

# Interactive Influence and Optimization of Reaction Parameters in the Hydrolysis of Olive and Coconut Triacylglycerols by *C. cylindracea* and *R. javanicus* Lipases in Reverse Micellar Media

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## ABSTRACT

The four reaction parameters—pH, reaction temperature, molar ratio (R) of water to surfactant, and Aerosol-OT concentration—have significant linear, quadratic, and interactive effects on the initial rate and the degree of hydrolysis for lipase-mediated hydrolysis of triacylglycerols (TAG) in Aerosol-OT/iso-octane reverse micellar media. Reaction temperature and pH had the most significant influence on the rate and the degree of hydrolysis, whereas Aerosol-OT concentration had the least influence on those parameters. The initial rate was most influenced by the interactive effect of pH with all other variables, whereas the degree of hydrolysis was the most influenced by the interactive effect of reaction temperature with other variables. Lower-level variable combinations were favorable over higher-level variable combinations to TAG hydrolysis in reverse micellar media. Regression models, developed for the initial rate and the degree of hydrolysis as a function of reaction variables, accounted for up to 96% of the variation in the two responses. The optimum reaction condition ranges were determined based on maximization of both the rate and the

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degree of hydrolysis. The differences in the physicochemical characteristics of substrates had no significant effect on the optimum condition ranges. However, the noticeable differences were observed for these ranges between the systems with *Rhizopus javanicus* and *Candida cylindracea* lipases. Lipase-catalyzed hydrolysis of TAG in Aerosol-OT/iso-octane reverse micellar media was optimum at about 22°C, 140 mM Aerosol-OT concentration, pH 6.8, and R value of 14.

**Index Entries:** Triacylglycerols; hydrolysis; reaction parameters; reverse micellar media.

## INTRODUCTION

Triacylglycerol acylhydrolase, EC 3.1.1.3 (lipase)-mediated hydrolysis of fats and oils is a promising alternative to the physicochemical processes for the production of fatty acids and glycerol. Until recently, the lipase reactions have been carried out in biphasic media, such as oil-in-water emulsion, which has limited commercial success because of higher reaction volume, enzyme cost, mass-transfer limitation, and substrate or product inhibition. Lipase catalysis in practically water-free media, such as reverse micellar solution (1-9), has received much attention. Such media offer several advantages over conventional aqueous emulsions and overcome some of the disadvantages associated with aqueous systems. In reverse micellar media, water and enzyme are solubilized in the core of micelles formed by surfactant in organic solvent. The water-insoluble substrate can be solubilized in bulk solvent, which provides an enormous interfacial area for biocatalysis with minimum energy input. The potential for the commercial applications of lipases has been increased with the development in this novel system, in conjunction with availability of low-cost commercial enzyme and possibilities of recovery of enzyme for reuse.

Rhee and his coworkers (10-14) and others (2,5,15,16) have conducted a number of studies on hydrolysis of triacylglycerols (TAG) in reverse micellar media by lipase enzymes. These studies have primarily focused on one variable at a time and held others at some constant value. Such an approach does not furnish important information regarding the interactive influence of reaction parameters on the enzymatic reactions. Also, information obtained by simultaneously varying the reaction parameters is more useful for the practical application. Therefore, the lipase-mediated hydrolysis of TAG in reverse micellar media was studied by a multivariable approach. The aim is to provide information useful in determining reaction conditions, which would increase lipase activity and degree of hydrolysis. In this article, the interactive influence of various reaction parameters on the hydrolytic reaction, optimization of reaction conditions, and models to predict the rate and degree of hydrolysis are presented.

## MATERIALS AND METHODS

### Materials

Lyophilized preparation of *Candida cylindracea* lipase (Type VII-S) was purchased from Sigma Chemical Co. (St. Louis, MO). Lipase from *Rhizopus javanicus* (FAP-15) was a gift from Amano International Enzyme Co. (Troy, VA). The specific activities of the two lipases, measured under standard aqueous conditions (29), were 21.4 and 58.9 U/mg, respectively. Olive and coconut TAG (saponification value, 190.5 and 255.6, respectively) were purchased from Sigma Chemical Co. Bis(2-ethylhexyl) sodium sulfocuccinate (Aerosol-OT) of >99% purity, purchased from Aldrich Chemical Co. (Milwaukee, WI), was used without further purification. HPLC-grade iso-octane was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Oleic acid of 99.9% purity was obtained from Sigma Chemical Co., and was used as a reference standard. Benzene and pyridine of spectrophotometric grade were purchased from Aldrich Chemical Co. All other reagents were of analytical grade.

### Preparation of Reverse Micellar Media

A 250-mM stock solution of Aerosol-OT in iso-octane was prepared and stored at room temperature after deaeration. Reverse micellar media of various compositions were prepared by mixing appropriate volumes of the stock solution of Aerosol-OT in iso-octane, substrate TAG and iso-octane in batch reactors of 10-mL volume. The desired enzyme concentrations and water contents were obtained by injecting appropriate volumes of buffer solution and the concentrated buffered enzyme stock solution using a positive displacement micropipeter. After adding the enzyme solution, the micellar solutions were mixed on a Vortex stirrer, until a clear single-phase medium was formed.

