

Bioesterification of carboxylic acids by immobilized esterase of *Pisum sativum*

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ABSTRACT

Semi-purified immobilized esterase, isolated from the seed of *Pisum sativum*, was found to be an efficient biocatalyst for the facile esterification of pharmaceutically important carboxylic acids. The partially purified esterase precipitated using ammonium sulfate was immobilized using sodium alginate method. After incubation, the immobilized beads so formed were found to be spherical with an average size 3 mm. Immobilized esterase (Km: 580 ug, Vmax: 350 ug/min) to be tested for the esterification of aromatic carboxylic acids was incubated separately with methanol containing benzoic acid, 4-amino-2-chlorobenzoic acid, salicylic acid, and ethanol containing 4-aminobenzoic acid, respectively, at room temperature with constant stirring. Activity of free and immobilized esterase was measured with synthetic substrate, 4-nitrophenyl acetate. The reaction progress was monitored by thin layer chromatography (Hexane: Ethyl acetate) with successive time intervals. The corresponding esters methylbenzoate, 4-amino-2-chloromethylbenzoate, methylsalicylate, and 4-aminoethylbenzoate were analyzed with spectroscopic techniques and determination of physical constants. This confirmed the ability of esterase to transform organic acids smoothly into desired esters. The present studies demonstrated the suitability of preparation and promising procedure for the green synthesis of aromatic esters. Highly purified esterase of *P. sativum* will afford wide futuristic scope for large scale production of pharmaceutically and/or industrially important products.

1. INTRODUCTION

Pisum sativum (Green peas) has a third large family of flowering plants offering an important ecological advantage due to its contribution to the development of low input farming system by fixing atmospheric nitrogen. The seeds of *P. sativum* are rich in protein, starch, sugars, fiber, minerals, and vitamins. Six detectable esterases from the germinating seeds of twelve varieties of this plant have been reported by Frankel and Garber using starch-gel zone electrophoresis [1]. Esterases are diverse group of hydrolyses catalyzing both way of reaction, the breaking as well as formation of ester bonds and ubiquitously distributed in microorganisms, plants, and animals [2]. In spite of this fact, they exhibit high regio- and stereospecificity, which makes them prominent and attractive biocatalysts for the preparation of optically pure compounds in chemicals synthesis [2,3]. These enzymes are stable enough to perform their action even in organic solvents and do not require cofactors. Highly specific feruloyl esterase [4] esterifies number of acids including fatty acids with alcohols and sugars. The desired modification in hydrophobicity or hydrophilicity of the original

compounds widens their applicability in the cosmetics, food, and pharmaceutical industries [5]. For commercial exploitation, attention has been directed toward the development of unique biocatalytic reaction. Biocatalysts have been the ideal choice for various bioprocesses, as chemical and pharmaceutical industries are always in pursuance for economically affordable and scalable processes with minimal waste generation.

The biocatalysts offer unique characteristics over chemical catalyst for regio-, stereo-, and chemo selectivity, high yield, and operate at mild conditions (temperature and pH). They work with minimum side reactions with ease in separation excluding environmental pollution [6]. Therefore, immobilization of such biocatalysts is necessary to make them recyclable, giving thermal and catalytic stability, resulting higher catalytic conversions [7].

In the present study immobilized esterase, isolated from *P. sativum* was employed for the biotransformation of some selected aromatic carboxylic acids.

2. MATERIALS AND METHODS

2.1. Substrate Synthesis

To test the activity of this biocatalyst, a synthetic substrate, 4-nitrophenyl acetate was prepared by acetylating 4-nitro phenol with acetic anhydride.

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2.2. Isolation and Semi-purification of Esterase

40 g of fresh *P. sativum* purchased from local market and crushed in mortar pestle. Crushed seeds were commingled in 400 mL of 0.05 M phosphate buffer (pH 4.5) under cold conditions. The resulting homogenized solution was centrifuged at 10°C for 15 min at 10,000 rpm. To precipitate the proteins containing the esterase, 50 % ammonium sulfate was mixed to the supernatant. The precipitate so obtained was dissolved in minimum quantity of water and dialyze against 0.2 M phosphate buffer (pH 7) at 5°C for 12 h. The dialyzed solution was incubated with acetone (1:10 v/v) at 4°C for precipitation [8]. The precipitate was collected separately and dissolved in minimum quantity of 0.2 M phosphate buffer. The resulting solution was then used as a source of an esterase. Activity of esterase was measured during isolation and semi-purified using synthetic substrate. Total protein content was determined during each step of purification by Bradford method [9] using 100 µg of BSA solution as a standard.

