

Enantioselectivity and Thermostability of a Novel Hyperthermotolerant Lipase from *Geobacillus Thermodenitrificans* nr68 (Lip.nr-68) on Secondary Racemic Alcohols Acetylation

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Abstract. In our previous work, a new lipase enzyme has been purified from a species identified as a Gram negative *Geobacillus thermodenitrificans* nr68, isolated from a hot spring in Malaysia with growth temperature of 48°C. This new lipase, called Lip.nr-68 has been characterized as a hyperthermotolerant protein with high stability at 65°C and has been showing excellent characteristics that are very much comparable yet better than some of those of well-known industrially-used lipases. It shows high activity against long-chain triglycerides with molecular weight of the purified enzyme estimated to be 33.5 kDa using SDS-PAGE analysis. This paper is focusing on hyperthermotolerant Lip.nr-68 performance in promoting for enantioselectivity activities towards three secondary racemic alcohols namely 1-phenylethanol, 1-cyclohexylethanol and 1-(naft-2-il) ethanol by acetylation with vinyl acetate. Lip.nr-68 has been confirmed to show high and usual enantioselectivity according to the Kazlauskas Rule towards all secondary racemic alcohols and has significantly approved as an enantiomer selective biocatalyst towards 1-phenylethanol and 1-cyclohexylethanol at 65°C. Lip.nr-68 has showed a reduction of (*R*) and (*S*) enantiomers as well as the production of 68-98% *ee* and almost 94% yield of 3-4 mg/ml for 1-cyclohexylethanol.

1. Introduction

The ability of most lipases to promote for the biotransformation process in the non-conventional medium like organic solvent and supercritical mediums is not to be underestimated. Lipases could greatly perform biotransformation in a macro-aqueous system with regio and enantioselective characteristics; making it a main pedestal that has become significant to relatively lots of present enzymatic study for the production of enantiomerically pure alcohols; which has been well recognized to be vital intermediates for the synthesis of pharmaceuticals compounds, agrochemicals composites, pheromones, flavours and fragrance, liquid crystals molecules and chiral auxiliaries in asymmetric synthesis [1],[2]. Therefore, enantioselectivity performance, enzymes stability and enzyme inhibition should be promoted to productively support the occurrence of pure racemic molecules. According to Itoh [3], the development of efficient methods for the synthesis of enantiomerically pure alcohols is of tremendous importance. The ability of lipases to dissolve racemic molecules has been adapted into lots of important applications such as in dairy and industrial biotechnology, oils processing, surfactants production as well as the production of pure enantiomeric molecules [4] and we are still looking into better performance of the technology. This paper is published to bring forward the excellent quality of our prime hyperthermotolerant *Geobacillus thermodenitrificans* nr68 lipase (Lip.nr-68) towards the



enantiomeric reactions on three types of secondary racemic alcohols listed as 1-phenylethanol, 1-cyclohexylethanol and 1-(naph-2-yl) ethanol. The main highlight that differ Lip.nr-68 from other most microbial lipases involved in acetylation of racemic mixtures is the stability at 65°C for 96 hours with an excellent *ee* and yield. Enantioselectivity of Lip.nr-68 towards the racemic mixtures was studied to accomplish and confirm the selectivity towards its enantiomer that has been applied as a model by Kazlauskas Rules [5].

2. Material and Methods

2.1 Source of enzyme, lipase activity test and source of racemic alcohols

The source of enzyme was a freeze-dried lipase from *Geobacillus thermodenitrificans* nr68 (Lip.nr-68) with activity of 30.50 U/g. The inorganic salts and materials for biomass production were purchased from Sigma, Aldrich or Fluka. Lipase activity was determined according to method by Nik Raikhan & Nurul [6] using *p*-nitrophenyl palmitate (pNPP, Sigma) as substrate. The 1-phenylethanol, 1-cyclohexylethanol and 1-(naph-2-yl) ethanol were the products of Aldrich.

