$ for the effective hindered diffusion coefficient and the threshold concentration of lipase, at which a halo forms, respectively. The procedures, delineated in this study, will be useful to understand the mechanism of halo formation and to decide quantitatively the activities of halo-forming enzymes as well.

ACKNOWLEDGEMENT

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NOMENCLATURE

A : total amount of enzyme in the agar plate [unit/cm^3 · cm^3]
C(r) : concentration at the radial distance of r [unit/cm^3]
$C_{i,j}$: concentration at the radial grid position i and the time grid of j [unit/cm^3]
C_0 : feed enzyme concentration [unit/cm^3]
D_e : effective hindered diffusion coefficient [cm^2/s]
l : thickness of agar plate [cm]
r : radial distance [cm]
R_w : radius of well [cm]
R_r : outer radius of absorption ring [cm]
R_h : radius of halo [cm]
t : time [s]

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