2.3. Immobilization

The semi-purified esterase was immobilized using sodium alginate method, for which 9 mL sodium alginate solution (1.5% w/v) in deionized water was merged with 1 mL of isolated enzyme solution and incubated for 30 min at room temperature [10]. Beads were formed by dropping the above mixture into 0.05 M calcium chloride solution. The isolation of beads so formed was achieved by means of filtration and careful cleansing with 0.2 M phosphate buffer (pH 7) followed by air drying for 1 h. The immobilized beads thus obtained were rounded in shape with an approximate diameter of 3 mm. It is worth to note that various parameters [11] such as polymer concentration, nozzle diameter, flow rate, and voltage are greatly affect the production of calcium alginate beads for immobilization.

2.4. Biotransformation-General Procedure

Dried immobilized beads were washed with methanol before use. 12 mL reaction mixture was prepared by adding 6 mL alcohol (methanol/ethanol) and 6 mL 0.2 M phosphate buffer containing 50 mg of carboxylic acid. To this 0.2 g, immobilized esterase was incubated with constant stirring at room temperature for 16–28 h [Figure 1]. Aliquots were examined time to time by means of TLC to monitor the progress of the biotransformation. The product formation was confirmed by its physical constant and spectral analysis. Four industrially important products were successfully prepared by immobilized esterase in presence of alcohol [Table 1].

Table 1: Efficiency of esterase in the biotransformation of carboxylic acids.

S. No.	R	R'	Time (h)	% Yield
1.	H	CH3	16	97
2.	4-NH2	CH3CH2	24	92
3.	4-NH2-2-Cl	CH3	28	88
4.	2-OH	CH3	22	85

Table 2: Partial purification of esterase from *Pisum sativum* seeds.

Steps	Volume (mL)	Purification (fold)	Total protein (mg)	Total activity [#]	Specific activity [*]
Homogenate	400	1	2960	117.332	0.0396
Supernatant (pH 4.5)	300	1.2	3360	151.998	0.0452
(NH4) 2SO4	300	0.9	3600	139.998	0.0388
Dialyzed solution	283	1.4	1867.8	98.104	0.0525

[#]mg/min, ^{*}units/mg of protein

2.5. Determination of Kinetic Vectors

Kinetic parameters of free and immobilized esterase were evaluated using increasing concentration (0.1–1.0 mg/ml) of 4-nitrophenyl acetate by incubating separately with free esterase and immobilized esterase (2 mg/ml).

The reaction mixture was diluted up to fix volume with 0.2 M buffer solution (pH 7) followed by incubation at room temperature for 15 min. Amount of product per minutes was determined using calibration curve of 4-nitrophenol by means of optical density measurement at 577 nm. The values of Michaelis constant (K_m) and maximal velocity (V_{max}) of free and immobilized esterase were determined through non-linear regression fitting of the Michaelis-menten plot equation using Microsoft office excel [12].

3. RESULTS AND DISCUSSION

1.4-fold purification of esterase was achieved after dialysis [Table 2]. Partial purified esterase found to be stable up to 45°C and pH 7.0 in phosphate buffer. It is observed that K_m value of the immobilized esterase is slightly higher to that of free enzyme [Table 3]. The K_m and V_{max} for partial purified free esterase are found to be 3.2 mM and 5.6 µg/min, respectively.