2.2 Nuclear magnetic resonance (NMR) analysis, Gas chromatography analysis (GC) and thin layer chromatography (TLC)

The NMR spectra were recorded in CDCl₃ on a Bruker DRX- 500 spectrometer (at 500MHz for ¹H and 125MHz for ¹³C spectra). The δ scale for the analysis was recorded in ppm. The IR spectra (film) were taken on a Specord 2000 Series Spectrophotometer and the wavenumbers of the absorptions are reported in cm⁻¹. GC analyses were carried out on HP 5890 or Agilent 4890D instruments equipped with FID detector and Hydrodex- β -PM 25m $\times$ 0.25 mm column (25m $\times$ 0.25 mm, 0.25 μ m film with permethylated-cyclodextrin; Macherey&Nagel) or 0.25 or Betadex 225 fused silica capillary column (30m $\times$ 0.25mm $\times$ 0.25 μ m film; Supelco) using H₂ carrier gas (oven: 100°C, injector: 250°C, detector: 250°C, head pressure: 10 psi, 50:1 split ratio). Optical rotations were determined on a Perkin-Elmer 241 polarimeter [4]. TLC was carried out on Kieselgel 60 F254 (Merck) sheets cut into 6.0 cm. Movement of solvent (hexane-acetone 10:4, v/v) was allowed up to 5.5 cm [7].

2.3 Enantiomer selective reaction by Lip.nr-68 towards racemic alcohol (rac-1a-c) at room temperature

20.0 mg freeze-dried Lip.nr-68 was added into a mixture of 1.0 ml hexane, 0.50 ml vinyl acetate and 0.50 ml tetrahydrofuran followed by 20.0 mg of racemic substrate *rac*-1a-c [8]. The sealed glass vials contained the catalyst was centrifuged at 1000 rpm at room temperature (20°C) using the times listed in Table 1. The conversions were checked by TLC (hexane-acetone 10:4, v/v). At the reaction time indicated in Tables 1, Table 2 and Table 3 below, the enantiomeric compositions and the ratio of the product and residual substrate fractions were analyzed by GC on chiral stationary phase (Tables 2 and Table 3). Tables 1, Table 2 and Table 3 list results only for those reactions which exhibited more than 5.0% of conversion after 24 hours.

Table 1. Chromatography Analysis for *rac*-1a: (*S*)-1a and (*R*)-2a

	Alcohol (<i>S</i>)-1a	Alcohol (<i>S</i>)-1a	Acetate (<i>R</i>)-2a	Acetate (<i>R</i>)-2a
Enzyme	Lip.nr-68	Lip.nr-68	Lip.nr-68	Lip.nr-68
Appearance	Colorless oil	Colorless oil	Colorless oil	Colorless oil
T (°C)	Room T	65	Room T	65
Time (hour)	96	24	96	24
Yield	129 mg, 65%; E.e.: 39% by GC; [α] _D ²² =18.8 (c1.0, CHCl ₃)	101 mg, 53%; E.e.: 65% by GC; [α] _D ²² = -57.7 (c 1.0, CHCl ₃)	63.5 mg, 25%; E.e.: 97% by GC; [α] _D ²² = +125 (c 1.0, CHCl ₃)	85 mg, 35%; E.e.: 97% by GC; [α] _D ²² = +123 (c 1.0, CHCl ₃)
IR	3252, 3118, 2976, 2952, 1476, 1430, 1378, 1304, 1180, 1008, 900, 750, 690	3352, 3008, 2976, 2952, 1666, 1290, 1368, 1204, 1080, 1208, 850, 760, 690	1740, 1456, 1376, 1244, 1208, 1064, 984, 760, 700	1740, 1456, 1376, 1244, 1208, 1964, 944, 770, 700
Other details	¹ H NMR: 1.513 (d, <i>J</i> = 6.9 Hz, 3H), 3.38	¹ H NMR: 1.513 (d, <i>J</i> = 6.9 Hz, 3H), 3.38	¹ H NMR: 1.574 (d, <i>J</i> = 7.4 Hz), 21.103	¹ H NMR: 1.574 (d, <i>J</i> = 7.4 Hz), 21.103 (s,

