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Ammonolysis of (5*S*)-*N*-(*tert*-butoxycarbonyl)-5-(methoxycarbonyl)-2-pyrroline with immobilized *Candida antarctica* lipase B (CAL B) in a packed bed reactor

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ABSTRACT

Conversion of (5*S*)-*N*-(*tert*-butoxycarbonyl)-5-(methoxycarbonyl)-2-pyrroline to its corresponding amide, an important intermediate in the synthesis of the dipeptidyl peptidase IV (DPP4) inhibitor Saxagliptin, was carried out by ammonolysis reaction catalyzed by immobilized *Candida antarctica* lipase B (Novozyme 435) in a packed bed reactor. The reaction proceeds smoothly at 50 °C in anhydrous *tert*-butanol containing 1.25 M ammonia and follows typical Michaelis-Menten kinetics with competitive product inhibition. The estimated kinetic parameters were V_{max} 40 ± 4.4 mM $h^{-1}g^{-1}$, K_m (216 ± 22 mM) and K_i 303 ± 31 mM. At substrate concentration of 30 mg/mL (132 mM) and flow rate of 0.1 mL/min, the product is obtained in > 98% yield and 98.5% purity at steady state in a column (100 cm × 1.2 cm) packed with 40 g immobilized CAL B.

1. Introduction

Lipases (EC Number 3.1.1.3) are the most commonly used enzymes in biocatalysis because of their wide substrate specificity coupled to a high regio- or enantioselectivity or specificity [1–3]. To overcome problems of operational stability, recovery and recycle, the enzymes are immobilized on solid supports [4–12] employing a variety of techniques ranging from simple adsorption, encapsulation and entrapment, to covalent bonding. The nature of immobilization support plays an important role since providing enough mechanical strength, resistance of chemical attack & microbial decomposition, maintaining the tertiary structure of the enzyme and preserving the catalytically active site of the enzyme in its immobilized state is absolutely essential [13]. The stability of the enzyme intensifies mostly due to several equivalent factors such as multipoint interactions, enclosure in an environment which is more favourable for its activity than free enzyme, protection from direct interactions with solvents etc. Another crucial factor is the choice of immobilization technique used. The enzyme may undergo structural changes during the immobilization process and this would affect its properties such as stability and enantioselectivity. In some cases, the enzyme may even get purified during immobilization [14], its enantioselectivity can improve and sometimes even it may even reverse [15] as amply demonstrated by groups of Fernandez-Lafuente and Guisan [15–17].

Among the commercially available lipases, *Candida antarctica* lipase B (CAL B) is probably the most popular [18–21]. It has a molecular weight of 33 kDa and isoelectric point of 6.0. The enzyme is sold in immobilized form as Novozyme-435. According to available information, the immobilization matrix consists of macroporous acrylic polymer beads of 500–700 μ diameter (Lewatit VP OC 1600, Bayer) [22–24]. The beads have BET surface area of 81.6 $m^2 g^{-1}$, Pore Volume of 0.45 $cm^3 g^{-1}$ and average pore diameter of 17.7 nm [25]. Investigations by the group of Prof. Roberto Fernandez-Lafuente have shown that the enzyme is mainly immobilized by adsorption via hydrophobic interactions in an “open-lid” form on the polymer surface [22]. The immobilized enzyme shows exceptional operational stability in organic solvents and temperatures up to 60 °C [26].

Saxagliptin is a well known dipeptidyl peptidase IV (DPP4) inhibitor and is marketed under the trade name ONGLYZA® by Bristol-Myers Squibb for the treatment of type 2 diabetes [27]. The amide (5*S*)-5-aminocarbonyl-4,5-dihydro-1H-pyrrole-1-carboxylic acid, 1-(1,1-dimethylethyl) ester **2**, is an important intermediate in its synthesis. The preparation of **2** by direct amidation with ammonia suffers from amide racemization and side-product formation while a two-step conversion via coupling reagents under mild conditions leads to low yields (57–64%) [26]. In comparison, several reports of enzymatic amidation without formation of side products and racemization have also been made [27–33]. CAL B-mediated ammonolysis of ester **1** without racemization in 96% yield has been reported by Patel and co-workers [26]. In this case the reaction was carried out in an autoclave under pressure

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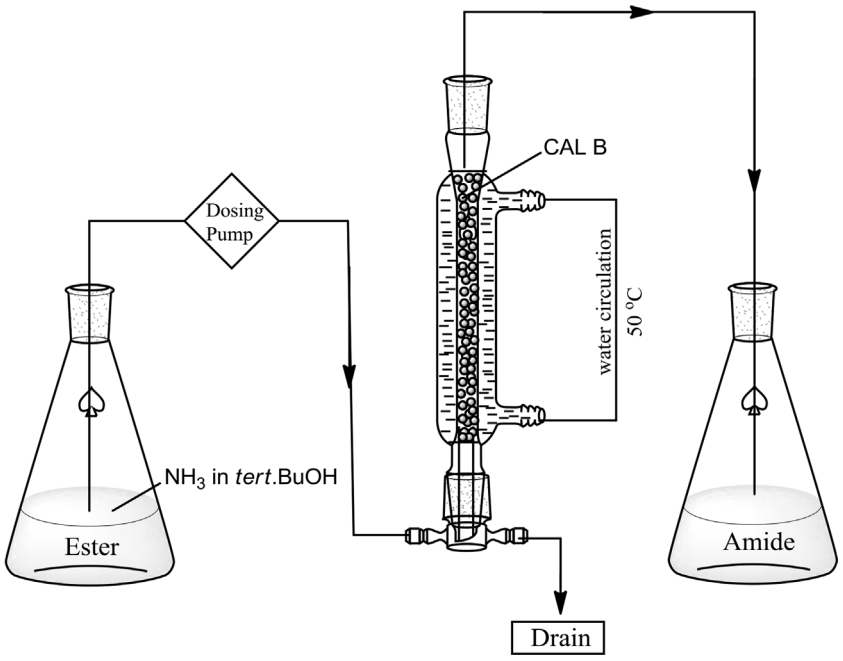