### Hydrolysis of TAG in Reverse Micellar Media

Reaction media containing the substrate were brought to the appropriate temperature before addition of the enzyme solution. Hydrolysis of TAG was initiated by injecting the enzyme solution and Vortex-mixing until the medium became clear. At predetermined time intervals, the appropriate volumes (10–400  $\mu$ L) of the reaction mixtures were transferred into 15-mL screw-cap tubes containing a predetermined volume of benzene, yielding a total volume of 5 mL. The tubes were immediately Vortex-mixed to stop the enzyme reaction, and were stored until later measurement of liberated fatty acids.

Table 1  
Variable-Level Combinations  
for Four Variable Rotatable Central Composite Design<sup>a</sup>

Coded levels	Reaction parameters, independent variables			
	pH	R, [water]/[surfactant]	Aerosol-OT concentration, mM	Temperature, °C
-2 (-α)	6	5.00	50.0	10.0
-1	7	8.75	87.5	17.5
0	8	12.50	125.0	25.0
1	9	16.25	162.5	32.5
2 (α)	10	20.00	200.0	40.0

<sup>a</sup>The center point ( $n_0$ ) was replicated six times and the entire experiment was replicated twice. The additional points were excluded from second-order model fitting.

### Determination of Initial Rate and Degree of Hydrolysis

The liberated free fatty acids were quantified using a method described by Lowry and Tinsley (17). The concentration of liberated fatty acids was calculated from the standard curve generated using oleic acid as the calibration standard.

Unless otherwise stated, the initial rate (activity) is expressed as  $\mu\text{mol Eq}$  of free fatty acid liberated/min under the assay condition based on 20 min of reaction time. The kinetics of hydrolysis were linear up to 30 min at the substrate concentration used in this study. The degree of hydrolysis or percent conversion was calculated as follows:

$$\% \text{ Degree of hydrolysis} = (\text{Liberated fatty acids} / \text{Total number of ester bonds}) \times 100 \quad (1)$$

The total number of ester bonds in TAG was estimated from the saponification value determined by standard procedure (18). Unless otherwise stated, the degree of hydrolysis is expressed for 24 h of reaction.

### Experimental Design

In order to examine the effects of temperature, pH, molar ratio of water to surfactant (R), and surfactant concentration on lipase-catalyzed hydrolysis in reverse micellar media, a rotatable central composite design (19) with four-variable and five-level pattern (Table 1) was adopted. The total number of observations was 30, which include 16 factorial (cubic) points, 8 axial points, and 6 center-point replicates. The axial points represent the extreme high and low values of independent variables (reaction conditions). The entire design was replicated twice. The data were fit to the second-order function as follows:

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where  $Y_i$  = initial rate of degree of hydrolysis;  $\beta_0$  = center point of system,  $\beta_i$  = coefficient of linear effects of reaction;  $X_i$  = pH, temperature,  $R$ , or surfactant concentration parameters,  $\beta_{ii}$  = coefficient of quadratic effects of reaction parameters, and  $\beta_{ij}$  = coefficient of interactive effects of reaction parameters.

In addition to these 30 experimental points, 8 additional points (Table 1) representing +1 and -1 coded levels of each reaction condition were studied to provide a complete set of 5 observations. These observations were made to assess the effect of each variable on the lipase reaction, as studied over the entire range of the variable levels at a fixed level of all the other variables. Also, these additional observations were excluded from the second-order model fitting, so that they can be used to check the efficacy of model by comparing the experimental and predicted values.

### Statistical Analysis

The coefficient of each parameter in the model was determined by General Linear Model (GLM) procedure using Minitab (20) statistical software. The three-dimensional surface plots, showing effects of two reaction conditions (holding all others at a constant value), were generated by Axum (20) graphical software using the GLM regression model. The optimum reaction condition ranges were determined by a four-factor Taylor polynomial optimization program using Design-Expert (21) statistical software.

## RESULTS AND DISCUSSION

In addition to enzyme and substrate concentration, temperature, molar ratio ( $R$ ) of water to surfactant, pH, and surfactant concentration are the key reaction parameters affecting lipase catalysis in reverse micellar media. The concentrations of the enzymes and substrates were kept constant for better assessment of the interactive effect of the other variables on the hydrolysis of olive and coconut TAG by *R. javanicus* and *C. cylindracea* lipases in Aerosol-OT/iso-octane reverse micellar media. The two enzymes were selected based on their positional specificity observed in aqueous emulsion. *R. javanicus* exhibits sn-1(3) specificity, whereas *C. cylindracea* exhibits no specificity in terms of position of fatty acid on the glycerol moiety. The two model substrates—olive and coconut TAG—were selected based on the significant difference in their physicochemical characteristics. The hydrolysis reactions were carried out at the substrate concentrations of 10%, v/v, and enzyme concentrations of 0.5 and 1.38 mg/10 mL reaction mixture for *R. javanicus* and *C. cylindraceae* lipases, respectively.