(s, 1H), 4.861 (q, $J = 6.3$ Hz, 1H), 7.40 (mc, 5H, Ar H); ^{13}C NMR: 25.034, 69.843, 125.071, 126.862, 127.970, 145.525	(s, 1H), 4.861 (q, $J = 6.3$ Hz, 1H), 7.40 (mc, 5H, Ar H); ^{13}C NMR: 25.034, 69.843, 125.071, 126.862, 127.970, 145.525	(s, 3H), 5.926 (q, $J = 6.9$ Hz, 1H), 7.30-7.40 (m, 5H); ^{13}C NMR: 21.416, 22.283, 72.241, 125.860, 127.632, 128.28, 141.422, 169.96	3H), 5.926 (q, $J = 6.9$ Hz, 1H), 7.30-7.40 (m, 5H); ^{13}C NMR: 21.416, 22.283, 72.241, 125.860, 127.632, 128.28, 141.422, 169.96
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Table 2. Chromatography Analysis for *rac*-**1b**: (S)-**1b** and (R)-**2b**

	Alcohol (S)- 1b	Alcohol (S)- 1b	Acetate (R)- 2b	Acetate (R)- 2b
Enzyme	Lip.nr-68	Lip.nr-68	Lip.nr-68	Lip.nr-68
Appearance	Colorless oil	Colorless oil	Colorless oil	Colorless oil
T (°C)	Room T	65	Room T	65
Time (hour)	96	24	96	24
Yield	330 mg, 96%; E.e.: 50% by GC; $[\alpha]_{\text{D}}^{22} = +1.6$ (c 1.0, CHCl_3)	215 mg, 80%; E.e.: 99.5 % by GC; $[\alpha]_{\text{D}}^{22} = +2.1$ (c 1.0, CHCl_3)	278 mg, 78%; E.e.: 91% by GC; $[\alpha]_{\text{D}}^{22} = +9.5$ (c 1.0, CHCl_3)	399 mg, 93.8%; E.e.: 92% by GC; $[\alpha]_{\text{D}}^{22} = +7.2$ (c 1.0, CHCl_3)
IR	3610, 3550-3250, 2960-2900, 1450	3610, 3550-3250, 2960-2900, 1450	2928, 1736, 1244	2928, 1736, 1244
Other details	^1H NMR: 0.73-1.88 (m, 12H), 1.16 (d, $J = 6.0$ Hz, 3H), 3.57 (dq, $J = 6.0$ Hz, 1H); ^{13}C NMR: 20.43, 26.21, 26.30, 26.60, 28.41, 28.82, 45.27, 72.22	^1H NMR: 0.73-1.88 (m, 12H), 1.16 (d, $J = 6.0$ Hz, 3H), 3.57 (dq, $J = 6.0$ Hz, 1H); ^{13}C NMR: 20.43, 26.21, 26.30, 26.60, 28.41, 28.82, 45.27, 72.22	^1H NMR: 1.09 (d, $J = 6.405$ Hz, 3H), 1.96 (s, 3H), 4.75 (q, $J = 6.405$ Hz, 1H), 1.65 (m, 11H); ^{13}C NMR: 17.05, 21.3, 26.04, 26.23, 28.5, 42.57, 74.56, 170.08.	^1H NMR: 1.09 (d, $J = 6.405$ Hz, 3H), 1.96 (s, 3H), 4.75 (q, $J = 6.405$ Hz, 1H), 1.65 (m, 11H); ^{13}C NMR: 17.05, 21.3, 26.04, 26.23, 28.5, 42.57, 74.56, 170.08.

Table 3. Chromatography Analysis for *rac*-**1c**: (S)-**1c** and (R)-**2c**

	Alcohol (S)- 1c	Acetate (R)- 2c
Enzyme	Lip.nr-68	Lip.nr-68
Appearance	White powder	Colorless oil
T (°C)	Room temperature	Room temperature
	96	96
Yield	342 mg, 71%; E.e.: 29% by GC; $[\alpha]_{\text{D}}^{22} = -15$ (c 1.0, CHCl_3)	342 mg, 16%; E.e.: 99% by GC; $[\alpha]_{\text{D}}^{22} = +125.5$ (c 1.0, CHCl_3)
IR	3610, 3550-3250, 3060, 3020, 2980, 2930, 1450, 860	1064, 1244, 1372, 1740, 2984
Other details	^1H NMR: 1.5 (d, $J = 6.5\text{Hz}$, 3H), 1.95 (br s, 1H), 4.95 (q, $J = 6.5\text{Hz}$, 1H), 7.4 (m, 3H), 7.72 (m, 4H); ^{13}C NMR: 24.52, 70.05, 123.7, 123.8, 125.8, 126.15, 127.7, 128.0, 128.4, 132.9, 133.36, 143	^1H NMR: 1.55 (d, $J = 6.59$ Hz, 3H), 2.02 (s, 3H), 5.98 (q, $J = 6.593$ Hz, 1H), 7.5 (d, 3H), 7.8 (t, 4H); ^{13}C NMR: 21.62, 22.17, 72.5, 124.09, 125.02, 126.06, 126.23, 127.66, 128.02, 128.35, 133.02, 133.18, 139.00, 170.5

