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Synergism of microwave irradiation and enzyme catalysis in kinetic resolution of *(R,S)*-1-phenylethanol by cutinase from novel isolate *Fusarium ICT SAC1*

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ABSTRACT

Enantiopure 1-phenylethanol is commercially used in food, pharmaceuticals, fine and agrochemical industries. Kinetic resolution of (*R,S*)-1-phenylethanol by cutinase from newly isolated fungal strain *Fusarium sp. ICT SAC1* was studied using vinyl acetate in non-aqueous media under both microwave irradiation and conventional heating. Effects of various parameters were studied systematically to propose a kinetic model and mechanism of the enzymatic reaction. The initial rate of reaction as well as product conversion increased synergistically with microwave irradiation (44.21% conversion of *R* enantiomer and 79.23% ee_s after 18 h) as compared to conventional heating (38.13% conversion with 61.85% ee_s at 40 °C in 24 h). Effect of various parameters such as solvent, speed of agitation, catalyst loading, substrate concentration, and temperature were studied. Lineweaver-Burk plots were generated to confirm that the ternary complex (ordered bi-bi) mechanism with inhibition by (*R,S*)-1-phenylethanol controlled the reaction. The experimental and simulated rates matched very well showing the validity of the proposed kinetic model. The results obtained in this study were good and comparable with earlier published data for cutinase from *Fusarium* sp.

1. Introduction

Enantiomerically pure chiral phenyl ethanol derivatives are in great demand due to their being essential middle compounds of chiral structure, building blocks and intermediates for synthesis of a variety molecules needed for manufacture of agrochemicals, natural products, flavours, fine chemicals, active pharmaceutical intermediates, drugs, asymmetric chiral ligands and auxiliaries [1–3]. Enzymatic approach for the kinetic resolution (KR) of such enantiomers is widely used in present times and has gained attention as a green, safe, clean and ecofriendly approach at industrial level [4].

Cutinase is an inducible enzyme belonging to serine hydrolase family which shows both lipase and esterase activity [5] with the catalytic triad of Ser-120, Asp-175 and His-188, and is similar to the lipase with serine centre at the middle of a sharp turn between β-strand and α-helix [6]. It does not require interfacial activation [7], and its unique structural orientation makes it an attractive and versatile biocatalyst over several lipases [5,6]. Hence several industrial houses have shifted towards utilizing this enzyme for a variety of enantioselective esterification and trans-esterification reactions of immense importance [6,7].

sec (*R,S*)-1-phenylethanol is an appropriate choice to set off KR by cutinase since its unique structure has a chiral centre that can be designed with sterically bulky groups such as methyl and phenyl. It finds applications in a variety of asymmetric transformations and also in the modelling of enzyme enantioselectivity [7]. Further, its esterified derivatives have many applications ranging from perfumery, soaps, detergents, cosmetics, room sprays, deodorants and flavors to imitate butter, apple, apricot, caramel, honey, vanilla, beer, etc.

The results available till date have shown cutinase to require longer reaction time for KR of (*R,S*)-1-phenylethanol [8]. The stereo preference of cutinase towards *R* isomer of *rac* 1-phenylethanol is well explained with the help of computational modelling [9]. For instance, cutinase from *Fusarium oxysporum* is reported to show 41% ester formation with 62% ee_s within 240 h [8]. Resolution of (*R,S*)-1-phenylethanol has been studied using *Candida rugosa*

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Nomenclature

[c]	Conversion, %
[R]	Concentration of *R* enantiomer, M
[S]	Concentration of *S* enantiomer, M
[A]	Initial concentration of (*R,S*)-1-phenylethanol, M
[B]	Initial concentration of vinyl acetate, M
V	Initial rate of the reaction, M min^{-1}
V_{max}	Maximum rate of the reaction, M min^{-1}
$K_{m(A)}$	Michaelis constant of (*R,S*)-1-phenylethanol, M^{-1}
$K_{m(B)}$	Michaelis constant of vinyl acetate, M^{-1}
$K_{i(A)}$	Inhibition constant of (*R,S*)-1-phenylethanol, M^{-1}

lipase, *Rhizopus oryzae* lipase in non-aqueous medium [10] and *Candida sp. 99-125* immobilized on silica nanotubes using amino propyl-grafted mesoporous silica nanotubes [11] (Table A1 in Supplementary information). Our earlier studies using a batch stirred reactor for KR of (*R,S*)-1-phenylethanol by cutinase produced by newly isolated *Fusarium sp. ICT SAC1* strain in solid state fermentation has shown 39.67% conversion of *R* enantiomer, *E* of >99% and ee_s of 65.05% in 24 h (Chaudhari et al., unpublished lab data). Such slow reactions need to be intensified in order to meet industrial requirements. A report by Parker et al. [12] highlights a 2–3 fold increase in the rate of transesterification reaction of butanol and ethyl butyrate using cutinase as catalyst. There are several studies on lipase-catalyzed KR of secondary alcohols under microwave irradiation with enhancements in both rate and/or enantioselectivity [1,3,13]. Hence it was decided to investigate ecofriendly and greener approach of microwave assisted enzyme catalysis to improve the rate of reaction, and for process intensification in KR of (*R,S*)-1-phenylethanol.

