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# Process characterization of a monoamine oxidase

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ABSTRACT

Redox biocatalysis is currently gaining focus because it offers exquisite selectivity using mild oxidants, such as oxygen (which is environmentally benign). However, it is often challenging to implement oxidative reactions at scale due to the low activity and stability of the biocatalyst under industrial conditions. Consequently, it becomes critical to identify the bottlenecks for specific oxidation reactions as a first step in scale-up. Subsequently, we can identify where research the effort is required when developing a biocatalytic reaction for implementation in an industrial reaction, i.e., on biocatalyst development (e.g. improvement of expression levels), process development (e.g. improved oxygen supply, product removal strategies) or biocatalyst stabilization (e.g. through immobilization or directed evolution). This paper presents a systematic method to identify the bottleneck of a potential biocatalytic process using a monoamine oxidase to synthesize an intermediate in the manufacture of a drug for treating Hepatitis C (Telaprevir).



## 1. Introduction

Biocatalysis for oxidation reactions is gaining focus because such reactions require less corrosive oxidants compared to chemical oxidations (e.g. replacing chromates with oxygen) [1]. Although advances towards green chemistry have helped in finding less corrosive oxidative catalysts (such as transition metals) for catalysis, selectivity still seems to be a limitation [2,3], also making biocatalysis in many cases more attractive. Several types of biocatalyst can be used for selective oxidation reactions including oxygenases [4–6], peroxidases [7], dehydrogenases [8], peroxygenases [9] and oxidases [10]. Among these enzymes, oxidases are attracting particular interest for their potential in industrial reactions. One such important example is the use of amino acid oxidase [11]. This paper deals with another type of oxidase–amine oxidase. Amine oxidases have been shown to be useful for obtain optically pure amines [12] (i.e. for deracemization) as well as for the synthesis of imines of particular interest in the pharmaceutical industry [13].

In the former case, a racemic mixture of amines is deracemized by combining an oxidation reaction catalyzed by monoamine oxidase (MAO) (EC 1.4.3.4) with a non-selective reduction using a chemical catalyst (represented by Scheme 1). By repeating this procedure, an optically pure amine can be obtained.

The latter synthetic process scheme is exemplified in a recent joint publication from Merck and Codexis [13]. They established the possibility of using MAO for the manufacture of Boceprevir-T intermediate, used for the treatment of Hepatitis C patients with HCV genotype [14]. Codexis was able to produce a 500 Kg batch of the Boceprevir intermediate with a 66% yield and >99% ee.

This paper deals with the synthetic process (as represented in Scheme 2). Here another potential MAO catalyzed oxidation of a secondary amine (also of pharmaceutical relevance) is examined [15,16]. The imine product is an intermediate in the production of telaprevir (a drug which has recently been approved by the FDA for the treatment of Hepatitis C) [17,18]. The conventional production of telaprevir consists of many steps (a sequence of nine steps is used to synthesize the central bicyclic pyrroline derivative), reducing the yield [19]. It has been shown that telaprevir can be produced with reduced number of steps when the imine product from Scheme 2 is used [17]. By replacing one of the key intermediate production steps by a selective reaction, the cost of the final drug can be lowered. However, the process for telaprevir production using MAO is not developed since most studies to date have been carried out as a proof of concept [20].

For process development, it is crucial to quantify the system and identify limitations and targets for the reaction. This paper indicates the first efforts towards quantitation of the target reaction system (Fig. 1). A systematic approach has been used to characterize the biocatalyst, which includes screening of different mutants as well as identifying the inhibition and inactivation caused by the substrate and products. A by-product of this reaction is hydrogen peroxide

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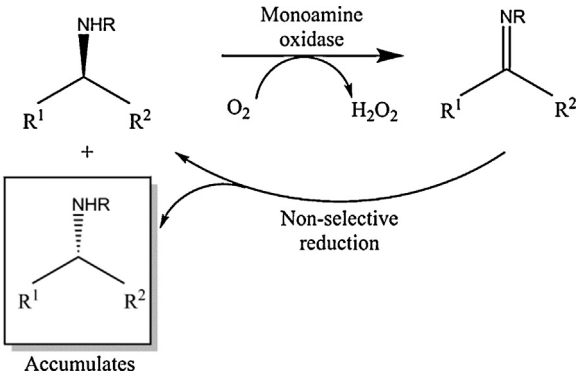
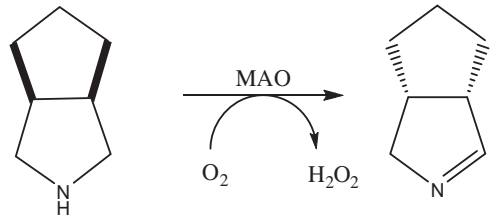



**Scheme 1.** Deracemization of an amine using an amine oxidase.




**Scheme 2.** Reaction scheme representing the oxidation of amine to imine catalyzed by monoamine oxidase.

which can potentially cause inactivation of the enzyme [21]. It is therefore necessary to remove the peroxide as it is formed to maintain the enzyme stability and it is hypothesized that this can be achieved by the use of another enzyme, catalase (EC 1.11.1.6). Following these studies, the constraints of the system were identified and compared to the desired target.