2.4 Enantiomer selective reaction and thermostability of catalytic reaction by Lip.nr-68 towards 1-phenylethanol (*rac*-**1a**) and 1-cyclohexylethanol (*rac*-**1b**) at 65°C

The above method was repeated with 20.0 mg of 1-phenylethanol (*rac*-**1a**) and 1-cyclohexylethanol (*rac*-**1b**) and temperature of 65°C using the times listed in Table 2. For the thermostability test, times in the Table 3 were used. Again, the conversions were checked by TLC (hexane–acetone 10:4, v/v). The enantiomeric compositions and the ratio of the product and residual substrate fractions were analyzed by GC on chiral stationary phase. GC retention time: R_t (Hydrodex- β -PM column, 65–118°C/min, 2.5°C/min, 12 psi): 17.6 min, (R)-**2a**; 18.5 min, (S)-**2a**; 18.9 min, (R)-**1a**; 19.5 min, (S)-**1a**; GC molar response factor of acetate/alcohol (*rac*-**2a**/*rac*-**1a**): 1.23 [was used to calculate c and E in Table 1, 2 and 3]; R_t (Hydrodex- β -PM column, 100–112°C, 1°C/min, 12 psi): 7.5 min, (R)-**1b**; 7.6

min, (S)-**2b**; 8.2 min, (R)-**2b**; 8.9 min, (S)-**1b**; GC molar response factor of acetate/alcohol (*rac*-**2b**/*rac*-**1b**): 1.25 [was used to calculate *c* and *E* in Table 1, 2 and 3]; *R*_i (Beta-Dex 225 column, 130–185°C, 2°C/min, 12 psi): 23.1 min, (R)-**1c**; 23.4 min, (S)-**1c**; 24.1 min, **2c**; GC molar response factor of acetate/alcohol (*rac*-**2c**/*rac*-**1c**): 1.21 [was used for calculations of *c* and *E* in Table 3]. Organic solvent was distilled out by using the rotary evaporation and residue was separated using vacuum chromatography (silica gel, hexane: acetone 10:0.5, v/v) to get the alcohol (S)-**1a-c** and acetate (R)-**2a-c**.

3. Results and Discussion

Kazlauskas *et al.* [5] has proposed and make used of a very impressive model by the active site of a hydrolase enzyme that has the capability to attach to groups of *smallR* and *bigR* in alcohols. Fig.1a show the site of the *smallR* and *bigR* on the secondary alcohols which commonly appear to be the favorable active sites for hydrolase enzyme. According to the Kazlauskas *et al.* [5], when the substitute of *smallR* and *bigR* differs greatly in their sizes, the resolution for enzyme activity will become absolute. Alcohols with empirical rules that shows hydroxyl group on the upper side will predict the favorable enantiomer for both lipase and esterase to be the one on the right side (Fig.1a). This means, the enzyme has to favor the (R)-selectivity when the priority is favoring the *bigR* compares to the *smallR*. Enantioselectivity of an enzyme give arise to the ability on the enzyme to recognize chiral substrate during the formation of enzyme-substrate complex. Fig.2 shows kinetic resolution for the secondary racemic alcohols *rac*-**1a-c** by the freeze-dried lipase enzyme from *Geobacillus thermodenitrificans* nr68 (Lip.nr-68). The (R)-selectivity of Lip.nr-68 reactions towards the 1-phenylethanol (*rac*-**1a**), 1-cyclohexylethanol (*rac*-**1b**) and 1-(naft-2-il) ethanol (*rac*-**1c**) will be using the model by Kazlauskas *et al.* [5]. The reaction will be decided as to follow the model or otherwise is named as Anti-Kazlauskas (R)-selectivity.