Microwave irradiation (MWI) has received importance in organic synthesis using biocatalysts as a green and clean process utilizing an alternative energy source with improved yields and reaction selectivity in shorter reaction times [2,3,14,15]. MWI has been successfully exploited earlier by our research team with better results than conventional reaction mode for enantioselective resolution of (*R,S*)-ketorolac [2], (*R,S*)-1-(1-naphthyl) ethanol [3], DL-3-phenyllactic acid [16], among many others.

The practical industrial application of every single chemical reaction relies on the knowledge of its kinetics established by using a suitable kinetic model. The kinetics of the reaction allows description of a chemical reaction and its optimization over an extensive range of reaction composition (presence of inhibitors and their concentrations), and reaction conditions [6]. The applicability of the model over a wide range of experimental conditions is based on the mechanistic scheme describing the fundamental steps involved in the reaction. However, investigations on detailed kinetic models that can ably illustrate the reaction insights taking place over an extended range of reaction conditions [17] are of principal importance for optimization of reaction parameters for its practical applications. Reaction kinetics also provides information on the rate of product formation with a change in various operational parameters for the design of suitable reactors for industrial scale up [18]. So far, kinetic mechanisms for cutinase in transesterification and esterification reaction are reported to proceed via Ping Pong bi–bi mechanism [6,19–21] and/or ternary complex ordered bi–bi mechanism [18]. However, the kinetic modeling of MWI *Fusarium sp. ICT SAC1* catalyzed KR (*R,S*)-1-phenylethanol has not yet been reported. Hence reaction kinetics using this enzyme system was studied in this work to understand the reaction mechanism and separation of racemates.

This paper reports on the process intensification of KR of (*R,S*)-1-phenylethanol via transesterification with vinyl acetate using lyophilized cutinase from a newly isolated *Fusarium sp. ICT SAC1* strain under MWI. Detailed studies on various parameters, *viz.* speed of agitation, nature of solvent, temperature, concentration of substrates and catalyst were performed under microwave irradiation to explicate KR of (*R,S*)-1-phenylethanol.

2. Materials and methods

2.1. Materials, chemicals and media components

(*R,S*)-1-phenylethanol, (*R*)-1-phenylethanol (*S*)-1-phenylethanol, and vinyl acetate were provided by Fluka (Buchs, Switzerland), all of which were dried using molecular sieve of 4 Å. HPLC grade *n*-hexane and all other solvents of analytical grade were purchased from s. d. Fine-Chem. Ltd (Mumbai, India). The media components used in the present study were procured from Hi-Media Laboratories, Mumbai.

2.2. Cutinase production

Cutinase was produced from a newly isolated strain of *Fusarium* sp. *ICT SAC1* (Genebank Accession No. KJ 522754). Multicolor watermelon rind (WMRs) was obtained from our Institute's cafeteria. The rinds were washed to remove any contaminant particulate matter, cut into small pieces of approximately 1 cm^3, and dried at 60 °C in a tray dryer for 12 h. Further, the dried WMRs were extracted with water to remove the inherent natural inhibitors/repressors (polyphenols and reducing sugars) and re-dried. Finally, they were ground in a laboratory grinding mill and fractionated using sieves of # 60 to a particle size of 250–400 μm.

Cutinase was produced by solid state fermentation using WMRs as a solid support as well as an inducer under the stated conditions. The water-extracted and dried powder (5 g, size-250–400 μm) was moistened with 10 mL of mineral media (g/L- 2.0 sodium nitrate, 1.0 dipotassium hydrogen phosphate, 0.75 magnesium sulfate heptahydrate, 0.5 potassium chloride). The flasks were plugged with cotton, sterilized, cooled at room temperature (30 ± 2 °C) and then inoculated with 2 mL of conidial spore suspension (pre-inoculum). Further, the contents of the flasks were mixed by gentle shaking and incubated under static conditions at 30 °C for 96 h.

2.3. Extraction, partial purification and lyophilization of cutinase

Crude enzyme was extracted by shaking a 96 h fermented WMRs substrate with 50 mL of 50 mM phosphate buffer (pH 7.2) on a rotary shaker (180 rpm) at 30 °C for 1 h. The slurry was squeezed through wet cheese cloth followed by vacuum filtration through Buchner funnel lined with Whatman No.1 filter paper. Further, the supernatant was centrifuged at 7840 × *g* for 10 min at 4 °C on Beckman Centrifuge (J2-MC, Ireland). The clear cell-free reddish brown colored transparent supernatant obtained was partially purified by ammonium sulfate precipitation at 50% (w/w) saturation at 10 °C, and allowed to stand for 1 h (4–8 °C). The precipitate was centrifuged at 7840 × *g* for 10 min at 4 °C. The solid pellet mass was redissolved in fresh 50 mL of phosphate buffer (50 mM, pH 7.2) and dialyzed overnight against the same buffer with three fresh buffer replacements, yielding five-fold purity of cutinase. From SDS-PAGE, the final molecular weight of cutinase cannot be precisely determined since there are multiple bands after partial purification. However, based on the literature reports, fungal cutinase (*Fusarium sp.)* appeared to be within 21–25 kDa. We also found the presence of bands within this molecular range (Fig. A1 in Supplementary information). The solution so obtained was kept in Freezone plus-6L (Labconco, Kansas) at −80 °C for 15 h and then freeze dried for