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Enantio-conversion and -selectivity of racemic atenolol kinetic resolution using free *Pseudomonas fluorescens* lipase (Amano) conducted *via* transesterification reaction

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In this report, effects of reaction parameters on kinetic resolution of racemic atenolol using *Pseudomonas fluorescens* lipase were investigated *via* transesterification for production of pharmacologically active eutomer (*S*)-atenolol with high enantiomeric purity. It was found that a temperature of 45 °C produced an acceptable enantioselectivity (*E*: 17). Good agitation speeds were found at 170–230 rpm producing *E* values of 12–15, whilst an enzyme activity of ≥2500 U gave 100% conversion of the (*S*)-atenolol. Substrate concentrations of 11.26–18.80 mM gave *E* values of 11.6–12.3. Variation of the substrate molar ratio yielded (*S*)-atenolol conversions of 44.67–61.58% with $E = 12–23$.

1. Introduction

Atenolol (Fig. 1) is a β-blocker compound used for treatment of high blood pressure, angina pectoris, and arrhythmia problems.[1,2] The compound is still sold as a racemic mixture. As the active isomer of atenolol is the (*S*)-enantiomer, replacing racemic atenolol with this optically active compound would give rise to reduced side effects. Two routes have been established to produce single enantiomer β-blocker compounds *i.e.* asymmetric syntheses and racemic resolutions.[3] But, there are limited methods applied to give (*S*)-atenolol.

(*S*)-Atenolol has been synthesized asymmetrically from achiral and/or racemic substrate(s) using chiral metal catalysts (*e.g.* cobalt salen complexes) *via* hydrolytic kinetic resolution of the substrate followed by ring opening reaction,[4,5] or simultaneous ring opening reaction and kinetic resolution of substrate[6,7] where the intermediate compound was finally aminated to give this enantiomer. Recently, Dwivedee *et al.*[8] developed a chemoenzymatic method where synthesis of the intermediates in aqueous medium and enzymatic kinetic resolution by CAL-A CLEA (*Candida antarctica* lipase A cross linking enzyme aggregate) in ionic liquid make the process greener than other reported methods. (*S*)-Atenolol has also been produced *via* addition of chiral addenda *e.g.* (*R*)- and (*S*)-epichlorohydrin catalysed by an alkali metal hydroxide and/or benzyltrimethylammonium chloride.[9–11] These asymmetric syntheses give an outstanding choice since they orientate to the development/exploration of raw materials and catalysts, give medium yields of the single enantiomer, have high enantiomeric excess, and use easily found chiral addenda. However, the chiral catalysts are expensive, and the asymmetric syntheses are associated with low temperatures, long reaction steps and long development periods.

Kinetic resolutions of the available racemic atenolol have been conducted *via* chromatography processes,[12,13] whole-cell

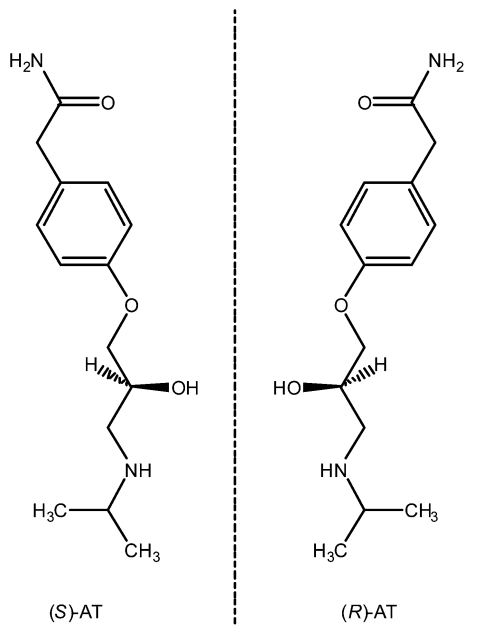

Fig. 1 Mirror images of atenolol enantiomers.

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fermentation (the racemate and its derivative),[14] and enzymatic processes using immobilised lipases.[15,16] The resolutions can operate continuously at mild temperatures and require the presence of chiral selectors, microorganisms, *etc.* Although liquid chromatography was a superior analytical technique, it needed tedious sample preparation, expensive chiral columns, large volumes of additives and was time consuming.[17] Even though the whole-cell approach was economical and operationally simple[14] and gave high yield and enantiomeric excess, the preparation and recovery of the cells should be considered. Enzymatic kinetic resolution produced high enantiomeric excess with maximum 50% conversion, but it was predominantly conducted in a one-pot batch reaction so that a sort of recycling system for the enzyme must exist if an expensive enzyme is used. Immobilisation of the enzyme is a valuable option as developed in previous experiments.

