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A new approach for determination of enzyme kinetic constants using response surface methodology

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Abstract

A statistical approach called response surface methodology (RSM) is used for the prediction of the kinetic constants of glucose oxidase (GOx) as a function of reaction temperature and pH. Lineweaver–Burk transformation of the Michaelis–Menten equation was utilized as the integral part of the RSM algorithm. The effects of variables, namely reciprocal of substrate concentration (0.033–0.5 mM^{-1}), reaction temperature (14.9–40.1 °C) and reaction pH (pH 4.4–8.5) on the reciprocal of initial reaction rate were evaluated and a second order polynomial model was fitted by a central composite circumscribed design (CCCD). It was observed that optimum reaction temperature and pH for the GOx reaction depended on the substrate concentration and varied between 27.8 °C and 6.4 pH and 32.7 °C and 6.1 pH in the investigated range of substrate concentration. The maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) of GOx were obtained for each reaction parameter by using the model equation. The maximum reaction rate varied between 3.5 μmol/min mg enzyme and 29.8 μmol/min mg enzyme. Michaelis–Menten constant was determined between 1.9 mM and 16.8 mM in the tested reaction parameters. The kinetic constants of GOx were also determined with the conventional method at six reaction parameters and compared with the results of the proposed method. The correlation coefficients (R^2) between the results of two methods were determined as 0.940 and 0.869 for V_{max} and K_m, respectively.

Keywords: Enzymes; Kinetic constants; Glucose; Glucose oxidase; Response surface methodology

1. Introduction

The characteristics of enzymes such as kinetic constants, activation energy, temperature, pH, water activity profiles, etc. determine the usability and productivity of enzymes. For that reason, these characteristics should be determined for a newly discovered enzyme, an enzyme used in different reaction mediums or an enzyme used in different forms (such as free or immobilized). In developing an enzyme based process, kinetic constants are the most important information which has to be determined. Simple enzyme kinetics is generally described by the Michaelis–Menten kinetics (Eq. (1)) [1].

$$V = \frac{V_{max}\, S}{K_m + S} \tag{1}$$

where V is the enzymatic reaction rate (mmol/min mg enzyme), S is the substrate concentration (mM), V_{max} is the maximum or limiting reaction rate (mmol/min mg enzyme) and K_m is the Michaelis–Menten constant (mM). By rearranging Eq. (1), the following equation can be derived:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\frac{1}{S} \tag{2}$$

Plotting of Eq. (2) as $1/V$ versus $1/S$ (known as a Lineweaver–Burk plot) gives an appropriate linear plot that is commonly used for determination of numerical values of V_{max} and K_m. Besides of Lineweaver–Burk plot, other plotting methods (Eadie–Hofstee plot, Hanes–Woolf plot) and non-linear regression methods have been also used for determination of kinetic constants [1–3].

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes [4]. RSM defines the effect of the independent variables, alone or in combination, on the process. In addition to analyzing the effects of the independent variables, this experimental methodology

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generates a mathematical model that accurately describes the overall process [5]. It has been successfully applied to optimizing conditions in food, chemical and biological processes [5–9].

In recent years, RSM has been used to determine the kinetic constants of enzymatic reactions as well as for the optimization of reactions [6,8]. Andersson and Adlercreutz [6] used RSM to determine the V_{max} and K_m values of horse liver alcohol dehydrogenase between pH 4 and pH 8 using a second order polynomial model, obtained from RSM, and the Michaelis–Menten equation. A quite good correlation between the kinetic constants obtained from conventional methods and the ones obtained from RSM was reported. Beg et al. [8] used RSM to determine V_{max}, K_m, catalytic power of enzyme (K_{cat}) and activation energy (E_a) of alkaline protease from *Bacillus mojavensis*. The kinetic constants were determined between 45 and 60 °C using a second order polynomial model, obtained from RSM, a Lineweaver–Burk plot and an Arrhenius plot. It was reported that values found were comparable to those obtained with conventional methods and RSM could successfully be adopted for determining kinetic constants for enzyme-catalyzed reactions.

