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Evaluation of Kinetic Parameters for Enzymatic Interesterification Synthesis of L-Ascorbyl Lactate by Response Surface Methodology

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Abstract

The kinetics of lipase-catalyzed interesterification synthesis of L-ascorbyl lactate was studied. To determine the enzyme kinetic constants of the interesterification, a three-factor and five-level central composite design was used. The factors studied were ethyl lactate concentration, reaction temperature (T), and water content (w). Moreover, a statistical approach called the response surface method (RSM) was used to predict the kinetic constants. Finally, the relationships between the kinetic constants (V_m and K_m) and the reaction parameters (T and w) were obtained.

To assess the accuracy of the RSM approach for determining V_m and K_m, detailed validation experiments were carried out by the conventional approach at four different reaction parameters(35°C, 10 μL; 45°C, 20 μL; 55°C, 15 μL; 65°C, 18 μL). The results indicated that the RSM approach gave reasonable results for the determination of Vm and Km in the range of tested parameters.

Index Entries: L-Ascorbyl lactate; interesterification; kinetic constants; response surface method; enzyme.

Introduction

L-Ascorbic acid (vitamin C) and its derivatives are valuable agents in the treatment of photoaging, skin cancer, and numerous skin disorders. Antioxidants stabilize reactive, potentially harmful free radicals generated

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after ultraviolet exposure and during normal and pathologic metabolic processes. If not quenched, these free radicals will contribute to photoaging and photocarcinogenesis by producing DNA mutations *(1)*. It is well known that therapeutic doses of topically applied vitamins frequently cause skin irritations, which interfere with treatment. To solve these problems, several researchers have conjugated L-ascorbic acid with carboxylic acids *(2,3)*.

Carboxylic acids are also useful in the treatment of aging skin, especially α-hydroxy acids. The most commonly used are glycolic acid, lactic acid, and salicylic acid, which seem to exert slight but significant effects in reducing skin discolorations and roughness when applied in a cream. These acids are known to improve skin function and appearance by accelerating desquamation and promoting renewal of the stratum corneum. Unfortunately, these acids penetrate too quickly into the deep epidermis and are irritating to the skin, particularly at concentrations of 5% or higher and for individuals with sensitive skin. These problems limit the availability of useful therapies for skin aging *(4)*.

L-Ascorbic acid conjugated to carboxylic acids is believed to be hydrolyzed in vivo and yield an active L-ascorbic acid and active hydroxy acid agent. Both compounds provide antiaging effects when made available to the skin. Esters of L-ascorbic acid and hydroxy acid are unusually effective as skin conditioners, with significant reductions in the irritation problems characteristic of L-ascorbic acid and hydroxy acids in nonesterified form.

Enzyme-catalyzed reactions are superior to conventional chemical methods, owing to mild reaction conditions, high catalytic efficiency, and the inherent selectivity of natural catalysts. Furthermore, the use of immobilized enzyme simplifies downstream processing. The number of reports concerning the use of immobilized lipase as catalyst in organic synthesis is increasing considerably, especially in the pharmaceutical and cosmetic industries, where regulatory pressure has encouraged the development and marketing of natural compounds. To develop an enzyme-based process, kinetic constants are extremely important information that has to be determined.

Previous studies have shown that the reaction of esterification immobilized lipase-catalyzed could be described by the Ping-Pong kinetic models *(5–7)*. The synthesis of ascorbyl derivatives catalyzed by immobilized lipase has been reported recently *(8–10)*. In these reactions, because L-ascorbic acid is slightly soluble, its concentration can be regarded as a constant. Hence, the initial reaction rate equation *(11)* can be simply expressed as

$$V = \frac{V_m[A]}{[A] + K_m} \tag{1}$$

in which V is the initial reaction rate, V_m is the maximum reaction rate, and K_m is the apparent Michaelis constant. In the present study, the interesterification kinetics of L-ascorbic acid and ethyl lactate for L-ascorbyl lactate

production were studied further. [A] is the concentration of ethyl lactate. By rearranging Eq. 1, the following equation can be derived:

$$\frac{1}{V} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{[A]} \tag{2}$$

Plotting Eq. 2 as $1/V$ vs $1/[A]$ (known as a Lineweaver-Burk plot) gives an appropriate linear fit that is commonly used to determine the numerical values of V_m and K_m.

Response surface methodology (RSM) is an effective statistical technique for the investigation of complex processes. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results. It is a faster and less expensive method for gathering research results than the classic method *(12)*. RSM has been successfully applied to study and optimize the enzyme synthesis of flavor ester.