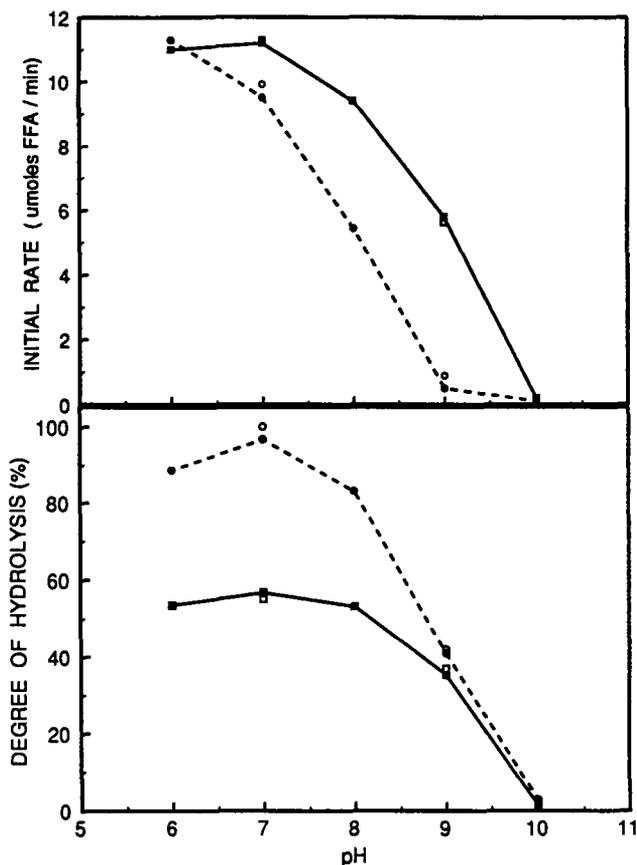


Fig. 1. Effect of pH on the initial rate and the degree of hydrolysis for the olive TAG hydrolysis in Aerosol-OT/iso-octane reverse micellar media by *R. javanicus* and *C. cylindracea* lipases. Reaction parameters: temperature = 25°C, [AOT] = 125 mM,  $R = 12.5$ , substrate concentration = 10% v/v; enzyme concentration: *R. javanicus* lipase (solid lines) = 0.5 mg/10 mL of reaction mixture, *C. cylindracea* lipase (dotted lines) = 1.38 mg/10 mL of reaction mixture; buffer: 20 mM glycine-glycine; open bullets represent predicted response.

### Effects of Reaction pH

The reaction pH is referred to the pH of the buffer that was used for the preparation of enzyme solution and reverse micellar system. The pH inside the water pool of reverse micelles could be different than the bulk pH, depending on the value of  $R$  and buffer used. Nevertheless, considering bulk pH as the reaction pH is a reasonable approximation for practical purposes. Figure 1 shows the effect of pH on the initial rate and degree of hydrolysis for olive TAG hydrolysis by *R. javanicus* and *C. cylindracea* lipases. As pH was increased from 6 to 10, the initial rate for *C. cylindracea* lipase-catalyzed hydrolysis of olive TAG gradually decreased to pH 9.0, with the highest rate at pH 6.0 and no detectable activity between pH 9–10.

However, *R. javanicus* lipase-catalyzed hydrolysis exhibited the maximum rate at pH 7.0 with considerable activity around pH 9.0. Han and Rhee (11) found the maximum activity of *C. cylindracea* at pH 7.1 in Aerosol-OT reverse micelles. The discrepancy may be owing to the difference in buffer components or enzyme concentration (13) used in both studies. In an aqueous emulsion system, the maximum activity of *C. cylindracea* lipase on olive TAG is found to be between pH 5.0 and 7.0 (24,25).

The degree of hydrolysis was maximum at pH 7.0 in pH range of 6–10 for the two enzymes. In the pH range of 6–9, olive TAG was hydrolyzed to a greater extent by *C. cylindracea* lipase than by *R. javanicus* lipase. The apparent difference could be ascribed to a difference in their specificity for the position of fatty acids on glycerol moiety. The rapid decrease in initial rate or degree of hydrolysis beyond pH 9.0 may be owing to the instability of Aerosol-OT under alkaline pH conditions.

### Effect of Molar Ratio of Water to Surfactant (*R*)

Water is indispensable for enzyme catalysis. It plays an important role in all noncovalent interactions for maintaining the active conformation of enzyme and in enzyme dynamics. Accordingly, it is reasonable that current research on enzyme reactions in organic solvent is focused on the effect of water (12,13,23,26). In reverse micellar media, one of the critical parameters that influences the reaction rate and the degree of hydrolysis for lipase-mediated hydrolysis of TAG is the water content, expressed as *R*. The value of *R* dictates the properties of water in aqueous pool within the reverse micelles, the nature of enzyme microenvironment, and the amount of water necessary for hydrolytic reaction and hydration of enzyme. So far, the role of water in enzymatic reactions in organic solvents is still obscure, although it is discussed in terms of hydration of enzyme (13,23,26–28). For hydrolytic reaction, the role of water is even more critical, because water is consumed during reaction and can affect the reaction equilibrium.

Figure 2 shows the effect of *R* on the initial rate and the degree of hydrolysis for olive TAG hydrolysis by *C. cylindracea* and *R. javanicus* lipases. With increase in *R* from 5 to 20, the initial rate gradually decreased, exhibiting highest rate at *R* = 5 for both enzyme systems. This observation is not in agreement with the results reported by other researchers (6,11), who observed bell-shaped dependence of the initial rate on *R*, with the maximum rate at *R* value of 10–12. However, this observation is in agreement with that of Han et al. (13). They observed a similar decrease in the initial rate with the increase in *R* from 5 to 20, exhibiting highest activity at *R* value of 5. At *R* value <5, lipase activity was significantly lower. They also demonstrated that the *R*-activity profile is greatly influenced by enzyme concentration. The discrepancy in the results between our study and the other studies (6,11) regarding *R*-activity profile may be the result

