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Fungal mediated kinetic resolution of racemic acetates to (*R*)-alcohols using *Fusarium proliferatum*

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ABSTRACT

Fungal mediated kinetic resolution of seven acyclic/aromatic acetates was achieved using *Fusarium proliferatum* to furnish (*R*)-alcohols in high enantiomeric excess (>95%). The kinetic resolution was established as one-pot two-step de-esterification/oxidation biocatalytic process. Further, the preparative scale synthesis of (*R*)-(+)-1-phenylethanol was accomplished through de-esterification/oxidation of (±)-1-phenylethyl acetate using the whole cell of *F. proliferatum* NCIM 1105.

Introduction

Enantiomerically pure low molecular weight alcohols and their corresponding acetates are widely useful chemicals in industry as well as academia. They are important ingredients in the perfumery industry due to their volatility and unique odor.[1–3] Furthermore, strategic utilization of these molecules as the chiral precursors for the asymmetric syntheses of complex organic molecules is well-known.[4,5] For example, acyclic alcohols such as (*R*)-(−)-lavandulol (**1a**) exist naturally as one of the major components of the lavender essential oil and is used in perfumery and cosmetic industry. (*R*)-(−)-Lavandulyl propionate has been investigated as the sex pheromone in mealy bug, whereas its (*S*)-enantiomer was found to be inactive (Fig. 1).[6] Both the enantiomers of 2-hexyl acetate (**2**) and 2-heptyl acetate (**3**) along with their corresponding alcohols are used in flavor and fragrance industry. On the other hand, (*R*)-(−)-2-hexanol (**2a**) and (*S*)-(+)-2-hexanol (**2b**) were used in the preparation of key intermediates in the total synthesis of anti-viral glycolipid cycloviracin B_1.[7] (*R*)-(−)-2-Heptanol (**3a**) was utilized in resolving the racemic mixture of a key intermediate in the synthesis of 2,3,4,5-tetrahydro-1*H*-1-benzdiazepine derivatives, known to be a strong vasopressin V_2 receptor agonist.[8]

Resolution of the racemic mixture of these alcohols/acetates is highly challenging and available chromatographic techniques are ineffective to achieve the desired resolution, especially in preparative scale separation. Enzyme mediated kinetic resolution of such alcohols using lipases, esterases, and alcohol dehydrogenases have been reported in the literature.[9–28] For example, resolution of (±)-lavandulol has been achieved previously with various lipases such as *Candida antarctica* lipase B (CAL B),[9] *Hog pancreas* lipase,[29] *Porcine pancreas* lipase,[26] and *Yarrowia lipolytica* lipases.[30] However, enzyme catalyzed resolution process is associated with several disadvantages such as higher cost, low substrate concentration, instability and cofactor dependency of the enzymes in several occasions. On the other hand, whole cell biocatalysis offers an inexpensive choice in which enzymes are stable within cellular environment and the microbial cells themselves act as the source of cofactors for the biocatalyst mediated conversion.[31–33] The present study describes one-pot two-step de-esterification followed by selective (*S*)-enantiomer oxidation of seven acyclic and aromatic acetates using *Fusarium proliferatum* (National Collection of Industrial Microorganisms/NCIM, catalog no. 1105). (*R*)-Alcohols produced using whole cells of *Fusarium proliferatum* showed >95% of ee. Substrate concentration and incubation time were optimized and the efficiency of resolution process [enantiomeric excess (ee)] was evaluated for each of these substrates. Furthermore, preparative scale resolution was successfully achieved on (±)-1-phenylethyl acetate (**6**) to isolate (*R*)-(+)-1-phenylethanol (**6a**).

