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The optimization of synthesis conditions for laccase entrapment in mesoporous silica microparticles by response surface methodology

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A B S T R A C T

This study investigated the optimization of in-situ laccase entrapment in mesoporous silica microparticles. The response surface methodology (RSM) based on a 3-level-4-factor Box–Behnken experimental design was employed to establish the relationships among the independent synthesis variables (ISV) as well as to search for an optimal synthesis condition for laccase entrapped mesoporous silica microparticles (LSM). The ISV comprise of H_2O/TEOS molar ratio (H_2O/TEOS), hydrochloric acid loading (HCl), triethylamine loading (TEA), and laccase loading (Lac) were evaluated towards the specific laccase catalytic activity (A_s) response as the dependent variable. The optimization results show that the optimal ISV obtained were (H_2O/TEOS) = 5.44, (HCl) = 2.52 × 10^{-6} mol, (TEA) = 0.39 × 10^{-3} mol and (Lac) = 3.83 mg/ml having the predicted and observed A_s of 301.7 and 298.36 U/g, respectively. A moderate degree of correlation between the A_s response and ISV was as well obtained by the determination coefficient (R^2) value of 0.89. These results demonstrated the applicability of RSM for the optimization of ISV in LSM synthesis. It was found that the LSM has a good reusability that holds potential industrial applications, even though it encounters the mass transfer kinetic limitations.

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1. Introduction

Silica materials have been used as support materials for enzyme immobilization with a wide range of applications including biocatalysis, biosensing, biofuels and enzyme-controlled drug delivery [1]. Their inert properties such as the chemical stability over a broad pH and temperature ranges stand out among other available support materials. The silica gels are generally prepared via sol–gel polymerization of silicon alkoxides. It mainly involves the hydrolysis of the alkoxide group and subsequent condensation reaction of the silanol groups to produce siloxane bonds and the by-products of alcohol and water. The formation of this polymer during condensation process is eventually manipulated for the immobilization of biomolecules such as enzymes and cells [2,3]. These biomolecules which are retained in the polymeric are unlikely to leach out with a relatively small perturbation to the native structure and function of the biomolecules [4]. Moreover, the mesoporous structure and high pore volume of the silica materials enhance the mass transfer limitation that is frequently associated with the immobilization by entrapment method.

In retaining stability and catalytic activity of the biomolecules such as enzymes during immobilization, the consideration on the characteristics of the supports, reactive groups, as well as the immobilization conditions should be taken into account. It is a multivariate process which involves many factors that could affect its immobilization efficiency [5]. Furthermore, in designing an enzyme immobilization, there is no ‘one size fits all solution’ since every enzyme is different [6]. The optimization of various factors to attain the optimal conditions could be achieved using the one-factor-at-a-time (OFAT) approach in which the experimental factors are varied one at a time, while keeping all other variables at a fixed condition. This conventional approach is unfortunately time consuming and incapable to consider any possible interaction between the factors [7]. The optimization through the OFAT leads to a complex assessment of other factors occurring simultaneously and misinterpretation of experimental results [8]. The application of response surface methodology (RSM), a kind of mathematical and statistical method used for modeling and analyzing the relationships between several independent variables, becomes therefore more favorable for optimization of the desirable response variable(s) [7]. The utilization of RSM for optimization purpose makes the process more authentic and rapid since it could prevent the experimental repetition which enables the work to be carried out with less reagent and labor consumption, thus making the process more economical and less time consuming [9].

The first step of multivariate optimization is screening of the independent variables in order to obtain their significant effects on the dependent variables. After determining the significant independent variables, the experimental matrix will be constructed according to the selected experimental designs such as full factorial design, Doehlert matrix (DM), central composite designs (CCD) and Box–Behnken design (BBD). The experimental data obtained will then be computed into statistical analysis to obtain a mathematical model and to predict the optimum response(s) [10]. The Box–Behnken design (BBD), a modified central composite experimental design produces an excellent predictability result over other experimental designs. It is more efficient than CCD and three-level

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factorial designs as well as offers fewer experiments than other RSM designs with the same number of factors [7].

It was reported that the BBD has been employed for optimization of various enzyme immobilization purposes [5,8,9]. Nonetheless, the previous reports mostly focused on the immobilization efficiency as their response of interest since they utilized the ex-situ immobilization procedure by adsorption or covalent binding. As compared to in-situ immobilization procedure via entrapment and encapsulation method, the ex-situ procedure becomes disadvantageous since the process is somehow time consuming and often result in lower immobilization yield as well [11,12]. In contrast, enzyme could be entirely entrapped in silica matrices via in-situ immobilization procedure as well as retained its initial catalytic activity after 1 month of storage duration [13].

To our knowledge, the application of RSM for optimization of the in-situ enzyme immobilization has not been reported elsewhere. Therefore, in this study, the RSM was performed to establish the relationships among the independent synthesis variables (ISV) as well as to search for an optimal synthesis conditions for the specific laccase catalytic activity (A_s) of the laccase entrapped mesoporous silica microparticles (LSM). The 3-level and 4-factor BBD was applied for this optimization study. The ISV optimization comprise of H_2O/TEOS molar ratio (H_2O/TEOS), hydrochloric acid loading (HCl), triethylamine loading (TEA), and laccase loading (Lac) followed by the characterization, kinetics and reusability of the LSM were investigated.