In recent years, RSM has been used to determine the kinetic constants of enzymatic reactions as well as to optimize reaction conditions *(13,14)*. A quite good correlation between the kinetic constants obtained from conventional methods and those obtained from RSM has been reported. The values found were comparable with those obtained with conventional methods, and RSM could be successfully adopted for determining kinetic constants for enzyme-catalyzed reactions.

In the present study, a new approach to determine the kinetic constants of L-ascorbic acid and ethyl lactate for L-ascorbyl lactate production was studied using RSM. Lineweaver-Burk transformation (Eq. 2) was utilized as a part of the RSM algorithm and a secondorder polynomial model was obtained for the reciprocal of enzymatic reaction rate ($1/V$) as a function of the reciprocal of ethyl lactate concentration ($1/[A]$), reaction temperature (T), and water content (w) using RSM. The accuracy of the calculated values was tested with V_m and K_m values that were obtained with the conventional method.

Materials and Methods

Lipase and Chemicals

Immobilized lipase from *Candida antarctica* (Novozym 435) was purchased from Sigma. L-Ascorbic acid was obtained from Aldrich. All other chemicals were commercially available products of reagent grade.

General Procedure for Enzymatic Reaction

Reactions were conducted in screw-cap glass vials. In standard conditions, different amounts of L-ascorbic acid and ethyl lactate (molar ratio of 5:1), 25 mg of lipase, followed by different amounts of water were added to 10 mL of tert-amyl-alcohol. The mixtures of L-ascorbic acid, ethyl lactate,

water, and Novozym 435 were stirred in an orbital shaking water bath (200 rpm) at different reaction temperatures. These conditions were used except when otherwise stated in the text.

High-Performance Liquid Chromatography Analysis

Quantitative analyses of reactants and products were conducted using a high-performance liquid chromatography system from Hewlett-Packard (processor, pump, ultraviolet [UV] detector, and injector model 1100). A reverse-phase column (Hewlett Packard XDB-C18, 250 × 4 mm, 5 μm) was used. A 20-μL vol of the proper dilution of the reaction mixture was injected, and a mixture of water/methanol/H_3PO_4 (95/5/0.1 [v/v/v]) was used as eluent with a flow rate of 0.7 mL/min. Products were detected using a UV detector at 280 nm and a differential refractometer.

Initial reaction rates were determined by plotting the product concentration as a function of reaction time and determining the slope of the line at times close to zero. The initial reaction rates were expressed as millimoles of product formed per minute per gram of immobilized lipase.

Determination Ranges of Variables

Before arranging an experimental design with a central composite design (CCD), the effects of ethyl lactate concentration, reaction temperature, and water content on enzymatic reaction rate were tested by varying one factor at a time while keeping the others fixed. The effects of reaction temperature, water content, and ethyl lactate concentration on enzymatic reaction rate are demonstrated in Figs. 1, 2 and 3, respectively. As shown in Fig. 1, an increase in temperature increased the initial reaction rate up to 50°C, and then the reaction rate decreased with a higher temperature. Therefore, 50°C was chosen as the center point temperature (T). Similarly, an increase in water content (w) caused an increase in initial reaction rate. When water content was up to 20 μL, the initial reaction rate began to decrease (Fig. 2). Thus, a 20-μL water content was chosen as the center point. Finally, an increase in ethyl lactate concentration caused an increase in initial reaction rate with a concentration of ethyl lactate up to 0.035 *M*, above which there was no significant change in the initial reaction rate (Fig. 3). Hence, an ethyl lactate concentration range of 0.015-0.035 *M* was chosen. In the CCD, the reciprocal of the substrate concentration (1/[A]) was used as well as [*A*] and, therefore, varied from 28.6 to 66.7 M^{-1}.

Experiments Using CCD

To determine the enzyme kinetic constants of the interesterification, experiments were conducted using a three-factor and five-level CCD. The factors studied were ethyl lactate concentration, reaction temperature (T), and water content (w). The assay conditions for the reaction parameters were taken at zero level (center point) and one level (+1 and (1). The design was extended up to a ±α (axial point) of 1.68. The center values for variables