Results and discussion

The fungal system, *Fusarium proliferatum* (NCIM, Catalog no. 1105) was screened for its ability to convert racemic acetates into corresponding (*R*)-alcohols in efficient manner. (±)-Lavandulyl

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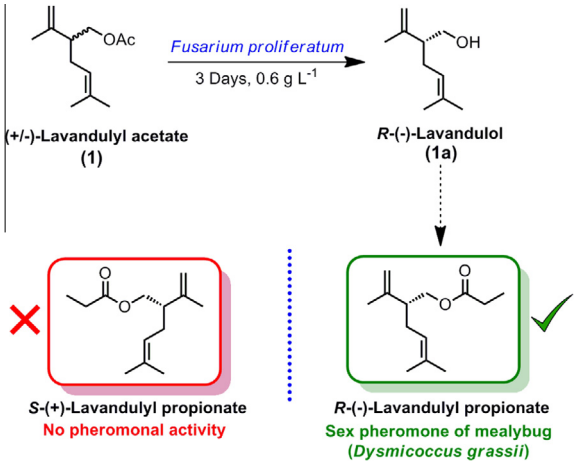


Figure 1. Kinetic resolution of (±)-lavandulyl acetate to (*R*)-(−)-lavandulol by *F. proliferatum*.

acetate (**1**), an acyclic racemic ester was used as the model substrate for the screening of fungal mediated kinetic resolution of esters. Fermentation procedures were carried out as reported earlier.[31,34] Among various fungal systems screened for hydrolytic kinetic resolution of (**1**), *Fusarium proliferatum* was able to convert it to (*R*)-(−)-lavandulol (**1a**) in an efficient manner with very high ee. Biocatalytic resolution was validated with two independent control experiments; substrate control (with substrate and without organism), and organism control (with organism and without substrate). Both the control experiments did not show any evidence for the formation of (*R*)-(−)-lavandulol (**1a**) as analyzed by GC-FID and GC–MS (see ESI).[34] In addition, the resolution achieved through the resting cells of *F. proliferatum* confirmed that the enzyme seems to be constitutive to the whole-cell used. Substrate concentration 0.6 g L^{-1} was found to be optimum to achieve the highest ee. Use of higher concentration of substrate led to the lower ee and conversion rate. After 3 days of incubation, (±)-lavandulyl acetate (**1**) was converted to (*R*)-(−)-lavandulol (**1a**) with 95.3% conversion and 99.6% ee. Time-course experiments (Fig. 2A) indicated that *F. proliferatum* was able to transform almost 95% of (**1**) in to (*R*)-lavandulol (**1a**) and (*S*)-lavandulol (**1b**) (*R*/*S* 45.9:49.4) after 12 h incubation. With prolonged incubation up to 3 days, the relative abundance of (*R*)-lavandulol (**1a**) increased to 99.8% and