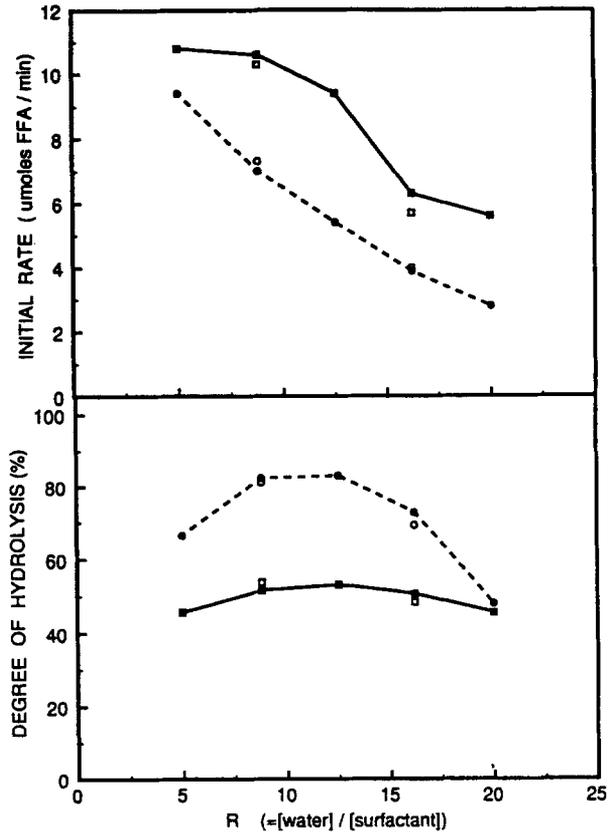


Fig. 2. Effect of molar ratio of water to surfactant ( $R$ ) on the initial rate and the degree of hydrolysis for the olive TAG hydrolysis in Aerosol-OT/iso-octane reverse micellar media by *R. javanicus* and *C. cylindracea* lipases. Reaction parameters: temperature = 25°C, [AOT] = 125 mM pH = 8.0 (20 mM glycine-glycine buffer), substrate concentration = 10% v/v; enzyme concentration: *R. javanicus* lipase (solid lines) = 0.5 mg/10 mL of reaction mixture, *C. cylindracea* lipase (dotted lines) = 1.38 mg/10 mL of reaction mixture; open bullets represent predicted response.

of differences in buffer components (29) or difference in enzyme concentration (13) used in the studies. The degree of hydrolysis exhibited bell-shaped dependence on  $R$  in the range of 5–20, with the maximum extent of hydrolysis at  $R \approx 11$  for *C. cylindracea* lipase and at  $R \approx 12.5$  for *R. javanicus* lipase. Olive TAG was hydrolyzed up to 85% by *C. cylindracea* lipase and up to 55% by *R. javanicus* lipase in the range of  $R$  value studied.

### Effect of Temperature

Figure 3 shows the effect of reaction temperature on the initial rate and the degree of hydrolysis for olive TAG hydrolysis by *R. javanicus* and *C. cylindracea* lipases. In the temperature range of 10–40°C, the rate and

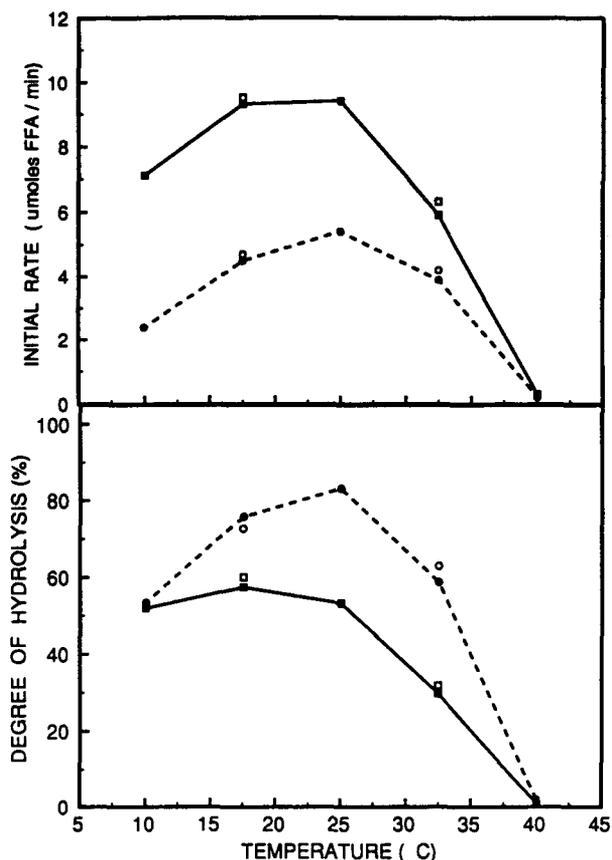


Fig. 3. Effect of temperature on the initial rate and the degree of hydrolysis for the olive TAG hydrolysis in Aerosol-OT/iso-octane reverse micellar media by *R. javanicus* and *C. cylindracea* lipases. Reaction parameters:  $R = 12.5$ ,  $[AOT] = 125$  mM,  $pH = 8.0$  (20 mM glycine-glycine buffer), substrate concentration = 10% v/v; enzyme concentration: *R. javanicus* lipase (solid lines) = 0.5 mg/10 mL of reaction mixture, *C. cylindracea* lipase (dotted lines) = 1.38 mg/10 mL of reaction mixture; open bullets represent predicted response.