2. Materials and methods

2.1. Materials

The reagent water type 1 was obtained using a nanopure deionizer purchased from Purite Ltd. (England). Other chemicals were of analytical grade reagents from various suppliers and used without further purification. 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) sodium salt (ABTS), sodium acetate and n-hexane were purchased from Sigma–Aldrich (USA). Triethylamine (TEA), isopropanol (IPA) and tetraethyl orthosilicate (TEOS) were purchased from Merck (Germany). Laccase from *T. versicolor* was purchased from Daiwa Kasei Co. Ltd. (Japan). The purity of the laccase powder was 30% (w/w) of pure protein and 70% (w/w) of dextrin, having molecular weight of 62 kDa, and the *p*I was 3.

2.2. LSM synthesis

Silica sols were prepared by hydrolysis and condensation reactions of TEOS as a main precursor. The starting materials consisting of TEOS, IPA, reagent water and 0.05 M HCl were stirred at a constant speed (500–600 rpm) for 2 h followed an addition of 1 ml of laccase solution and base catalyst (TEA). The silica gel was then formed due to the condensation process. The gel was left to age at room temperature (30 °C) for 1 h to strengthen the silica framework structure. The gel underwent solvent exchange treatment in silane: isopropanol: hexane solution (1: 1: 2 by volume) for 2 h. The sample was then washed with IPA and dried at room temperature (30 °C) until a constant weight was obtained. The LSM powder was then collected and stored in an airtight Teflon bottle at 5 °C.

The residual protein in the solution (supernatant and/or washes) was determined by using the Biuret method [14]. In order to determine the leaching potential of the LSM, the accurate weight of the LSM was placed in the 10 ml, 0.4 M, and pH 7 phosphate buffer solution, stirring for 30 min using a magnetic stirrer at 500 rpm. The leached laccase in the phosphate buffer solution was then determined by using the Biuret method.

2.3. LSM characterization

A JSM-6390LV (JEOL, USA) scanning electron microscope (SEM) was used to examine the surface morphology of LSM. The LSM was sputter-coated with a thin layer of gold to avoid electrostatic charging during the examination with an accelerating voltage of 10–15 kV. The microstructure of the LSM sample was carried out by transmission electron microscopy (TEM) Tecnai G2 200 kV (Fei, USA). The specific surface area of the LSM was measured by the nitrogen adsorption–desorption technique carried out at a temperature of 77 K using a Micromeritics ASAP 2020 (USA). The pore size distribution was calculated on the basis of desorption branch of the isotherm plot by Barret–Joyner–Halenda (BJH) method [15].

2.4. Specific laccase catalytic activity assay

The specific laccase catalytic activities (A_s) of free laccase and LSM were determined using ABTS ($\varepsilon_{436} = 29\,300\ M^{-1}\ cm^{-1}$) as a substrate according to Leoniwicz et al. [16]. The reaction mixture contained 1 mM ABTS in 100 mM sodium acetate buffer (pH 5). An accurately weighed 0.05 g of sample (free laccase or LSM) was placed into a reaction mixture and the change in absorbance at 436 nm was recorded over 5 min using a Perkin Elmer LAMBDA 35 UV/VIS spectrophotometer (USA). The laccase catalytic activity, A (U) was calculated using Eq. (1) derived from the Beer–Lambert law;

$$A(U) = \frac{10^6 \cdot \Delta A \cdot V}{\varepsilon \cdot l \cdot \Delta t} \tag{1}$$

where ΔA is the change of absorbance at 436 nm, V is the reaction volume (L), ε is the molar absorption coefficient ($M^{-1}\ cm^{-1}$), l is the UV/VIS path length (cm) which is 1.00 cm for a standard UV/VIS cuvette, Δt is the reaction time (min.), and $\Delta A/\Delta t$ is the change of absorbent over time (slope). One unit of A (U) is defined as the amount of laccase or LSM that releases 1 μmole per minute of oxidized product, whereas the specific laccase catalytic activity, A_s (U/g) is obtained by dividing A (U) with the actual mass (g) of laccase used in the assay.

The kinetic parameters of the Michaelis–Menten equation (K_m and K_{cat}) for free and LSM were calculated from the reaction rates obtained from the batch experiments using initial ABTS concentrations ranging from 0.5 to 5 mM. For each run, the absorbance measured at 436 nm was recorded over time from which the initial reaction rate was then calculated. The kinetic parameters were estimated by fitting the kinetic data to the Michaelis–Menten equation using a non-linear regression method (Origin Pro 7.5). In order to investigate the reusability of the LSM, the experiment was conducted for ten-reaction cycle under fixed conditions for 1 h of reaction time.

2.5. Experimental design and statistical analysis

A 3-level-4-factor Box–Behnken design, requiring 27 experiments, was employed in this study. The independent synthesis variables (ISV) and their selected levels comprising of: the molar ratio of water to TEOS, (H_2O/TEOS) (2.5–7.5); hydrochloric acid loading, (HCl) ($0.5–3.5 \times 10^{-6}$ mol); triethylamine loading, (TEA) ($0.5–2.5 \times 10^{-3}$ mol); and laccase loading, (Lac) (0.5–5.5 mg/ml) are shown in Table 1. All experiments were repeated twice to reduce the experimental errors. The second-order polynomial

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