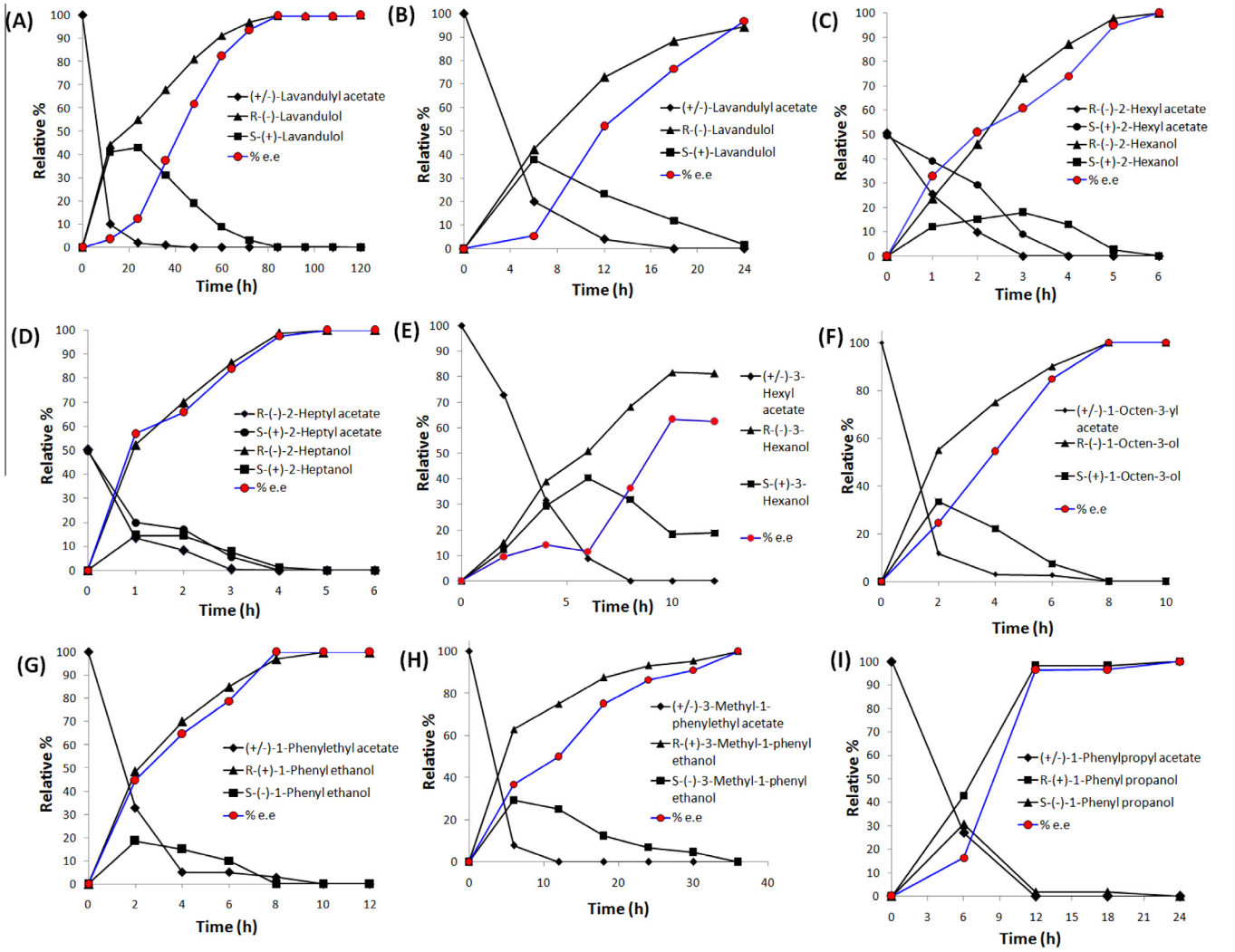
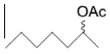
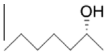
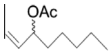
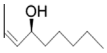
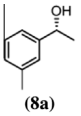


Figure 2. Time-course experiment of kinetic resolution using *F. proliferatum* in graphical representation: (A) Whole cells incubated with (±)-lavandulyl acetate at 0.6 g L^{-1} concentration; (B–I) resting cells incubated with (±)-lavandulyl acetate (B), (±)-2-hexyl acetate (C), (±)-2-heptyl acetate (D), (±)-3-hexyl acetate (E), (±)-1-octen-3-yl acetate (F), (±)-1-phenylethyl acetate (G), (±)-3-methyl-1-phenylethyl acetate (H), (±)-1-phenylpropyl acetate (I) respectively with 0.1 g L^{-1} substrate concentration.

Table 1
Hydrolytic kinetic resolution achieved on various racemic acyclic/aromatic acetates through resting cells of *F. proliferatum*

Entry	Substrate[a]	Time (h)	Product	Configuration and % ee
1	 (**1**)	24	 (**1a**)	*R*, 96.8
2	 (**2**)	6	 (**2a**)	*R*, 99.9
3	 (**3**)	6	 (**3a**)	*R*, 99.9
4	 (**4**)	12	 (**4a**)	*R*, 62.5
5	 (**5**)	8	 (**5a**)	*R*, 99.9
6	 (**6**)	12	 (**6a**)	*R*, 97.5
7	 (**7**)	48	 (**7a**)	0
8	 (**8**)	36	 (**8a**)	*R*, 99.9
9	 (**9**)	24	 (**9a**)	*R*, 98.2