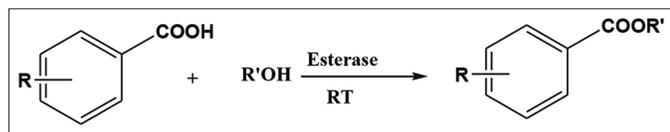
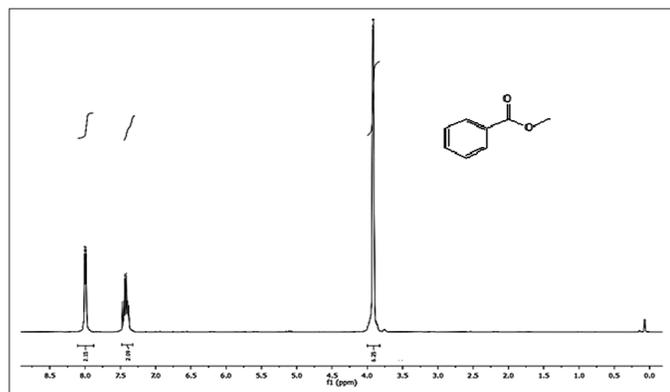
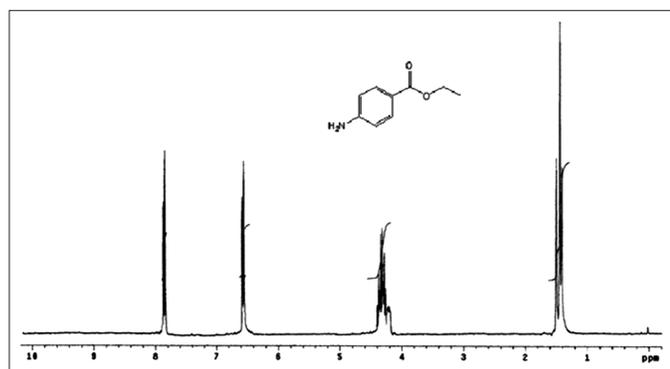
In case of immobilized enzyme, it was observed 4-fold increase in the K_m value in comparison to free enzyme. Michaelis constant values for immobilized *Thermomyces lanuginosus* lipase [13] on hydrophobic and hydrophilic mesoporous supports were reported as 3.4 and 1.5 mM, respectively, in contrast with free enzyme (0.8 mM). These variations in the kinetic parameters indicated that covalent binding of lipase onto the glutaraldehyde-activated functionalized nanotubes resulting change of affinity toward the substrate [14]. This could possibly be due to slightly decreased association between the substrate and the active site of enzyme in the bound lipase. The significant rise in k_m and V_{max} after immobilization might be due to exposure of substrate binding site precisely orientated for interaction with substrate on immobilized esterase in calcium alginate beads.

The IR and ¹HNMR spectra for methyl benzoate, ethyl 4-aminobenzoate, methyl-4-amino-2-chlorobenzoate, and methyl salicylate are in accordance to that of reported [Figures 2 and 3]. Accordingly, it was concluded that the immobilized esterase in 3 mm size calcium alginate gel works satisfactory.

Mahajan *et al.* [15], successfully, prepared three products in a single reactor using immobilized enzymes with calcium alginate beads. In the present study, we wish to highlight the use of single biocatalyst for preparation of four products separately. It has been verified the advantages of gel entrapment method for the production of these three products in one step. However, Vaija *et al.* [16] noted the preparation of citric acid with alginate bead captured *Aspergillus niger* in fermentation process and observed 5 times more product than obtained in classical batch fermentation. Faccio [17] reviewed the analysis

Table 3: Kinetics of free and immobilized esterase.

Parameters	Free esterase	Immobilized esterase
Km (uM)	2.7	3.2
Vmax (u/min)	2.5	5.6

**Figure 1:** General reaction for biotransformation.**Figure 2:** ¹H NMR spectrum of Methyl benzoate.**Figure 3:** ¹H NMR spectrum of ethyl-4-aminobenzoate.

of surface before the immobilization to improve the stability and applicability. Therefore, it is important to evaluate all the parameters including immobilization method, purity, and characterization of esterase, porosity of calcium alginate gel, and its effect on production rate.

4. CONCLUSION

The present study proposed a facile approach for the esterification of aromatic carboxylic acids. Immobilized enzyme from *P. sativum* found to be efficient biocatalyst for the small scale production of pharmaceutically important esters. Although immobilized esterase shown low-to-moderate activity, it has retained same activity even after prolonged storage at 4°C. Optimization of the biotransformation conditions and highly concentrated purified enzyme may increase the rate as well as yield of the product. This also opens futuristic scope toward wide range of acids to be useful for large scale preparation of industrially important derivatives.

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6. AUTHORS' CONTRIBUTIONS

We the authors of the manuscript titled, "Biotransformation of aromatic acids by immobilized esterase of *Pisum sativum*" as mentioned on title page undertake that we have made substantive intellectual contributions to the whole content of this manuscript.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data supporting to this study are available on request.

11. PUBLISHER'S NOTE

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