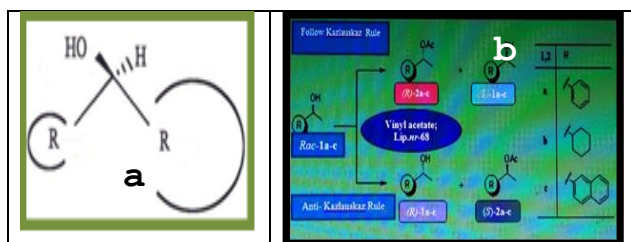


Figure 1. (a) Group of *smallR* and *bigR* in the alcohol base on the Kazlauskas *et al.*, (1991). [5]. (b) Kinetic resolution for racemic secondary alcohols *rac*-**1a-c** by Lip.nr-68 (Modified from [8]).

3.1 Lip.nr-68 reactions towards *rac*-**1a-c** at room temperature

Room temperature was recorded as 20°C. The objective of this reaction is to record the favorable substrate of the Lip.nr-68 among the given secondary racemic alcohols (*rac*-**1a-c**) and to determine the ability of the Lip.nr-68 to follow/not-to-follow the Kazlauskas Rules during the acetylation. Thin layer chromatography results of the Lip.nr-68 reactions towards all three *rac*-**1a-c** have confirmed acetate production. Methods by Poppe and Novák [7] were applied to measure the acetate production from *rac*-**1a-c**. The reaction has applied the Lip.nr-68's potential towards the acetylation by replacing the hydrogen atom of a hydroxyl group with an acetyl group (CH₃CO). All *rac*-**1a-c** were then going through biocatalytic reaction by the Lip.nr-68 using method by Nagy [8] at 20°C for 96 hours. Results from gas chromatography confirmed the acetylation by Lip.nr-68.

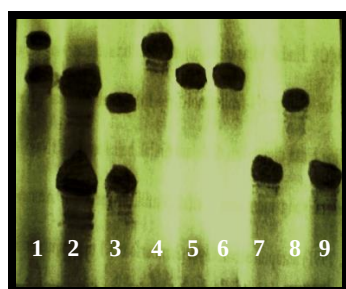


Figure 2. Thin layer chromatography (TLC) for reaction of Lip.nr-68 on the racemic substrates (*rac*-**1a-c**) after 96 hours at room temperature (20°C). Lane 1: 1-phenylethanol (*rac*-**1a**); Lane 2: 1-cyclohexylethanol (*rac*-**1b**); Lane 3: 1-(naft-2-il) ethanol (*rac*-**1c**); Lane 4: Phenylethanol acetate marker; Lane 5: 1-phenylethanol marker; Lane 6: cyclohexylethanol acetate marker; Lane 7: 1-cyclohexylethanol marker; Lane 8: 1-(naft-2-il) ethanol acetate marker; Lane 9: 1-(Naft-2-il) ethanol marker.

Table 4 shows the kinetic resolution for the racemic substrate *rac*-**1a-c** by the Lip.*nr*-68 at room temperature; 20°C. Retention time for the product analysis and details on the alcohol (*S*)-**1a-c** and acetate (*R*)-**2a-c** are listed in Table 1-3. The percentage of substrate to product conversion (*c*) was 27.5%, 50.7% and 18% for each of 1-phenylethanol (*rac*-**1a**), 1-cyclohexylethanol (*rac*-**1b**) and 1-(naft-2-il) ethanol (*rac*-**1c**), respectively. The highest enantioselectivity (*E*) was recorded with *rac*-**1a** (76) followed by *rac*-**1b** (65) and finally *rac*-**1c** (2). According to Heinsman *et al.* [9], the high number of ties between the reactions with chiral centers will increase the potential of both enantiomers to come together into the active site due to its high conformational of flexibility. Therefore the value of enantiomer selectivity, *E* for both compounds is expected to be low. *R* enantiomers were recorded to be higher than *S* enantiomers in all *rac*-**1a-c**. For *rac*-**1a**, the *ee* value was recorded as 57.6%, *rac*-**1b** as 10.4% and *rac*-**1c** as 27%. This results shows that Kazlauskas Rules [5] has been chosen by Lip.*nr*-68 during the selectivity towards the secondary alcohols. The enzyme that acts as a chiral catalyst will usually react faster with one of the enantiomers. In terms of thermodynamics, the isomer (*R*)- and isomer (*S*)- found in the racemic mixtures are representing two kinds of reagents (A and B) which are competing for a single type of enzyme. Thus, the distinction of the Gibbs free energy ($\Delta\Delta G^\ddagger$), among degrees of removal for enantiomer either to act faster or slower will determine the effectiveness of the potential of the kinetic resolution. The purity of the yield commonly depends so much on the (*c*) value [4].

