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Kinetic characteristics of biodegradation of methyl orange by *Pseudomonas putida* mt2 in suspended and immobilized cell systems

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ABSTRACT

Azo dyes, which are considered the most recalcitrant and persistent among all groups of dye, were biodegraded by various types of bacteria. In this study, the decolorization of methyl orange was determined under various conditions by non-immobilized and immobilized *Pseudomonas putida* mt2. In both systems, the decolorizations were more favorable under anoxic condition. Optimal conditions for decolorization were investigated. The optimal temperature for suspended cell operation is in the range between 33 and 35 °C and the decolorization seems not to be suitable in acidic condition. However, the optimal range of pH and temperature in the immobilized cell system were obtained at 7–9 and 35–37 °C, respectively. Kinetics parameters of ν_{max} and k_m were predicted up to 7.5 mg/(g h) and 283 g L^{-1}, respectively, for non-immobilized system; and 6.3 mg/(g h) and 257 g L^{-1}, respectively, for immobilized Ca-alginate system. Mass transfer coefficients were calculated as 0.75×10^{-5} and 6.20×10^{-5} for each system.

1. Introduction

Azo dyes are the most important and diffuse group of synthetic dyes, and have azo bond as the color-producing group, which are classified as the most recalcitrant and persistent group. Over 7×10^5 metric tons of synthesis dyes are produced worldwide every year for dyeing and printing [1]. It has been estimated that approximately 10% of the dye stuff used during dyeing processes does not bind to the fibers and is discharged with wastewater [2]. Several studies indicated that most azo dyes are toxic, carcinogenic, and mutagenic, which have substantial effects on environment and results in a threat to public health [3–5]. It was challenging to decolorize wastewater with recalcitrant synthetic dye compounds released from industrial effluents [6].

Several physical and chemical methods were used and indicated as efficient approaches for degradation of azo dyes, such as precipitation, adsorption, filtration, reverse osmosis, and advanced oxidation processes (reduction, complexometric methods, ion exchange and neutralization) [7–10]. However, such complex procedures may have relatively high cost and lead to sludge formation or secondary pollution. Biological treatments were discovered as the satisfaction of spontaneous demand to offer the complete clean-up of pollutants in a natural and economically feasible manner [11]. For aerobic decolorization, bacteria must be acclimatized for a long period under aerobic chemostatic growth in the presence of a simple azo compound. Subsequently, the bacteria synthesize an azoreductase that specifically corresponds to the azo compound added in the presence of oxygen. In contrast, decolorization under anaerobic activity is relatively nonspecific with respect to the included azo compounds. This indicates that an anaerobic process is more useful for the removal of mixed azo dye in wastewater [12].

It was reported that free cells degradation is inhibited at a high concentration of dye [13]. The application of immobilization can protect microbial cells from possible toxic effects caused by pollutant metabolites or changes in the environment conditions. The rapid development of this technology can be explained by its inherent advantages over the use of free cells, such as improved biocatalysts stability. It allows continuous operation without washout, separation of suspended biomass from the effluent, cell reuse for extended period of time, and protection of cells from inhibitory-products or contamination [14–16]. In a large number of studies using immobilized cells on azo dye decolorization, entrapment was the most widely used technique for immobilization, and alginate was chosen as the suitable matrix material. Such a mild method is characterized by preservation viability of cells to achieve all of the advantages of heterogeneous catalysis because of its nontoxic characteristic [17,18]. However, the diffusion limitation can reduce the degradation efficiency because substrates must

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be transported across external boundary layers (external mass transfer) and within the matrix (internal mass transfer) [19–21]

In this study, *Pseudomonas putida* mt2 was applied as the biological carrier to degrade methyl orange at both non-immobilized and immobilized Ca-alginate bead systems to investigate the decolorization properties and degradation limitation of this resistant dye. The optimality of each system was studied. Kinetics parameters and mass transfer coefficient were also evaluated and discussed.

2. Materials and methods

2.1. Materials and cell cultivation

The dye, methyl orange ($C_{14}H_{14}N_3NaO_3S$), was obtained from Merck Chemicals (Taiwan). Solutions were made by dissolving requisite quantity of the dye without further purification in distilled water. All other chemicals used in this study were purchased from Sigma (USA).

One colony of *P. putida* mt2 on agar medium was cultivated in 50 mL of medium 1 ($(NH_4)_2HPO_4$ 3 g/L, KH_2PO_4 1.2 g/L, NaCl 5 g/L, $MgSO_4.7H_2O$ 0.2 g/L, yeast extract 0.5 g/L, glucose 3 g/L and methyl orange 0.05 g/L) for cell growth, in which the carbon source consisted of 3 g/L glucose concentration and 50 mg/L methyl orange concentration. Biomass was harvested at late-exponential phase and subsequently transferred to 100 mL of medium 2 for biodegradation study. The medium 2 consisted the identical composition to medium 1, except that the dye concentration was doubled and the glucose concentration was considerably decreased to 100 mg/L. The fresh medium 2 in the 500 mL flask were flushed with filtered O_2 or N_2 before cells added. The suspended cell culture was sealed with parafilm on the top of flask to avoid penetration of air. Subsequently, the culture was performed under several of experimental conditions in an incubator at 200 rpm or without agitation (static condition).