[a] Substrate concentration: 0.1 g L^{-1} and substrate conversion: 100% for each entry.

the percentage of (*S*)-lavandulol (**1b**) steeply diminished to 0.18% in the fermentation broth. Continuing the incubation up to 5 days didn't alter the relative abundance of the individual enantiomer in the reaction mixture. Resting cell experiments were carried out as reported earlier[31] by incubating well washed *F. proliferatum* with 0.1 g L^{-1} concentration of (±)-lavandulyl acetate (**1**). Time-course experiment with the resting cells revealed that after 6 h of incubation, the abundance of (*R*)-lavandulol (**1a**) starts to increase in the fermentation broth, further reaching up to 94.4% and 96.8% ee at 24 h of incubation with quantitative consumption of the acetate (Fig. 2B). To investigate the substrate scope of the whole cell biocatalyst four racemic acyclic esters [(±)-2-hexyl acetate (**2**), (±)-2-heptyl acetate (**3**), (±)-3-hexyl acetate (**4**), and (±)-1-octen-3-yl acetate (**5**)] and four aromatic esters [(±)-1-phenylethyl acetate (**6**), (±)-2-methyl-1-phenylethyl acetate (**7**), (±)-3-methyl-1-phenylethyl acetate (**8**) and (±)-1-phenylpropyl acetate (**9**)] were chosen as the substrates (Table 1). The kinetic resolution of these substrates was assessed by incubating it with resting cells of *F. proliferatum* and carrying out time course study (Fig. 2C–I). Resting cell experiments were carried out with acyclic esters [(**2**), (**3**), (**4**), and (**5**)] at substrate concentration of 0.1 g L^{-1} for different time interval of incubation. Esters (**2**) and (**3**) yielded enantiomerically pure (*R*)-alcohols (**2a**) and (**3a**) with 100% conversion and 99.9% ee respectively after 6 h of incubation (Fig. 2C and D).

While esters (**4**) and (**5**) were converted into corresponding (*R*)-alcohols (**4a**) and (**5a**) with 100% conversion and 62.5%, 99.9% ee at the end of 12 h and 8 h of incubation period respectively (Fig. 2E and F). Similar experiments were performed with the racemic aromatic esters [(**6**), (**7**), (**8**), and (**9**)] using resting cells of *F. proliferatum* at the concentration of 0.1 g L^{-1}. Time course study experiments indicated that the fungal systems converted (±)-1-phenylethyl acetate (**6**) and (±)-3-methyl-1-phenylethyl acetate (**8**) to (*R*)-(+)-1-phenyl ethanol (**6a**) and (*R*)-(+)-3-methyl-1-phenyl ethanol (**8a**) in quantitative yields with 100% conversion and 99.9% ee at the end of incubation periods 12 h and 36 h respectively (Fig. 2G and H).There was no enantioselectivity observed with aromatic ester (**7**) with whole cells of *F. proliferatum*, which may be due to presence of 2C-methyl group on phenyl ring of the compound (**7**). On the other hand, the fungal system efficiently converted the acetate (±)-1-phenylpropyl acetate (**9**) to (*R*)-(+)-1-phenylpropanol (**9a**) with 100% conversion and 98.2% ee after 24 h of incubation in the resting cell experiment (Fig. 2I).

Time-course study experiments have indicated that during initial period of incubation racemic acetates were converted into individual enantiomeric alcohols at the same rate and accumulation of (*R*)-alcohols with decrease in (*S*)-alcohols levels in the assay mixture was observed with continued incubation (Fig. 2). Careful analysis of the GC and GC–MS chromatograms (see ESI)[34] of the extracted fermentation broth revealed the presence of corresponding ketones in case of all seven substrates [(**2**), (**3**), (**4**), (**5**), (**6**), (**8**), and (**9**)] during later stage of incubation (Fig. 3B). These results indicate that there is a selective oxidation of (*S*)-alcohol (see ESI) to the corresponding ketone with faster rate in comparison to (*R*)-alcohol, leading to kinetic resolution with high ee (Fig. 3A). To further substantiate this pathway of hydrolytic kinetic resolution, racemic mixture of alcohols was incubated with *F. proliferatum* which resulted in decrease in the relative abundance of (*S*)-alcohol in the reaction mixture along with the formation of corresponding prochiral ketone. Similar case studies were performed using individual (*R*) and (*S*)-alcohol with *F. proliferatum*, which showed