the extent of hydrolysis showed a typical bell-shaped dependency. Both enzymes exhibited remarkable activity in reverse micelles at temperature as low as 10°C with rapid decrease in activity beyond 32°C, presumably because of thermal denaturation of the enzymes. The rate and the extent of hydrolysis were maximum at around 25°C, when olive TAG was hydrolyzed by *C. cylindracea* enzyme. As reported by Han and Rhee (11), the activity of *C. cylindracea* lipase in Aerosol-OT reverse micelles was maximum at 30–35°C, which is slightly higher than that found in our study. This difference may be owing to the difference in the other reaction conditions, particularly pH and  $R$ . For *R. javanicus* lipase-catalyzed hydrolysis of olive TAG, the initial rate was maximum around 20°C, whereas the extent of hydrolysis was maximum around 17°C. The temperatures at

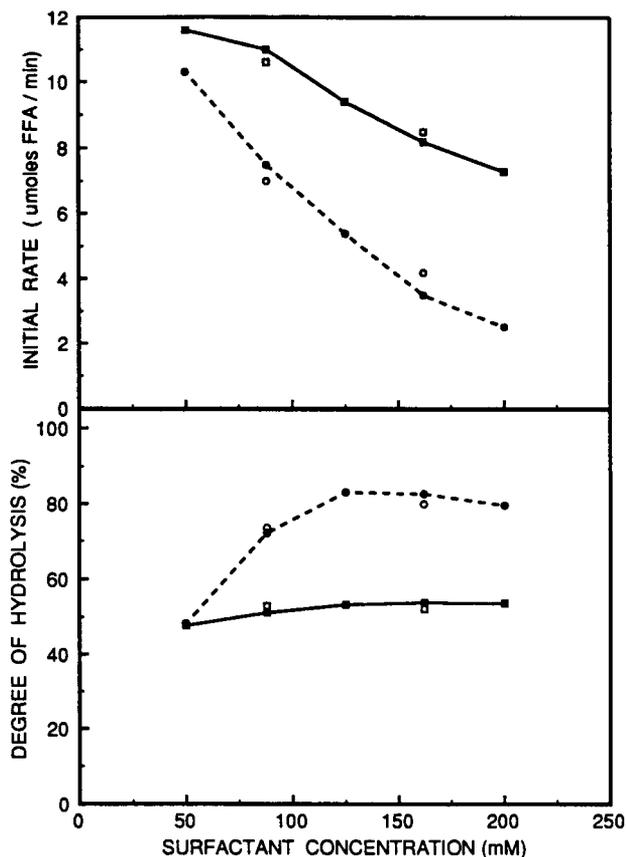


Fig. 4. Effect of surfactant concentration on the initial rate and the degree of hydrolysis for the olive TAG hydrolysis in Aerosol-OT/iso-octane reverse micellar media by *R. javanicus* and *C. cylindracea* lipases. Reaction parameters: temperature = 25°C,  $R = 12.5$ , pH = 8.0 (20 mM glycine-glycine buffer), substrate concentration = 10% v/v; enzyme concentration: *R. javanicus* lipase (solid lines) = 0.5 mg/10 mL of reaction mixture, *C. cylindracea* lipase (dotted lines) = 1.38 mg/10 mL of reaction mixture; open bullets represent predicted response.

which the activity of the enzymes is maximum were lower in reverse micellar media than those in aqueous emulsion. According to the enzyme suppliers, the optimum temperature in aqueous system for *C. cylindracea* lipase is about 35–37°C and for *R. javanicus* lipase is about 40°C.

### Effect of Surfactant Concentration

The influence of surfactant concentration on the initial rate and the degree of hydrolysis for olive TAG hydrolysis by *C. cylindracea* and *R. javanicus* lipases is shown in Fig. 4. With an increase in Aerosol-OT concentration from 50 to 200 mM, the initial rate gradually decreased exhibiting maximum rate at 50 mM Aerosol-OT for the two enzymes. On the other hand, the extent of hydrolysis increased with increase in Aerosol-OT

Table 2  
Regression Coefficients and Coefficients of Determination ( $R^2$ ) of Second-Order Fitted Equation<sup>a</sup> for Initial Rate and Degree of Hydrolysis for *R. javanicus* Lipase-Catalyzed Hydrolysis of TAG in Aerosol-OT/iso-octane Reverse Micellar Media

Variable	Initial rate		Degree of hydrolysis	
	Olive TAG	Coconut TAG	Olive TAG	Coconut TAG
Intercept ( $\beta_0$ )	9.383	53.131	15.213	49.433
Linear				
P ( $\beta_1$ )	-2.840 <sup>c</sup>	-3.603 <sup>c</sup>	-8.642 <sup>c</sup>	-8.202 <sup>c</sup>
R ( $\beta_2$ )	-1.189 <sup>c</sup>	-1.147 <sup>c</sup>	-2.682	-1.255
A ( $\beta_3$ )	-0.960 <sup>c</sup>	-0.672	-0.403	-0.238
T ( $\beta_4$ )	-1.544 <sup>c</sup>	-2.044 <sup>c</sup>	-14.495 <sup>c</sup>	-12.331 <sup>c</sup>
Quadratic				
P × P ( $\beta_{11}$ )	-0.937 <sup>c</sup>	-1.625 <sup>c</sup>	-6.793 <sup>c</sup>	-6.388 <sup>c</sup>
T × T ( $\beta_{44}$ )	-1.435 <sup>c</sup>	-1.644 <sup>c</sup>	-6.849 <sup>c</sup>	-5.915 <sup>c</sup>
Interaction				
P × R ( $\beta_{12}$ )	-0.681 <sup>b</sup>	-0.772	-0.501	-1.424
P × A ( $\beta_{13}$ )	-0.822 <sup>c</sup>	-1.346 <sup>c</sup>	-1.631	-2.527
P × T ( $\beta_{14}$ )	-0.935 <sup>c</sup>	-2.157 <sup>c</sup>	-5.659 <sup>c</sup>	-6.338 <sup>c</sup>
A × T ( $\beta_{34}$ )	0.058	-0.476	-7.784 <sup>b</sup>	-4.162 <sup>b</sup>
$R^2$	0.96	0.94	0.93	0.92

P = pH, R = [water]/[surfactant], A = surfactant Aerosol-OT, T = temperature.