Table 4. Kinetic resolution for racemic substrates *rac*-**1a-c** by Lip.*nr*-68 at 20°C.

Substrates	Reaction Times (h)	<i>c</i> ^a (%)	Alcohol (<i>S</i>)- 1a-c ; <i>ee</i> ^a (%)	Acetate (<i>R</i>)- 2a-c <i>ee</i> ^a (%)	<i>E</i> ^b
1-phenylethanol (<i>rac</i> - 1a)	96	27.5	38.7	96.3	76
1-cyclohexylethanol (<i>rac</i> - 1b)	96	50.7	79.8	90.2	65
1-(naft-2-il) ethanol (<i>rac</i> - 1c)	96	18	7	34	2

Note for Table 1. *ee* is an enantiomeric excess or non-reacted substrate. ^aValues for *ee* of (*R*)-**2a-c**, (*S*)-**1a-c** and percentage of product formation (*c*) was determined by the Hydrodex β -PM column on GC. ^bValues of the enantiomeric selection or enantioselectivity (*E*) was calculated from *c* and *ee*_{2b} using method by Chen *et al.* [11]. It was confirmed by calculation of enantiomeric excess between substrate and product (*ee*_{1a-c} and *ee*_{2a-c}; [12]). Freeze-dried Lip.*nr*-68 activity was recorded as 30.5 U/g.

The vinyl acetate in the study of acetylation of racemic secondary alcohols *rac*-**1a-c** by Lip.*nr*-68 was used as a chiral acyl donor. The enantioselectivity process taking place during the transesterification of amine or racemic alcohols (*R* and *S*) in the organic solvent, generally rely on chiral acyl donor such as vinyl and isopropenyl acetate (IPA), or anhydride to avoid the problem of reverse reaction. For the production of esters in the kinetic resolution through transesterification by lipase on alcohol in a particular organic solvent, the reverse reaction of the optical active compound with the non-chiral alcohol at the acyl donor reagent has been reported to be a major issue since the purity of the optical yield will decrease significantly [4]. Thus, acyl donor reagent with non-reversible quality such as enol-acetate (vinyl acetate and isopropyl acetate) is introduced and has been widely used for the acetylation of the chiral alcohols by lipases in the organic solvents. In addition to vinyl acetate and isopropyl acetate, 1-etoksiviny ester which is a result of new donors in the process of resolution of racemic biotacalys was superior than most of the main reagents such as vinyl ester and ester isopropenyl [10]. Moreover, many empirical studies have shown that the kinetic resolution in biocatalysis is also affected by the type of organic solvent used in the system.

3.2 Lip.*nr*-68 reactions towards *rac*-**1a** and *rac*-**1b** at 65°C

Acetylation of racemic secondary alcohols *rac*-**1a** and *rac*-**1b** were performed at 65°C. Fig. 4 shows the thin layer chromatography (TLC) for reaction after 4 and 24 hours. Acetate production was observed through the clear lanes on Fig.3, by comparing with the given markers. Positive results tell the potential of Lip.*nr*-68 on acetylation reaction of H₂ where atom was substituted at the hydroxyl group with an acetyl group (CH₃OH) to produce acetate. Therefore, acetylation of *rac*-**1a** and *rac*-**1b** has been carried at 65°C based on the method by Nagy [8]. Gas chromatography results (GC) shows

that both of the alcohols are suitable for the acetylation using Lip.nr68.



Figure 3. Thin layer chromatography (TLC) for reaction of Lip.nr-68 on rac-1a and rac-1b after 4 and 24 hours at 65°C. Lane 1: 1-phenylethanol (rac-1a, 4 hours); Lane 2: 1-cyclohexylethanol (rac-1b, 4 hours); Lane 3: 1-phenylethanol (rac-1a, 24 hours); Lane 4: 1-cyclohexylethanol (rac-1b, 24 hours); Lane 5: phenylethanol marker; Lane 6: cyclohexylethanol acetate marker; Lane 7: 1-phenylethanol marker; Lane 8: 1-cyclohexylethanol marker.