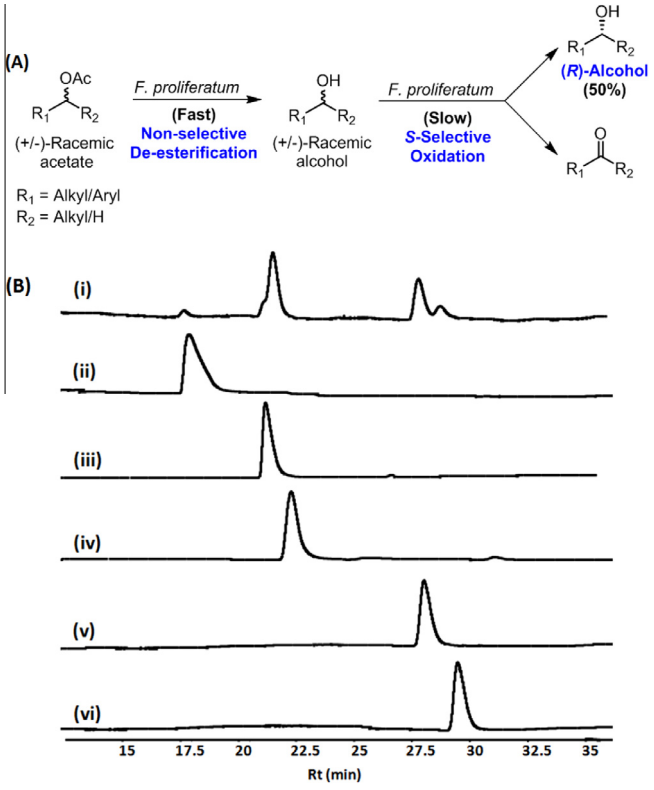


Figure 3. (A) Schematic representation of de-esterification/oxidation one-pot two-step kinetic resolution of acyclic/aromatic acetates by *F. proliferatum*; (B) GC-FID chromatograms of (i) extracted reaction mixture of (±)-2-hexyl acetate with resting cell of *F. proliferatum* after 2 h of incubation period, (ii) standard 2-hexanone (iii) standard (*S*)-(+)-2-hexanol, (iv) standard (*R*)-(−)-2-hexanol, (v) standard (*S*)-(+)-2-hexyl acetate and (vi) standard (*R*)-(−)-2-hexyl acetate.

selective oxidation of (*S*)-alcohol to corresponding ketone while (*R*)-alcohol was remained as it is in fermentation broth during similar incubation conditions. GC and GC–MS analysis of the assay extract obtained by incubating corresponding prochiral carbonyl compounds did not show presence of corresponding reduced alcohol metabolites. GC and GC–MS analyses of the assay extract obtained by incubating (±)-lavandulyl acetate (**1**) with *F. proliferatum* did show the presence of the corresponding aldehyde metabolite.

Scale-up studies were carried out in shake flasks (100 mL media) containing (±)-1-phenylethyl acetate (**6**) as substrate.

Substrate concentration studies carried out using varied substrate concentration (0.2, 0.4, 0.8, 1.2, 2.0 and 2.4 g L^{-1}) indicated that the microorganism could transform (**6**) in to (*R*)-(+)-1-phenylethanol (**6a**).