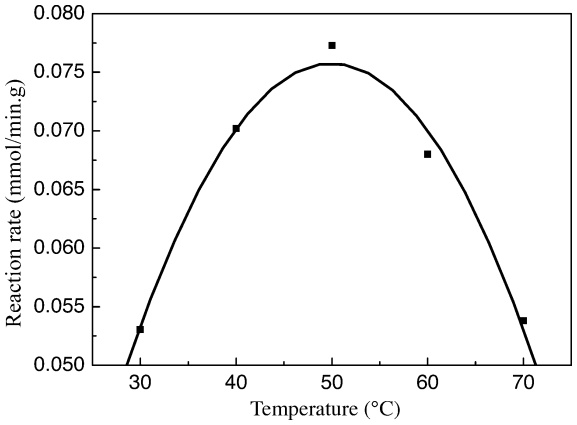

Fig. 1. Effect of reaction temperature on enzymatic reaction rate. Reaction conditions: 0.02 *M* ethyl lactate concentration, 5:1 molar ratio of L-ascorbic acid and ethyl lactate, 20 mg of lipase, 10-μL water content.

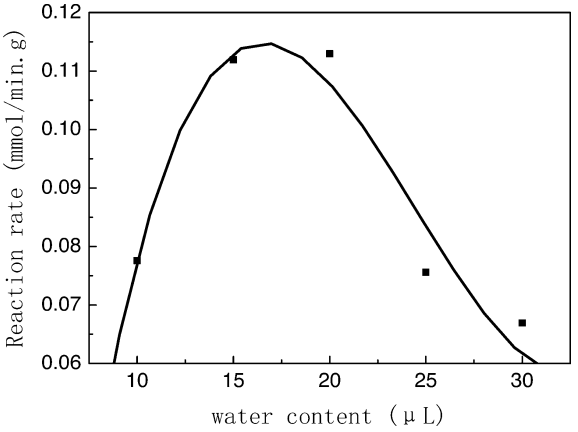

Fig. 2. Effect of water content on enzymatic reaction rate. Reaction conditions: 0.02 *M* ethyl lactate concentration, 5:1 molar ratio of L-ascorbic acid and ethyl lactate, 20 mg of lipase, 50°C.

were carried out at least six times for the estimation of error and single runs for each of the other combinations; twenty runs were done in a totally random order. Table 1 provides the independent variables and their levels.

The variable levels X_i were coded as x_i according to Eq. 3 such that X0 corresponded to the central value:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad i = 1, 2, 3...k \tag{3}$$

in which x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and ΔX_i is the step change.

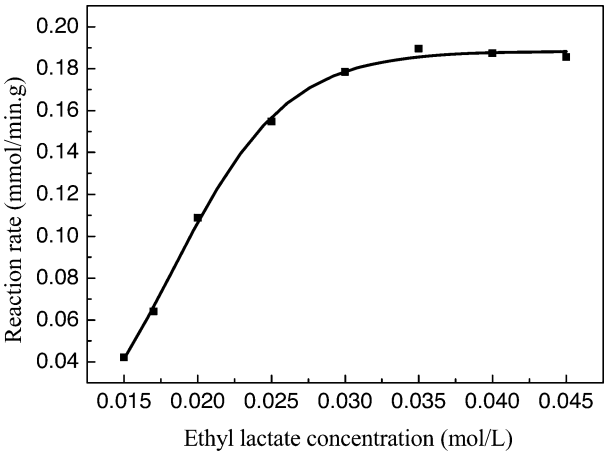

Fig. 3. Effect of ethyl lactate concentration ([A]) on enzymatic reaction rate. Reaction conditions: 5:1 molar ratio of L-ascorbic acid and ethyl lactate, 20 mg of lipase, 50ºC.

Table 1
Range of Variables for CCD

Variable	Value				
	$-\alpha$	−1	0	1	$+\alpha$
Reciprocal of substrate concentration (M^{-1})	28.6	36.2	47.6	59.0	66.7
Reaction temperature (°C)	33.2	40	50	60	66.8
Water content (μL)	8	12.9	20	27.1	32

Data analysis, analysis of variance (ANOVA), and multiple linear regression were performed using SAS8.0 software. A quadratic model, including interaction terms, was assumed to describe relationships between the responses and the experimental factors X_i:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_{ii} X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \quad (4)$$

in which Y is the response, β_0 is the constant coefficient (intercept), β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the second-order interaction coefficient.

Determination of Simple Enzyme Kinetics With Conventional Method

To assess the prediction of the kinetic constants based on RSM analysis, a detailed validation in support of RSM was carried out by the conventional method at four different reaction parameters (35°C, 10 μL; 45°C, 20 μL; 55°C, 15 μL; 65°C, 18 μL). The ethyl lactate concentration ranged from 0.015 to 0.045 *M*. The V_m and K_m values of Michaelis-Menten kinetics were determined using a Lineweaver-Burk plot at defined parameters.