<sup>a</sup>  $Y = \beta_0 + \beta_1 P + \beta_2 R + \beta_3 A + \beta_4 T + \beta^2 P + \beta_{22} R^2 + \beta_{33} A + \beta_{44} T^2 + \beta_{12} PR_{13} + \beta PA_{14} + \beta PT + \beta_{23} RA + \beta_{24} RT + \beta_{34} AT$ .

<sup>b</sup> Significant ( $P < 0.05$ ).

<sup>c</sup> Significant ( $P < 0.01$ ).

concentration up to 125 mM, followed by no significant change up to 200 mM. The influence of Aerosol-OT concentration on the initial rate and the degree of hydrolysis were more drastic in case of *C. cylindracea* lipase than that in case of *R. javanicus* lipase. It is observed from Fig. 4 that the response in the initial rate and in the degree of hydrolysis as a function of surfactant concentration is different. Thus, it is obvious from these observations that emphasis only on the initial rate will not adequately demonstrate the productivity of a lipolytic process.

### Interactive Influence of Reaction Parameters on TAG Hydrolysis

As discussed earlier, the reaction rate or the degree of hydrolysis has either linear, quadratic, or both—linear and quadratic—dependence on the individual variable in the range studied. Table 2 shows such relationships that are statistically significant for the reaction systems with the two substrates. This relationship can provide some useful intrinsic properties of the reaction system; however, it fails to provide important information from a practical application standpoint. Interactive effects indicate how the

dependence of the lipolytic processes on a individual reaction condition is affected when other conditions are changed. It is evident that a number of variables had significant interactive effects (Table 2). The initial rate was most influenced by the interactive effect of pH with all other variables, whereas the degree of hydrolysis was most influenced by the interactive effect of reaction temperature with other variables. The overall hydrolytic reaction in reverse micellar media was most influenced by the interactive effect of pH and temperature.

Table 3 shows the initial rate and the degree of hydrolysis for *R. javanicus* lipase-catalyzed hydrolysis of olive TAG at various combinations of reaction conditions. The data clearly demonstrate the interactive effect of various reaction parameters on the rate and extent of hydrolysis, such as pH and temperature on the initial rate, and *R* and temperature on the degree of hydrolysis. For example, the initial rate was higher at 32.5°C (10.83  $\mu\text{mol}/\text{min}$ ) than that at 17.5°C (9.57  $\mu\text{mol}/\text{min}$ ), when the reaction was carried out at pH 7.0 (*R* = 8.8, Aerosol-OT = 162.5 mM), whereas the rate was lower at 32.5°C (no detectable activity) than that at 17.5°C (7.19  $\mu\text{mol}/\text{min}$ ), when the reaction was carried out at pH 9.0 (*R* = 8.8, Aerosol-OT = 162.5 mM). Similarly, the degree of hydrolysis was lower at *R* = 8.8 (53.7%) than that at *R* = 16.2 (57.9%), when reaction was conducted at 17.5°C (pH = 7.0, Aerosol-OT = 162.5 mM), whereas the degree of hydrolysis was higher at *R* = 8.8 (42.5%) than that at *R* = 16.2 (18.1%), when reaction was conducted at 32.5°C (pH = 7.0, Aerosol-OT = 162.5 mM). It is also observed from the Table 3 that the lower-level variable combinations were favorable over higher-level variable combinations for TAG hydrolysis in reverse micellar media.

Response surface diagram illustrating the effect of two variables on olive TAG hydrolysis by *R. javanicus* lipase is shown in Fig. 5. The interactive effect of pH and temperature on the initial rate is apparent from the shift in the optimum temperature toward higher temperature, as reaction is carried out at lower pH. Similarly, the response for the degree of hydrolysis as a function of *R* was totally opposite if reactions were carried out at 10–20 vs 30–40°C, illustrating the interactive effect of *R* and temperature.

### Statistical Models for TAG Hydrolysis in Reverse Micelles

Table 2 shows the coefficients of linear, quadratic, and interactive effects of the reaction variables, and the coefficients of determination ( $R^2$ ) of second-order fitted GLM regression model for the initial rate and the degree of hydrolysis for lipase-catalyzed TAG hydrolysis in Aerosol-OT/iso-octane reverse micellar media. It is observed from the table that up to 96% of variations in the response were accountable to the reaction variables studied. The adequacy of the models is further validated by the nonsignificance of the "lack of fit" test. Eight additional experimental

Table 3  
 Initial Rate and Degree of Hydrolysis  
 for *R. javanicus* Lipase-Catalyzed Hydrolysis of Olive TAG in  
 Aerosol-OT/iso-octane Reverse Micellar Media at Various Reaction Conditions<sup>a</sup>