2.2. Decolorization in non-immobilized system

The decolorization was operated under static and shake conditions for comparison of aerobic and anaerobic biodegradation. The static condition was chosen for the following experiments with various conditions of pH, temperature, cell mass, and initial dye concentration. Samples were collected at regular intervals and analyzed. Residual dye was determined through the absorbance of filtered supernatant at optical density of 465 nm, and biomass was measured at 600 nm by Spectrophotometer [22].

2.3. Preparation of immobilized Ca-alginate cell beads and decolorization by immobilized cell beads

The immobilization was performed according to the method described by Keweloh and Heipieper [23]. Biomass was harvested from medium 1 at stationary phase and subsequently re-suspended in 20 mL of 2% (w/v) sodium alginate. The final mixture was dropped into 200 mL $CaCl_2$ 2% (w/v) solution by gravity through syringe and needle. The drops of alginate solution were gelled to form 3 mm diameter spheres upon contact with $CaCl_2$ solution. The immobilized *P. putida* mt2 beads were stored in 2% $CaCl_2$ solution at 30 °C for 1 h to 2 h complete gel formation.

The beads with immobilized cells after 2 h storage in $CaCl_2$ were washed by deionized water and adapted to 50 mL fresh medium 1 at 30 °C and shaken at 100 rpm for 3 h. Subsequently, beads were rewashed by deionized water and transferred to medium 2 with limited concentration of glucose to obtain dye removal. Experiments were operated under various conditions, including dissolved oxygen, pH, temperature, cell mass and initial dye concentration. The determination of residual dye is described in Section 2.2.

2.4. Model description

2.4.1. Diffusion effects on nonporous support material

It was assumed that all cells are equally active, substrate diffuses through a thin liquid film surrounding the support surface to reach the reactive surface, and the intrinsic kinetic parameters ν'_{max} and k_m are unaltered.

At steady state, the reaction rate is equal to the mass transfer rate (J_s; based on Fick's law) and can be defined using Eq. (1):

$$J_s = k_L([S_b] - [S_s]) = \frac{\nu'_{max}[S_s]}{k_m + [S_s]} \quad (1)$$

where k_L is the liquid mass transfer coefficient (cm/s), S_b is the substrate concentration in bulk liquid, S_s is the substrate concentration at surface, ν'_{max} is the maximal reaction rate per unit of external surface area (g/(cm^2 s)), and k_m is the Michaelis Menten constant. Because the value of $[S_s]$ is not amenable to direct experimental observation, (1) can be solved graphically according to the method reported by Shuler *et al.* [24].

2.4.2. Diffusion effects in a porous matrix

It was assumed that the porous structure is isothermal and spherical in shape, and microorganisms are uniformly distributed in the particle. In addition, the substrate between the exterior and interior of the support was not partitioned. Therefore, Eq. (2) can be written in the basic of Fick's law stating that diffusion rate is equal to reaction rate at steady state.

$$\frac{d^2C}{dr^2} + \frac{2}{r}\frac{dC}{dr} = \frac{\rho_p}{D_e}\nu \quad (2)$$

where C is substrate concentration within the immobilized particles (mg/dm^3); r is the radical position within the bead; ρ_p is the density of dried microorganism (g/dm^3); D_e is effective diffusion coefficient of dye within the bead (cm^2/s) and ν is the actual biodegradation rate.

The biodegradation kinetics can be expressed by the first order kinetics if low concentration of dye substrate was introduced, and biodegradation rate (ν) can be defined in Eq. (3)

$$\nu = k * C \quad (3)$$

where k is the first order biodegradation rate constant (dm^{-3} solution g^{-1} dried cell weight h^{-1}) and C is substrate concentration within the immobilized particles (mg/dm^3). To solve Eq. (2) with boundary conditions which $C = C_s$ at $r = R$ and $dC/dr = 0$ at $r = 0$. Consequently, Eq. (4) can be yielded:

$$\frac{C}{C_S} = \frac{\sinh(3\varphi \cdot r/R)}{(r/R)\sinh 3\varphi} \quad (4)$$

where R is the radius of the bead (cm), C_s is the substrate concentration at the bead surface and φ is the Thiele modulus for a spherical particle which can be obtained by the following Eq. (5).

$$\varphi = \frac{R}{3}\sqrt{\frac{k'}{D_e}} \quad (5)$$

where k' is equaled to $\frac{k}{\rho} * 3.6$, and ρ is density of bead.

The effectiveness factor, η, was defined as the ratio of the actual reaction rate to the rate evaluated at outer surface conditions (ν_s) (see Eq. (6)).

$$\nu = \eta * \nu_s \quad (6)$$