Table 2
Experimental Design and Results of CCD

	Independent variable			Response
Run no.	$X_1(1/[A])$	X_2(T)	$X_3(w)$	$Y(1/V)$
1	0	–1.682	1	13.473
2	1	–1	–1	15.45
3	–1	(1	1	9.77
4	–1	(1	–1	9.54
5	1	(1	1	15.37
6	0	0	0	9.52
7	0	0	1.682	10.946
8	–1.682	0	0	5.18
9	0	0	0	9.54
10	0	0	0	9.44
11	0	0	0	9.46
12	0	0	–1.682	11.039
13	0	0	0	9.42
14	1.682	0	0	16.44
15	0	0	0	9.466
16	–1	1	–1	9.536
17	1	1	1	15.384
18	1	1	–1	15.324
19	–1	1	1	9.51
20	0	1.682	0	13.23

Results and Discussion

Evaluation of Kinetic Constants by Response Surface Modeling

The effects of $1/[A]$, T, and w on the reciprocal of the initial reaction rate $1/V$ were investigated by using RSM. The results obtained after using a CCD were then analyzed by using ANOVA. Table 2 summarizes the reciprocal of the initial reaction rate $(1/V)$ of the CCD for each individual run along with the predicted response. Because the inverse of the Michaelis-Menten equation (Eq. 2) does not contain a quadratic term for $1/[A]$, the following quadratic polynomial equation was obtained:

Table 3 presents the model coefficients obtained from multiple linear regression. The analysis showed that the model is significant ($Pr >$ F-value $<.001$). The regression coefficient of determination (R^2), a measure of how well a model can be made to fit the raw data, was 0.9568. This implied a satisfactory fitting of the quadratic model to the experimental data, and approx 96% of the variability in the dependent variable (response) could be explained by the model. The adjusted R^2, which is more suited for comparing models with different numbers of independent variables, was 0.9255. The coefficient of variation (CV) was 7.22%. Hence, the model was found to be adequate for prediction within the range of variables employed.

Table 3
Regression Coefficients and R2 Values for Response of Predicted Quadratic Polynomial Model

Term	Coefficient estimate	Standard error
β_0	38.34463	9.93197
β_1	0.26976	0.14717
β_2	–1.46349	0.26100
β_3	(0.45533	0.31661
β_{22}	0.01454	0.00215
β_{33}	0.01206	0.00423
β_{12}	0.00016667	0.00254
β_{13}	–0.00034594	0.00358
β_{23}	–0.00020423	0.00408
$Pr >$ F-value	<.001	
R^2	0.9568	
Adjusted R^2	0.9255	
CV	7.22	

The effects of $1/[A]$, T, and **w** on the reciprocal of the initial reaction rate $1/V$ can be observed from the predicted polynomial model (Eq. 5). To aid visualization, three-dimensional (3D) response surface graphs for all interactions are shown in Figs. 4–6. The third independent variables were kept at the zero level to obtain these figures. To obtain the critical values (optimum) of T and w, the derivatives $\partial Y/\partial T$ and $\partial Y/\partial w$ of Eq. 5 were set to zero, and then Eqs. 6 and 7 were derived:

$$\frac{1}{V} = Y = 38.34463 + 0.26976 \times \frac{1}{[A]} - 1.46349 \times T + 0.4553 \times w + 0.01454 \times T^2 \quad (5)$$
$$+0.01206 \times w^2 + 0.00016667 \times \frac{1}{[A]} \times T - 0.00034594 \times \frac{1}{[A]} \times w$$
$$-0.00020423 \times T \times w$$

$$\frac{\partial(1/V)}{\partial T} = \frac{\partial Y}{\partial T} = -1.46349 + 0.02908 \times T + 0.00016667 \times \frac{1}{[A]} - 0.00020423 \times w = 0 \quad (6)$$

$$\frac{\partial(1/V)}{\partial w} = \frac{\partial Y}{\partial w} = 0.45533 + 0.02412 \times w - 0.00034594 \times \frac{1}{[A]} - 0.00020423 \times T = 0 \quad (7)$$

These results showed that optimum values of reaction parameters (T, w) depended on the concentration of ethyl lactate. That is to say, each concentration of ethyl lactate has its own optimum values of reaction parameters (T, w). It should be related to the 3Dstructural changes of enzyme in the presence of different amounts of substrate. However, the mechanism of the substrate's effect on enzymatic structure is unknown. Further study on this topic is needed, and the results will be important for industrial application of the enzymes.