Table 5 shows the kinetic resolution of racemic substrate *rac-1a-b* by Lip.nr-68 at 65°C. The retention time for the analysis of the yield of *rac-1a-b* using gas chromatography and details on the alcohol (*S*)-**1a-b** and acetate (*R*)-**2a-b** are listed in Table 1 and Table 2. For *rac-1a*, the conversion of substrate-product; (c) was recorded as 21.7% and 39.7% after 4 hours and 24 hours, respectively. As for *rac-1b*, the percentage of substrates promoted by the Lip.nr-68 after 4 hours was 39.2% and was 51.1% at the 24th hour. Results after 4 hours of reactions indicated that *rac-1a* was going through better acetylation to compare to *rac-1b* with *E* of 65 over 55 but the enantiomeric selection or enantioselectivity (*E*) of *rac-1a-b* at 65°C was both very high after 24 hours with value over 100. Again, *R* enantiomer is shown to exceed over the *S* enantiomer in all the GC results. For 1-phenylethanol (*rac-1a*), the enantiomeric excess (*ee*) or (*S*)-**1a** was 26.8% and 65.8%, respectively at 4h and 24h, while for (*R*)-**2a** was 96.1% and 97.3%. The enantiomeric excess (*ee*) of (*S*)-**1a** at 4 and 24 hours was 67.8% and 99.5%, the rest of the data can be accessed from Table 5.

These results have clearly shown that the Lip.nr-68 has promoted for acetylation of the both alcohols at 65°C following the Kazlauskas Rule [5]. According to research by Bouzemi *et al.* [13], primary alcohols having hydroxymethyl group on the secondary stereogenic *S* alcohol and the *R* configuration is preferred for the production of acetic acid by acylation catalyzed by most lipases. This group has been experimenting a series of arylalkylcarbinol derivatives, where the substrates were deracemized through sequential combination of *Candida antarctica* lipase B (CAL-B) and the (*S*)-acetates were obtained in 71%–99% *ee* and 76%–89% yields. Another excellent research was the use of YS lipase from *Pseudomonas fluorescens* with isopropenyl acetate as acylation agent in diisopropylether by Naemura *et al.* [14]. Based on the feature selection enantiomers, active site model of lipase YS has been proposed to identify the primary or secondary enantiomer which reacts more quickly to the acetylation process. Escorcia [15] reported that about 300 microorganisms were analyzed for the production of chiral secondary alcohols by enantioselective reduction of an alkyl aryl ketone and about 60 cultures showed a reduction of ketones with *R* and *S* enantiomers as well as the production of 92-99% *ee* and 95% yield of 1-4 g/L.

Table 5. Kinetic resolution for racemic substrates *rac-1a* and *rac-1b* by Lip.nr-68 at 65°C.

Substrates	Reaction time (h)	c ^a (%)	Alcohol (<i>S</i>)- 1a-b ; <i>ee</i> ^a (%)	Acetate (<i>R</i>)- 2a-b , <i>ee</i> ^a (%)	<i>E</i> ^b
1-phenylethanol (<i>rac-1a</i>)	4	21.7	26.8	96.1	65
1-phenylethanol (<i>rac-1a</i>)	24	39.7	65.8	97.3	>100
1-cyclohexylethanol (<i>rac-1b</i>)	4	39.2	67.8	93.5	55
1-cyclohexylethanol (<i>rac-1b</i>)	24	51.1	99.5	92.8	>100

Note for Table 2. *ee* is an enantiomeric excess or non-reacted substrate. ^aValues for *ee* of (*R*)-**2a-c**, (*S*)-**1a-c** and percentage of product formation (c) was determined by the Hydrex β-PM column on GC. ^bValues of the enantiomeric selection or enantioselectivity (*E*) was calculated from c and *ee*_{2b} using method by Chen *et al.* [11]. It was confirmed by calculation of enantiomeric excess between substrate and product (*ee*_{1a-c} and *ee*_{2a-c}; [12]). Freeze-dried Lip.nr-68 activity was recorded as 30.5 U/g.

