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Kinetic mathematical model for ketone bioconversion using *Escherichia coli* TOP10 pQR239

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HIGHLIGHTS

- A non-reported pseudo intrinsic kinetic model was developed on basic reactions.
- The reaction mechanism proposed followed the formalism of Langmuir–Hinshelwood.
- Complexes formation of substrate inhibition and oxygen inactivation are accounted.
- Affinity and inhibition constants were obtained from the kinetic model.
- This is the first report for oxygen inactivation constant (22.3 μM).

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ABSTRACT

The aim of the current work was to develop a pseudo intrinsic kinetic model, based on elementary reactions, to describe the behavior of the bioconversion of ketones using *Escherichia coli* TOP10 pQR239. Since there are no reports of the oxygen inactivation constant in the literature, this study gave new insights to find optimal conditions of a suitable oxygen supply during the bioconversion. In this model the reaction mechanism proposed followed the formalism of Langmuir–Hinshelwood and considered both substrate inhibition and oxygen inactivation by the formation of intermediary complexes. Therefore, approximations of the pseudo equilibrium of reaction rates or steady state intermediary species were not considered, which allowed for identifying the role of each reaction step involved in the bioconversion. This kinetic model adequately described the observations with and without substrate inhibition and/or oxygen inactivation. And the regression and the estimated parameters were statistically significant, making these analyses reliable regarding the kinetic behavior of CHMO. Then, substrate and oxygen affinity and inhibition constants were obtained from the kinetic parameters of the model. It was observed that oxygen and substrate presented similar affinity constant values. The substrate inhibition (K_{IS}) and oxygen inactivation (K_{IO2}) constants were determined to be 9.98 μM and 22.3 μM, respectively, showing that the CHMO enzyme was twice more sensitive to inhibition by an excess of substrate than oxygen.

1. Introduction

Lactones have wide applications in flavorings, as precursors of anticancer and antihypertension drugs and in the pharmaceutical industry [1]. Lactones have been obtained by Baeyer–Villiger reactions using catalytic process [2] or whole cell bioconversion with a cyclohexanone monooxygenase (CHMO) expressed in *Escherichia coli* TOP10 pQR239 [3]. The use of whole cells allows enzyme cofactor regeneration for the production of enantiomerically pure compounds [4]. However, it has been reported [5,6] that ketone bioconversion using CHMO is inhibited by the substrate and product at concentrations above 0.4 and 4 g L^{-1}, respectively. Additionally, Bennett [7] reported that enzyme inactivation may occur due to residue oxidation of two serines close to the active site. A number of strategies have been proposed to avoid these types of inhibition, such as substrate feeding and *in situ* product removal using Lewatit resin [8], biocatalyzer encapsulation to prevent CHMO oxidation [9], the use of ionic liquids as an immiscible phase substrate reservoir and *in situ* product removal and maintaining

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Nomenclature

CHMO	cyclohexanone monooxygenase
N_T	enzyme total concentration (μg g^{-1} *of biomass*)
θ_E	free enzyme fraction
θ_{EO_2}	enzyme–oxygen complex fraction
θ_{EO_2S}	enzyme–oxygen-substrate complex fraction
θ_{EO_2SS}	substrate inhibition complex fraction
$\theta_{O_2EO_2}$	oxygen inactivation complex fraction
[*P*]	product concentration (g L^{-1})
[O_2]	oxygen concentration (g L^{-1})
[*S*]	substrate concentration (g L^{-1})
r_n	reaction rate of each enzymatic complex (g L^{-1} h^{-1})
k_j	kinetic constant of the reaction (s^{-1})
k_La	oxygen mass transfer coefficient (h^{-1})
K	affinity or inhibition constant of substrate and/or oxygen (g L^{-1})

the biocatalyzer (whole cells) in the aqueous phase [4], a stirred tank partitioning bioreactor using ionic liquids as the dispersed phase [10] and CHMO molecular structure changes to fold the serines susceptible to oxidation inside the enzyme [11].

Despite several experimental studies on the molecular structure of CHMO, its catalytic activity and reaction rates of the intermediate steps in the overall reaction in order to propose a basic bioconversion mechanism [12–14], there have been few studies on kinetic modeling considering simultaneous substrate, product and oxygen inhibition phenomena. Some pseudo empirical kinetic models have been reported following Michaelis–Menten approach to describe product formation and substrate consumption in monooxygenase kinetics [15,16,17]. However, they do not take into account elementary reactions accounting for oxygen as a second substrate for bioconversion. The use of this kind of kinetic models reduces the number of required kinetic parameters; nevertheless, the estimated kinetic values depend on the catalyst concentration and become independent of the reactor size and its geometrical configuration, providing uncertainties for scaling-up ketone bioconversion. In this sense the development of a kinetic model based on an elementary reaction mechanism describing Baeyer–Villiger bioconversion will make it possible to describe, understand and find optimal conditions for carrying out this kind of bioconversion but mainly for design and scale-up.