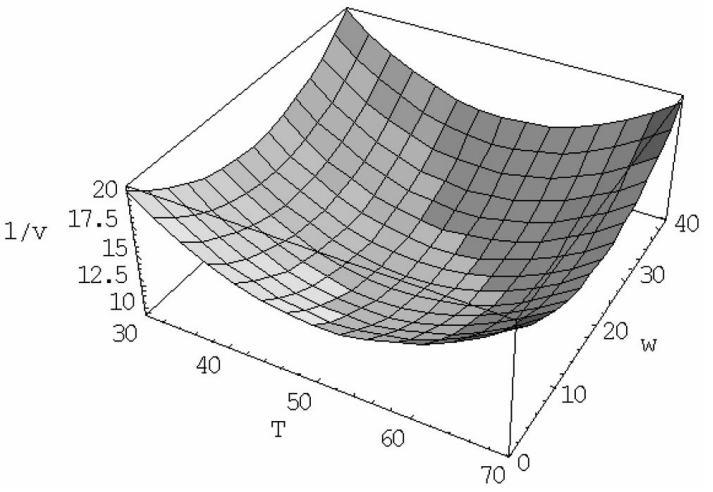

Fig. 4. Response surface plot for reciprocal of enzymatic reaction rate as function of reaction temperature and water content (1/[A] =47.6)

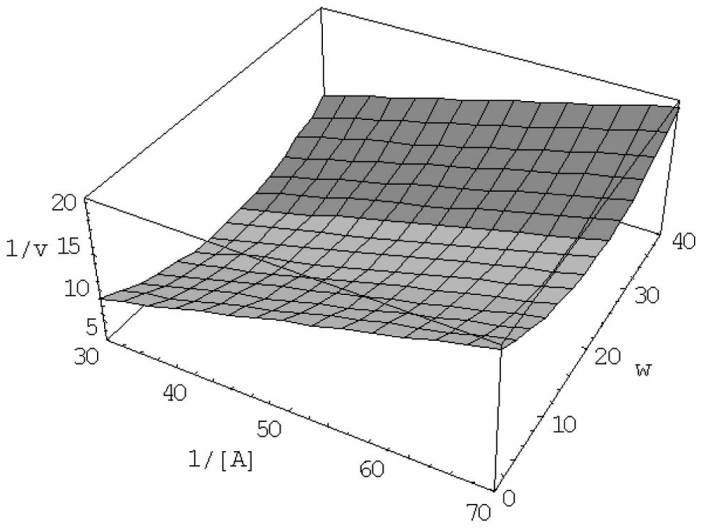

Fig. 5. Response surface plot for reciprocal of enzymatic reaction rate as function of reaction temperature and water content (T = 50°C).

It can evaluate Eq. 5 under the condition $1/[A]=0$, which immediately leads to Eq. 8. In other words, when the concentration of ethyl lactate $[A]$ is up to the maximum, the initial reaction rate will be the maximum V_m.

$$V_{\max} = \frac{1}{38.34463 - 1.46349 \times T + 0.45533 \times w + 0.01454 \times T^2 + 0.01206 \times w^2 - 0.00020423 \times T \times w} \quad (8)$$

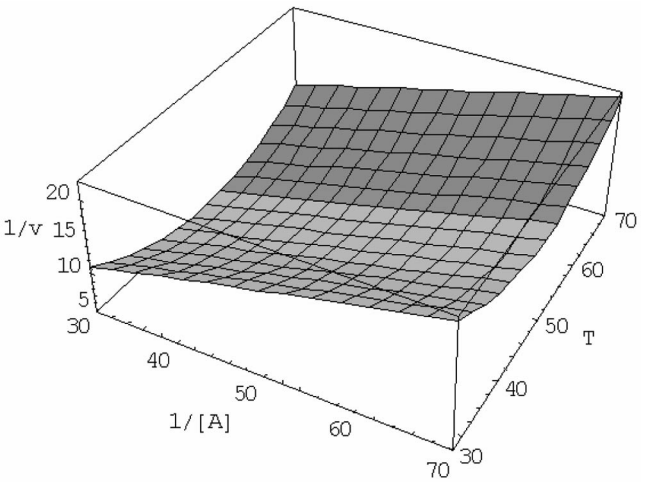

Fig. 6. Response surface plot for reciprocal of enzymatic reaction rate as function of reciprocal of ethyl lactate concentration and reaction temperature (w = 20 μL).