Increase in the substrate concentration decreased the level of metabolite formation as well as enantiomeric excess (ee). Time course experiments indicate that *F. proliferatum* could transform 100% of (**6**) into (**6a**) at the end of 3 days of incubation period. The fermentation volume was scaled up to 1.0 L with substrate concentration 0.4 g L^{-1}.[34] Purification of the alcohol fraction from 1.0 L fermentation medium containing 0.4 g racemic acetate (**6**) resulted in the isolation of 0.14 g pure (*R*)-(+)-1-phenylethanol (**6a**) with a yield of 47% and 99.9% ee. Purified (*R*)-(+)-1-phenylethanol (**6a**) was further characterized by analytical techniques such as NMR (^{1}H, ^{13}C) and optical rotation. (*R*)-1-Phenylethanol: colorless liquid; $[\alpha]_D^{25}$ = +36.2. ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 7.25 (5H, m, Ar), 4.82 (1H, m, H-1), 1.43 (3H, d, J = 6.4, H-2); ^{13}C NMR (CDCl3, 50 MHz) δ: 145.78, 128.39, 127.62, 125.33 70.25, 25.05.

Thus, the fungal system, *F. proliferatum* can be used for large-scale production of corresponding (*R*)-alcohols through kinetic resolution of acyclic and aromatic acetates with fine tuning of the fermentation conditions.

Conclusion

In conclusion, an efficient one-pot two step de-esterification/oxidation biocatalytic technique was developed for the kinetic resolution of acyclic and aromatic acetates by using the whole-cells of *Fusarium proliferatum*. The fungal system was able to carry out the kinetic resolution of four racemic acyclic esters [(±)-lavandulyl acetate (**1**), (±)-2-hexyl acetate (**2**), (±)-2-heptyl acetate (**3**) and (±)-1-octen-3-yl acetate (**5**)] and three aromatic esters [(±)-1-phenylethyl acetate (**6**), (±)-3-methyl-1-phenylethyl acetate (**8**) and (±)-1-phenylpropyl acetate (**9**)] into corresponding (*R*)-alcohols in an efficient manner with high ee. Enantioselective hydrolysis of (±)-1-phenylethyl acetate (**6**) to (*R*)-(+)-1-phenylethanol (**6a**) was successfully scaled up to preparative scale, which indicated great potential of the developed process to be applied in large scale preparation of enantiopure (*R*)-alcohols.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.08.084.

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34. *Conditions for biotransformation:* Fermentations were carried out in modified Czapek Dox (C.Z) medium. In brief, Flasks (500 mL) containing 100 mL of sterile medium were inoculated with 3 mL (3% v/v) of a spore suspension from a 2–3-day-old culture grown on potato dextrose agar (PDA) slants and were incubated at 29–30 °C on a rotary shaker (200 rpm) for 48 h. After this growth period, substrate (0.6 g L^{-1}) in acetone was added to each flask, and the incubation was continued for an additional period as required. Control experiments were also run with the substrate but without microorganism and with microorganism but without substrate. In time course experiments, incubations were carried out for 1–5 days and the metabolites formed at the end of each incubation period were monitored by GC and GC–MS analyses.
In time course experiments, incubations were carried out for varied times and the metabolites at the each incubation period were monitored by GC analysis. Quantification was made comparing with standard graph drawn for individual compounds. Resting cell experiments were carried out by incubating 3 g of thoroughly washed mycelial pellet with substrate 0.1 g L^{-1}) in 50 mL phosphate buffer (0.1 M, pH 7.2 with 0.2% of glucose). For scale up studies, (±)-1-Phenylethyl acetate (0.4 g L^{-1}) was added to the flask (2.8 L) containing well grown microorganism in 1 L modified C.Z media. After an incubation period of 3 days, mycelia were filtered using muslin cloth. Separated mycelia and filtered media were extracted with dichloromethane (DCM) thrice (1 L × 3 times) and filtrate was then passed though anhydrous sodium sulphate. Filtrate obtained was concentrated to get crude extract which was further purified over silica gel column (230–400 mesh) to obtain enantiopure (*R*)-(+)-1-phenyl ethanol.