Reaction parameters					
Temp., °C	AOT, <sup>b</sup> mM	R <sup>c</sup>	pH	Initial rate, <sup>d</sup> μmol FFA/min	Degree of hydrolysis, <sup>d</sup> %
17.5	87.5	8.8	7.0	10.42 ± 0.21	47.3 ± 1.4
			9.0	9.56 ± 0.10	46.0 ± 1.0
		16.2	7.0	10.22 ± 0.20	49.8 ± 0.2
			9.0	6.81 ± 0.03	49.1 ± 1.2
			7.0	9.57 ± 0.24	53.7 ± 0.6
			8.8	7.19 ± 0.22	55.3 ± 1.1
	162.5	7.0	10.73 ± 0.23	57.9 ± 0.6	
		16.2	9.0	1.83 ± 0.01	53.3 ± 1.1
		7.0	9.96 ± 0.25	41.4 ± 0.2	
		8.8	9.0	6.44 ± 0.01	37.1 ± 1.0
		7.0	8.67 ± 0.19	31.0 ± 0.7	
		16.2	9.0	0.00 ± 0.00	0.0 ± 0.1
32.5	162.5	7.0	10.83 ± 0.03	42.5 ± 0.1	
		8.8	9.0	0.00 ± 0.00	0.2 ± 0.1
		7.0	7.49 ± 0.08	18.1 ± 0.3	
	16.2	7.0	7.49 ± 0.08	18.1 ± 0.3	
		9.9	0.00 ± 0.00	0.1 ± 0.0	

<sup>a</sup>Reaction parameters: enzyme concentration = 0.5 mg/10 mL reaction mixture, substrate concentration = 10% v/v, buffer 20 mM glycine-glycine buffer.

<sup>b</sup>Surfactant concentration, Aerosol-OT (sodium dioctyl sulfosuccinate).

<sup>c</sup>Molar ratio of water to surfactant.

<sup>d</sup>Mean ± SD.

points representing +1 and -1 coded levels of each variables were studied, and were excluded from the model fitting to check the efficacy or the prediction power of the model. The closeness of the predicted and actual responses indicates efficacy of the models in predicting the responses (Figs. 1-4).

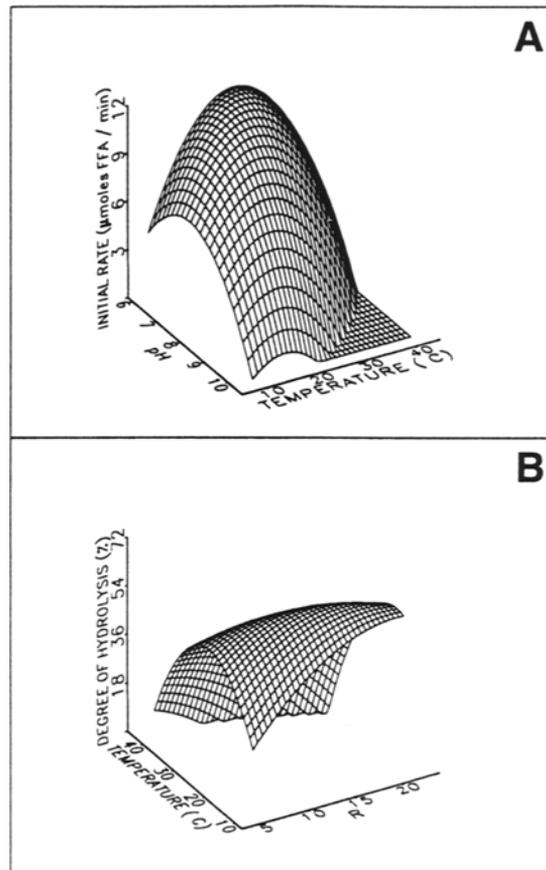


Fig. 5. Response surface diagram illustrating the interactive effect of various reaction parameters on the hydrolytic reaction of *R. javanicus* lipase on olive TAG in Aerosol-OT/iso-octane reverse micellar media. Reaction parameters: [AOT] = 125 mM, substrate concentration = 10% v/v, enzyme concentration = 0.5 mg/10 mL of reaction mixture. (A) Initial rate as a function of pH and temperature at  $R = 12.5$ . (B) Degree of hydrolysis as a function of  $R$  and temperature at pH 8.0.

### Optimization of Reaction Conditions

Normally, an optimum reaction condition can be mathematically computed using the GLM regression equation derived from such factorial experiments as central composite design. However, the computation becomes tedious with a four-factor design, and the optimum condition can be determined only on the basis of one response, i.e., either reaction rate or degree of hydrolysis. It is desirable to determine the maximum rate as well as maximum degree of hydrolysis, i.e., maximum conversion in shortest time. With the availability of computer software, it is possible to determine optimum reaction condition based on multiple responses.

Table 4  
Optimum Reaction Condition Ranges Determined by Four-Factor Taylor Polynomial Optimization for Hydrolysis of Olive and Coconut TAG by *R. javanicus* and *C. cylindracea* Lipases in Aerosol-OT/iso-octane Reverse Micellar Media

Reaction conditions	<i>R. javanicus</i> lipase		<i>C. cylindracea</i> lipase	
	Olive TAG	Coconut TAG	Olive TAG	Coconut TAG
pH	6.8-7.6	6.8-7.6	6.0-6.8	6.0-6.8
0	11-14	14-17	8-14	8-11
AOT (mM) <sup>b</sup>	110-170	110-170	110-140	140-170
Temperature (°C)	16-22	16-22	22-28	22-28

<sup>a</sup>Molar ratio of water to surfactant.