The K_m/V_m ratio is assigned from the slope of the Lineweaver-Burk plot (Eq. 2). As shown in Eqs. 9 and 10, the values of K_m/V_m and K_m can be determined by the derivatives of $\partial(1/V)/\partial(1/[A])$ in Eq. 5:

$$\frac{K_m}{V_m} = \frac{\partial(1/V)}{\partial(1/[A])} = 0.26976 + 0.00016667 \times T - 0.00034594 \times w \quad (9)$$

$$K_m \frac{0.26976 + 0.00016667 \times T - 0.00034594 \times w}{38.4463 - 1.46349 \times T + 0.45533 \times w + 0.01454 \times T^2 + 0.01206 \times w^2 - 0.00020423 \times w} \quad (10)$$

The V_m and K_m values could be calculated for each combination of T and w within the investigated ranges using Eqs. 8 and 10. In addition, the values of V_m and K_m changed at different temperature and water content.

We have demonstrated that it is easy to estimate the kinetic constants and the influence of T and w on the rate and interaction between the substrate concentration and T, w from a few experiments by using response surface modeling. By changing the variables such as T and w, the information on how these parameters are dependent on or independent of each other should also be possible to extract. This is now under further evaluation.

Evaluation of Kinetic Constants by Conventional Approach

The kinetic constants of the interesterification were determined by conventional approach at four different reaction parameters. Based on the measured experimental data, the kinetic constants (Vm and Km) were determined from a linear regression using a Lineweaver-Burk reciprocal plot (Fig. 7). At the same reaction parameters, Vm and Km were calculated using Eqs. 8 and 10. The values obtained were found to be very close to

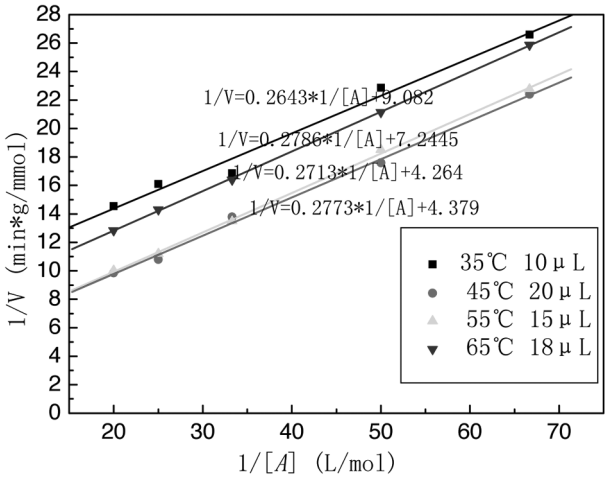

Fig. 7. Lineweaver-Burk plot for determining kinetic constants Vm and Km.

Table 4
Comparison of Kinetic Constants Obtained From RSM and Conventional Approach

Water content (μL)	Temperature (°C)	V_m (mmol/[min × g])		K_m (mmol/L)	
		Conventional	RSM	Conventional	RSM
>10	35	0.110	0.117	0.0291	0.0319
20	45	0.234	0.224	0.0636	0.0608
15	55	0.228	0.219	0.0633	0.0601
18	65	0.138	0.140	0.0385	0.0393

those obtained by the conventional approach (Table 4). The results of the present investigation conclusively suggest that RSM could successfully determine Vm and Km values in the range of tested parameters.

Conclusion

For most enzyme-catalyzed reactions, Vm and Km are important parameters and depend on several rate constants, each of which may be affected differently by reaction parameters. The conventional approach used for the estimation of kinetic constants is well known. However, the number of experiments required to determine enzyme characteristics is cumbersome and time-consuming. In the present study, RSM was used to predict the kinetic constants. With a minimal amount of experiments, this method can give as much information as possible. Equations 6 and 7 showed that optimum values of reaction parameters (T,w) depended on substrate concentration. At the same time, the relationships (Eqs. 8 and 10) between

the kinetic constants (Vm and Km) and the reaction parameters (T and w) were derived. To confirm the accuracy of the RSM approach for determining Vm and Km, detailed validation experiments were carried out by the conventional approach at four different reaction parameters. The results indicate that the RSM approach gave reasonable results for the determination of Vm and Km in the range of tested parameters. The results conclusively suggest that RSM could be successfully adopted for evaluation of the kinetic constants for enzyme-catalyzed reactions. However, the mechanism of how the 3D structural changes of enzyme in different parameters, how the reaction parameters affect kinetic constants should be further studied.

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