Fig. 1. Schematic diagram of packed bed reactor (100 cm × 1.2 cm).

using free enzyme suspended in toluene with enzyme to ester ratio of 1:2 (w/w). The reaction was complete in 3 days, and after workup, the product of 98% purity was obtained in 81% yield. It was however not mentioned whether the enzyme was recovered and reused.

CAL B immobilized on different solid supports was also used by the same authors [34]. In this case, the reaction was carried out in a packed bed reactor with recycle. The overall procedure as reported in the patent is however quite cumbersome. The enzymatic reaction is carried out in a mixture of *tert*-butyl methyl ether (MTBE) and *tert*-butanol containing ammonia. Although, it was not specifically mentioned, the reaction appears to suffer from product inhibition and for that reason, the reaction is stopped at approximately 50% conversion. The product is separated by column chromatography and the substrate is recycled. This process needs to be repeated three times to obtain product in 66% yield which decreases to 50% after crystallization. For large scale production, such a complicated procedure is not desirable.

In present work, we have simplified the overall process by performing the ammonolysis reaction with immobilized CAL B (Novozyme 435**)** in a catalytic packed bed reactor (PBR) (Fig. 1). The reactor essentially consists of a jacketed glass column maintained at 50 °C with a circulating water bath. The column is filled with immobilized enzyme beads and substrate dissolved in anhydrous *tert*-butanol containing 1.25 M ammonia is pumped at a fixed flow rate from the bottom of the reactor. The product stream is collected from top and analysed for monitoring the reaction and the kinetic parameters were studied in order to overcome product inhibition and process optimization.

2. Materials and methods

2.1. Materials

Novozyme 435 was a gift from Novozymes, Bangalore, India. Gum arabic (cat. no. 51198) was obtained from Sigma-Aldrich, Bangalore, India. The ester **1** was a gift from M/s Lee Pharma Ltd, Hyderabad. Glyceryl tributyrate (tributyrin), calcium acetate, and Tris-HCl buffer were obtained from Hi-Media, India. All solvents were HPLC grade purchased from Merck India and used as received. All other reagents were of analytical grade obtained from Qualigens, India and used as received.

2.2. Methods

2.2.1. Ammoniacal solution

The solvent (1 L) was taken in a 2 L jacketed vessel cooled to 15 °C with water circulation bath and ammonia gas was slowly bubbled for 1 h. Temperature of the ammoniacal solution was slowly raised to 50 °C before use. Ammonia concentration in the solvent was determined by mixing 100 µL of the solvent containing ammonia with 10 mL of 0.1 N HCl and back titrating residual HCl with 0.1 N NaOH using phenolphthalein indicator. Required ammonia concentrations (1–1.25 M) were prepared by appropriate dilution of the stock solution with the solvent. Water content of *tert*-butanol solutions was adjusted by addition of appropriate volumes of distilled water to commercially available dry solvent (water content 0.05%, Karl-Fisher titration).

All enzyme assays were performed at 30 °C. All experiments were repeated 3 times and were reproducible within (± 5%). Analysis of experimental data was performed using Graph Pad Prism 5 (www.graphpad.com).

2.2.2. CAL B assay

Enzyme activity was measured at 30 °C using tributyrin as substrate. A well sonicated emulsion consisting of 10 mM Tris-HCl buffer, 3% gum arabic, 0.25 M calcium acetate, 10 mM calcium chloride and 0.43 M tributyrin was used as assay solution. The free enzyme solution (100 µL of 5 mg/mL) or polymer bearing immobilized enzyme (50 mg) was added to substrate solution (15 mL) and stirred at 200 rpm. Acid produced during hydrolysis was continuously titrated with 0.2 N NaOH maintaining the pH at 7.5 for 10 min. Enzyme activity (tributyrin hydrolysis units, TBU) is expressed in terms of µmoles of NaOH consumed in 1 min. The native enzyme from Sigma showed activity of 5000 TBU g^{-1} while the immobilized enzyme had activity of 200 TBU g^{-1} under present assay conditions. Based on these values, the enzyme loading on the support was 40 mg g^{-1}. The enzyme activity and loading were calculated by using the following Eqs. (1) and (2),

$$\text{Specific activity (units g}^{-1}) = \frac{\{[\text{Vol. of NaOH consumed in 10 min}]\text{x}[\text{Normality of NaOH}]\text{x 1000x 100}\}}{[\text{mg of polymer used}]} \quad (1)$$