The objective of this work was to develop a pseudo-intrinsic kinetic model to describe the behavior of the bioconversion of ketones using whole cells. The mathematical model was based on an elementary reaction mechanism that followed the Langmuir–Hinshelwood formalism, considering inhibition and inactivation by the formation of substrate or oxygen enzyme complexes in the active site of the CHMO. The mathematical model was adjusted and kinetic parameters were estimated for three possible cases of the bioconversion of ketones: (1) without any inhibition, (2) inactivation by oxygen excess and (3) inhibition by substrate excess. The mathematical model was validated through the comparison of experimental data obtained for simultaneous substrate inhibition and oxygen inactivation versus calculated values using the estimated kinetic parameters. The kinetic parameters obtained yielded valuable information to determine which may be the limiting step in the bioconversion reaction.

2. Materials and methods

2.1. Microorganism and chemicals

The *E. coli* strain TOP10 pQR239 was kindly provided by Professor John M. Ward (University College London, London, United Kingdom) for research and academic purposes, and is referred to hereafter simply as *E. coli*. To prepare inocula for bioconversion experiments, *E. coli* cells were cultured in Erlenmeyer flasks of 250 mL containing 70 mL of a complex media (in g L^{-1}): tryptone 10.0, yeast extract 10, NaCl 10.0, in phosphate buffer 50 mM pH 7.0, supplemented with 10 g L^{-1} glycerol. Culture media was sterilized in an autoclave at 120 °C for 15 min and supplemented with 100 mg L^{-1} ampicillin (previously filter sterilized using a 0.25 μm filter). Erlenmeyer flasks were incubated at 150 rpm for 16 h at 37 °C. After this 16 h growth period, cyclohexanone monooxygenase expression was induced by adding the necessary amount of arabinose solution (100 g L^{-1}) to reach a final concentration of 2 g L^{-1}. After 3 h of induction, cells were harvested by centrifugation at 5000 rpm for 10 min.

Bicyclic ketone bicycle[3.2.0]hept-2-to-6-one (⩾ 98%) and bicyclic lactone (1S,5R)-(-)-2-oxabiciclo[3.3.0]oct-6-en-3-ona (⩾ 99.0%)) (Fluka, Switzerland) were used as the substrate and product standards, respectively. Tryptone, yeast extract, NaCl and glycerol were purchased from Sigma Aldrich (EUA).

2.2. Stirred tank bioreactor description

A module with two glass 100 mL stirred tank bioreactors (MMBR100, UAM-I, Mexico) was used for all bioconversion studies. The jacketed bioreactors had an internal diameter of 4.75 cm and an operating volume of 70 mL (H_L/D_T = 0.87). The bioreactors were fitted with a single, six flat blade Rushton turbine, D_i = 1.9 cm (D_i/D_T = 0.40), located 1.9 cm from the flat base of the vessel. The bioreactor was equipped with four equidistant baffles, 0.5 cm in width, to enhance mixing.

2.3. Oxygen mass transfer coefficient (k_La) determination

Optical fiber dissolved oxygen mini sensors (PreSens, GmbH Germany) were used for k_La determinations. The oxygen sensors were coupled to an OXY-4 mini four-channel oxygen meter (PreSens, GmbH Germany). Oxygen mass transfer coefficients (k_La) were calculated according to the dynamic method and mathematical model proposed by Fuchs et al. [18], which takes into account the electrode response time and the dimensionless dissolved oxygen concentrations in the bioreactor (Eq. (1)). The effect of operating conditions, including agitation (750, 1350 and 1950 rpm) and aeration (0.75, 1.0 and 1.4 vvm) rates on k_La was studied.

$$Y_P = \frac{K_P \cdot e^{-k_L a t} - k_L a \cdot e^{-K_P t}}{K_P - k_L a} \quad (1)$$

where Y_P is the dimensionless dissolved oxygen concentrations in the bioreactor defined by Eq. (2).

$$Y_P = \frac{C_P^* - C_P}{C_P^* - C_{P0}} \quad (2)$$

where C_P^* is the saturated oxygen concentration (7.2 mg L^{-1}, which was determined with the OXY-4 module software using the atmospheric pressure of Mexico City, 1016.9 hPa), C_P is the dissolved oxygen concentration, C_{P0} is the initial dissolved oxygen concentration the bioreactor. K_P is the electrode constant defined as the inverse of the response time, k_La is the oxygen mass transfer