In this study, a new approach to determine the kinetic constants of glucose oxidase (GOx) with RSM was studied. In the traditional approach for predicting a polynomial model, the response, which is the enzymatic reaction rate, is defined as a function of substrate concentration and the other independent variables. This method needs a further step such as a non-linear regression method [6] or a Lineweaver–Burk plot [8] for determination of kinetic constants. In this study, Lineweaver–Burk transformation (Eq. (2)) was utilized as a part of the RSM algorithm and a second order polynomial model was obtained for the reciprocal of enzymatic reaction rate ($1/V$) as a function of reciprocal of substrate concentration ($1/S$), reaction temperature (T) and pH (pH) using RSM. The polynomial model was used for calculation of V_{max} and K_m of GOx enzyme at different reaction parameters (T and pH). The accuracy of the calculated values was tested with V_{max} and K_m values which were obtained in conventional method at six reaction parameters.

2. Experimental

2.1. Chemicals

Glucose oxidase (EC 1.1.3.4 from *Aspergillus niger*), peroxidase (POD) (EC 1.11.1.7 from horseradish) were received from Biozyme Laboratories (Blaenavon, Gwent, UK). Phenol, D-glucose and 4-aminoantipyrine were obtained from Sigma Co. (St. Louis, MO, USA). All other chemicals were commercially available products of reagent grade.

2.2. Equipment

A UV–vis spectrophotometer (Shimadzu 1201 PC, Shimadzu Corporation, Japan) was used in the determination of the enzymatic reaction rate. A circulating water bath (PolyScience 910 Refrigerated Circulator, Polyscience Div. of Preston Ind. Inc., Niles, IL, USA) was used to maintain constant experimental temperature with an accuracy of ±0.1 °C.

2.3. Determination of enzymatic reaction rate

The enzymatic reaction rate of GOx enzyme was determined by measuring the hydrogen peroxide production from D-glucose as described elsewhere [10,11]. The reaction was done in 100 ml of a 0.67 mM phosphate buffer solution (pH varies between 4.4 and 8.5 depending on the tested reaction pH) containing 10 mg GOx at defined reaction parameters. The initial substrate concentration was adjusted to the tested concentration by using a stock D-glucose solution (500 mM). The reaction medium was mixed continuously during the incubation period with a magnetic stirrer, and the reaction temperature was kept constant using a water bath. A 2.5 ml aliquot of 0.1 mM phosphate buffer (pH 7.0) containing POD (0.1 mg), 4-aminoantipyrin (0.5 mg) and phenol (2.5 mg) was the assay solution. A 1.0 ml sample of the GOx/D-glucose reaction solution was goended with 4 ml dilute acid solution (sulphuric acid, 0.2%, v/v), and then 100 μl of this solution was added to the assay mixture. After incubation at 37 °C for 15 min, hydrogen peroxide produced from glucose was determined spectrophotometrically at 505 nm and the remaining glucose concentration in the reaction medium was calculated stoichiometrically. The graph of glucose concentration versus incubation period was plotted and the initial reaction rate $(dC/dt)_{t=0}$ was determined.

2.4. Experimental design with response surface methodology

Before arranging an experimental design with response surface methodology, the effects of substrate concentration, reaction temperature and pH on enzymatic reaction rate were tested by varying one factor at a time while keeping the others constant.

A three-factor and five-coded level central composite circumscribed design (CCCD) was used to determine the GOx enzyme kinetic constants. Three independent variables (reciprocal of substrate concentration, $1/S$; reaction medium temperature, T; reaction medium pH) were varied simultaneously relative to the chosen center point (reciprocal of substrate concentration, 0.267 mM^{-1}; reaction medium temperature, 27.5 °C; acidity, pH 6.5), there being six replicates at the center points and single runs for each of the other combinations, 20 runs were done in a totally random order. Duplicate experiments were carried out at all design points. The independent variables and their levels are given in Table 1. The relationship between the natural variables (x_i) and coded variables (X_i) is

$$X_i = \frac{x_i - [x_{max} + x_{min}]/2}{[x_{max} - x_{min}]/2} \quad (3)$$