## 2. Materials and methods

All chemicals unless specified were purchased at Sigma–Aldrich (Steinhiem, Germany) and used as purchased. The solvents were GC grade while the salts were analytical grade.

### 2.1. Plasmid

Plasmids (pET 16b vector) containing 3 different mutants of MAO genes derived from *Aspergillus niger* (wild-type) [22] were kindly donated by University of Manchester (Professor N.J. Turner).

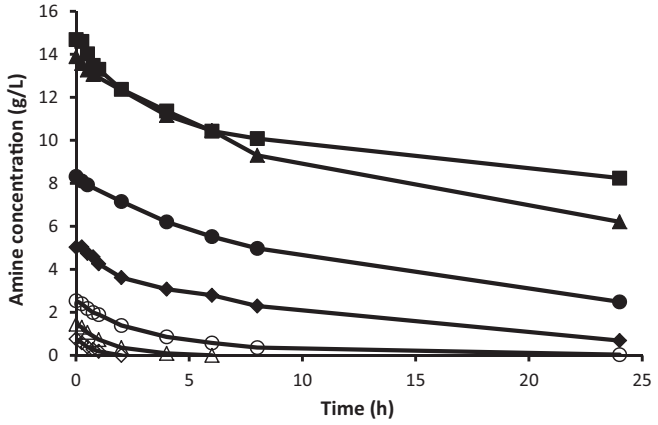



**Fig. 1.** Reaction progress curves for MAO-N-D5 catalyzed reaction at different substrate concentrations. (■) 15 g/L, (▲) 14 g/L, (●) 8 g/L, (♦) 5 g/L, (○) 2.5 g/L, (△) 1.5 g/L, (◊) 0.78 g/L.

### 2.2. LB broth and plates

LB broth was made with 10 g/L tryptone (Nordic biolabs AB, Täby, Sweden), 5 g/L yeast extract (Nordic biolabs AB, Täby, Sweden) and 10 g/L sodium hydroxide. To this, 2% (w/v) agar was added for making plates. The broth was autoclaved and ampicillin (filter-sterilized) was added to the broth (100 μg ampicillin/mL media) prior to use and the agar just prior to plating (100 μg ampicillin/mL media).

### 2.3. Preparation of competent cells

A starter culture was used to inoculate 5 mL of LB broth and the cells were incubated at 37 °C to grow the cells to an $OD_{600}$ of ~0.6. 2 mL of cells were transferred to Eppendorf tubes and incubated in ice for 20 min. The cells were then centrifuged at 6000 rpm for 10 min and the pellet was re-suspended in ice-cold water. The centrifugation step followed by re-suspension in water was repeated and centrifuged to obtain pellet. The pellet was re-suspended in 500 μL of 10% glycerol and centrifuged for 10 min. The supernatant was discarded, the pellet re-suspended in glycerol. The cells were stored as 100 μL aliquots until further use.

### 2.4. Plasmid transformation

Plasmids (3 mutants) obtained from University of Manchester were transformed into competent *E. coli* BL21 by electroporation. To 50 μL of electro-competent cells, 0.5 μL of the respective plasmid was added and transferred to an electroporation cuvette (BIORAD, Copenhagen, Denmark) and exposed to a short pulse of high voltage. Following the electroporation, 950 μL of LB medium was added to the cuvette and incubated for 1 h at 37 °C. 50 μL of the cell suspension was plated onto a LB plate containing ampicillin.

A colony was picked from the transformation plate, streaked onto a new plate and, incubated at 37 °C overnight. 5 mL of LB broth containing ampicillin was inoculated with a colony and allowed to grow to an $OD_{600}$ of 0.6. This culture was then stored in 10% glycerol to obtain glycerol stocks. The stocks were stored at −80 °C until further use.

### 2.5. Fermentation

#### 2.5.1. LB plates and pre-culture

From the glycerol stock, cells were streaked onto an LB plate containing 100 μg/mL ampicillin. The plate was incubated at 37 °C overnight. 5 mL of LB broth containing 100 μg/mL of ampicillin was inoculated with a colony from the LB-Amp plates. The culture tube was incubated at 30 °C in a shaker at 150 rpm for about 4 h.

#### 2.5.2. Fermentation

All fermentations were carried out in un-baffled shake flasks. To 500 mL of LB broth containing ampicillin, 5 mL of pre-culture was added (1% inoculum). The flask was incubated at 30 °C, 150 rpm. Cells were harvested after 18 h of growth by centrifugation at 4000 rpm, 20 min. The harvested cells were re-suspended in 100 mM phosphate buffer containing mono and di-basic potassium salts at pH 7.6 (25 °C) and used for biocatalysis. Fermentation was carried out prior to each biocatalytic reaction.

### 2.6. Biocatalysis

Biocatalysis was carried out in baffled shake flasks with a final working volume 20 mL of reaction mixture (substrate and biocatalyst suspended in 100 mM phosphate buffer, pH 7.6) incubated at 37 °C and 150 rpm. The substrate, aza-bicyclo-octane HCl was