<sup>b</sup>Surfactant concentration, Aerosol-OT (sodium dioctyl sulfosuccinate).

The optimum ranges for the reaction conditions determined by four-factor Taylor polynomial optimization for olive and coconut TAG hydrolysis in Aerosol-OT/iso-octane reverse micelles by *R. javanicus* and *C. cylindracea* lipases are shown (Table 4). These optimum ranges were determined based on the maximization of both the rate and the degree of hydrolysis. There were no significant differences in the optimum reaction condition ranges for hydrolysis of olive vs coconut TAG, except for a slightly higher range of *R* for coconut TAG when hydrolyzed by *R. javanicus* lipase and a slightly higher range of Aerosol-OT concentration when hydrolyzed by *C. cylindracea* lipase. This could be owing to an increased requirement of water for coconut TAG, since this substrate has a higher number of ester bonds to be hydrolyzed per unit amount. The difference between the systems with the two enzymes was considerable in terms of optimum ranges for pH, *R*, and temperature. When compared to the reaction system with *C. cylindracea* lipase, the system with *R. javanicus* lipase exhibited a higher optimum range for pH and *R*, and a lower optimum range for temperature. These differences may be related to the intrinsic characteristics of the enzymes. In general, the optimum reaction conditions determined for lipase-catalyzed hydrolysis of TAG in Aerosol-OT/iso-octane reverse micellar system are: pH around 6.8, *R* around 14, Aerosol-OT concentration around 140 mM, and temperature around 22°C.

## REFERENCES

1. Bello, M., Thomas D., and Legoy, M. D. (1987), *Biochem. Biophys. Res. Commun.* **46**, 361-367.
2. Holmberg, K. and Osterberg, E. (1987), *Prog. Colloid & Polymer. Sci* **74**, 98-102.
3. Holmberg, K. and Osterberg, E. (1988), *J. Am. Oil Chem. Soc.* **65**, 1544-1548.
4. Hann, D., Kwon, D. Y., and Rhee, J. S. (1988), *Kor. J. Appl. Microbiol. Bioeng.* **6**, 250-258.

5. Holmberg, K., Lassen, B., and Stark, M. B. (1989), *J. Am. Oil Chem. Soc.* **66**, 1796-1800.
6. Kim, T. and Chung, K. (1989), *Enzyme Microb. Technol.* **11**, 528-532.
7. Hayes, D. G. and Gulari, E. (1990), *Biotechnol. Bioeng.* **35**, 793-801.
8. Nagao, A. and Kito, M. (1990), *Biocatalysis* **3**, 295-305.
9. Osterberg, E., Blomstrom, A. C., and Holmberg, K. (1989), *J. Am. Oil Chem. Soc.* **66**, 1330-1333.
10. Han, D. and Rhee, J. S. (1985), *Biotechnol. Lett.* **7**, 651-656.
11. Han, D. and Rhee, J. S. (1986), *Biotechnol. Bioeng.* **28**, 1250-1255.
12. Han, D., Rhee, J. S., and Lee, S. B. (1987), *Biotechnol. Bioeng.* **30**, 381-388.
13. Han, D., Walde, P., and Luisi, P. L. (1990), *Biocatalysis* **4**, 153-161.
14. Chang, P. S. and Rhee, J. S. (1990), *Biocatalysis* **3**, 343-355.
15. Schmidli, P. K. and Luisi, P. L. (1990), *Biocatalysis* **3**, 367-376.
16. Stark, M. B., Skagerlind, P., Holmberg, K., and Carlfors, J. (1990), *Colloid Polym. Sci.* **268**, 384-388.
17. Lowry, R. R. and Tinsley, I. J. (1976), *J. Am. Oil Chem. Soc.* **53**, 470-472.
18. AOAC (1984), "*Official Methods of Analysis*," 14th ed. Association of Official Analytical Chemists, Washington, D. C.
19. Box, G. E. P. and Wilson, K. B. (1951), *J. Royal Statistical Soc. Series B* **13**, 1-9.
20. Minitab (1990), *Minitab Reference Manual*. Minitab, Inc., State College, PA.
21. Axum (1989), *Axum Technical Graphics and Data Analysis*. TriMetrix, Inc., Seattle, WA.
22. Design-Expert (1988), *Design-Expert Experimental Design*. Stat-Ease, Inc., Minneapolis, MN.
23. Zaks, A. and Klibanov, A. M. (1988), *J. Biol. Chem.* **263**, 8017-8021.
24. Linfield, W. M., O'Brien, D. J., Serota, S., and Barauskas, R. A. (1984), *J. Am. Oil Chem. Soc.* **61**, 1067-1071.
25. Wang, Y. J., Sheu, J. Y., Wang, F. F., and Shaw, J. F. (1988), *Biotechnol. Bioeng.* **31**, 628-633.
26. Hirata, H., Higuchi, K., and Yamashina, T. (1990), *J. Biotechnol.* **14**, 157-168.
27. Klibanov, A. M. (1986), *Chemtech.* **16**, 354-359.
28. Yamane, T., Koima, Y., Ichiryu, T., and Shimizu, S. (1988), *Ann. NY Acad. Sci.* **542**, 282-293.
29. Patel, M. T (1993), PhD Dissertation, The Pennsylvania State University, University Park, PA.