3.3 Thermostability of Lip.nr-68 reactions towards *rac-1a* and *rac-1b* at 65°C

Acetylation study on the secondary racemic alcohols *rac-1a-b* by Lip.nr-68 was accomplished with exposure of Lip.nr-68 at 65°C for the purpose of to investigate the effect to the acetylation. Acetylation was maintained to take place for 24 hours after the enzyme was exposed at different hours

to the temperature. A total of 20.0 mg of free-substrate freeze dried Lip.nr-68 in 1.0 ml of hexane, 0.50 ml of vinyl acetate and 0.50 ml tetrahydrofuran was incubated at 65°C in a small glass container with a lid at 1000 rpm for 24, 48, 72, 96 and 120 hours. After each of the subsequent hours, 20.0 mg of racemic *rac-1a* and *rac-1b* was added. The analysis by gas chromatography (GC) has been evaluated and the results are listed in Table 6.

Table 6. Kinetic resolution for substrates *rac-1a* and *rac-1b* for thermostability of Lip.nr-68 at 65°C.

Substrate	Free-substrate enzyme exposure (h)	Reaction time (h)	c ^a (%)	(S)-1a-b ee ^a (%)	(R)-2a-b ee ^a (%)	E ^b
1-phenylethanol (<i>rac-1a</i>)	24	24	36.7	54.3	92.1	42
1-cyclohexylethanol (<i>rac-1b</i>)	24	24	44.1	76.7	88.4	34
1-phenylethanol (<i>rac-1a</i>)	48	24	37.7	68.7	96.3	>100
1-cyclohexylethanol (<i>rac-1b</i>)	48	24	49.6	64.0	96.1	>100
1-phenylethanol (<i>rac-1a</i>)	72	24	40.7	66.7	98.3	>100
1-cyclohexylethanol (<i>rac-1b</i>)	72	24	51.9	65.2	97.6	>100
1-phenylethanol (<i>rac-1a</i>)	96	24	49.1	67.8	93.9	>100
1-cyclohexylethanol (<i>rac-1b</i>)	96	24	65.5	66.9	94.1	>100
1-phenylethanol (<i>rac-1a</i>)	120	24	51.1	69.5	95.2	77
1-cyclohexylethanol (<i>rac-1b</i>)	120	24	60.2	68.4	93.2	65

Note for Table 3. *ee* is an enantiomeric excess or non-reacted substrate. ^aValues for *ee* of (R)-2a-c, (S)-1a-c and percentage of product formation was determined by the Hydrodex β-PM column on GC. ^bValues of the enantiomeric selection or enantioselectivity (*E*) was calculated from *c* and *ee*_{2b} using method by Chen *et al.* [11]. It was confirmed by calculation of enantiomeric excess between substrate and product (*ee*_{1a-c} and *ee*_{2a-c}; [12]). Freeze-dried Lip.nr-68 activity was recorded as 30.5 U/g.

As expected, (R) enantiomer was shown to exceed over the (S) enantiomer in all the GC results (Table 6). However, Lip.nr-68 has been performing a very excellent thermostability in organic solvent. This enzyme has been exposed for 120 hours and the enantiomeric selection or enantioselectivity values (*E*) were recorded as more than 100 for both *rac-1a* and *rac-1b* acetylated by Lip.nr-68 exposed to 65°C at 48-96 hours (Table 6). The highest conversion rate (*c*) for both *rac-1a* and *rac-1b* by Lip.nr-68 was 49.1% and 65.5%, achieved after 24 hours by lipase exposed for 96 hours. These results indicated that the enzyme Lip.nr-68 has higher enantioselectivity against the substrate *rac-1a* over *rac-1b* when exposed beforehand without a substrate at the same temperature and time. The GC results also showed that the number of (R) enantiomer was found to exceed over (S). Enzymatic transformations for organic substrates are often used for the provision of optical active material [16],[17].

4. Conclusion

Research shows that Lip.nr-68 is an effective biocatalyst with high enantioselectivity towards all three (3) secondary racemic alcohols namely 1-phenylethanol (*rac-1a*), 1-cyclohexylethanol (*rac-1b*) and 1-(naphthalen-2-yl) ethanol (*rac-1c*) by acetylation with vinyl acetate. The thermostability was recorded maximum at 65°C for 96 hours, while the reaction has followed the Kazlauskas Rules.

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