As enzymatic membrane reactors (EMR) can operate continuously and be used repeatedly, these reactors could give better prospects in the kinetic resolution of racemic atenolol. However, the proper enzyme(s) for the kinetic resolution process must firstly be chosen as the EMR require the free enzyme(s) to be attached onto the membrane surfaces. Hence, a selection process for the candidate free enzymes for the kinetic resolution of the racemic atenolol must be conducted before the EMR process proceeds since no report on the free enzyme-based kinetic resolution of racemic atenolol was found.

Of all the methods used in the kinetic resolutions of racemic mixtures, enzymatic transesterification is the most dominant technique.[18] Therefore, the kinetic resolution of racemic atenolol *via* an enzymatic transesterification reaction was studied with the aim of gaining knowledge for (*S*)-atenolol preparation using free *Pseudomonas fluorescens* lipase (Amano) and vinyl acetate (acetyl donor) in tetrahydrofuran.

2. Materials and methods

2.1 Materials

(*R*,*S*)-Atenolol (99% USP, Nanjing Chemlin Chemical Industry (Nanjing, China)), (*S*)-atenolol (97%, Tocris Bioscience (Bristol, England)) and (*R*)-atenolol (99%, Sigma-Aldrich (Malaysia)) were used. The chemicals were of analytical grade except for analysis (of HPLC grade) and bought from EOS Scientific (Malaysia), Fisher Scientific (Malaysia) and Merck (Malaysia). *Pseudomonas fluorescens* lipase (Amano, product no. 534730, unit activity: 20 U mg^{-1}) was purchased from Modern-Lab Chemicals Sdn Bhd (Malaysia). The racemate, single enantiomers, lipase and all chemicals were used without pre-treatment.

2.2 Experimental procedure

2.2.1 Enzyme screening. Enzymes as listed in Table 1 were used to convert one of the atenolol enantiomers into an atenolol acetate enantiomer. A number of enzymes activity (Table 1) was added to a 25 mL solution of 18.80 mM racemic atenolol in solvent that had been mixed with vinyl acetate in the stoichiometric ratio of 1 : 1.5–2.4 (mol mol^{-1}). Initial samples were taken before the reaction was started. The mixtures were incubated in orbital shakers (Max Q 4000 Barnstead Lab-Line or Infors HT Ecotron) for 24 h at 40 °C and 200 rpm (or 155 rpm, see Table 1). 1 mL aliquots were taken at a certain time interval and transferred into 2 mL glass vials prior to analysis.

Table 1 Comparison of prospective enzymes

Enzyme	X_S (%)	X_R (%)	ee_S (%)	E	Chirality
Lipoprotein *Burkholderia* sp.[a]	100	0	100	∞	*S*
Lipoprotein *Pseudomonas* sp.[b]	16.87	50.75	37	13	*R*
PFL Amano[c]	46.36	0	24	15	*S*
PFL Fluka[d]	70.73	8	54	28	*S*
PFL Sigma[d]	80.80	25.17	58	5	*S*

[a] 200 rpm, 40 °C, 18.80 mM racemic atenolol in THF, 1.5 molar ratio, 2000 U. [b] 200 rpm, 40 °C, 18.80 mM racemic atenolol in DMSO, 1.5 molar ratio, 2200 U. [c] 200 rpm, 40 °C, 18.80 mM racemic atenolol in THF, 2.4 molar ratio, 1000 U PFL-Amano. [d] 155 rpm; 40 °C, 30.04 mM racemic atenolol in THF, 2.4 molar ratio, PFL-Fluka: 800 U, PFL-Sigma: 3200 U; 6–24 h observations.

2.2.2 Factor of agitation speed. The effect of agitation on the production of (*S*)-atenolol acetate by the biocatalyst was determined by varying the agitation speed from 140 rpm to 260 rpm. The substrate (18.8 mM) and vinyl acetate were supplied at a molar ratio of 1 : 2.4 (25 mL of 18.8 mM substrate was used). The temperature was set at 40 °C. 1000 U PFL-Amano was added into the mixture at each agitation speed.

2.2.3 Diversity of substrate concentration. The effect of substrate concentration was studied by varying the racemic atenolol concentration from 3.75–37.60 at fixed substrate to acetylating agent ratio of 1 : 2.4 (25 mL solution of substrate was used), while other parameters (solvent, temperature, agitation speed, enzyme activity) were kept constant at 40 °C, 200 rpm and 1000 U PFL-Amano.

2.2.4 Effect of reaction temperature. Since temperature governs the catalytic activity of the enzyme in the reaction, it was investigated in the range of 25–50 °C. Other reaction conditions were set as follows: substrate concentration of 18.8 mM, molar ratio of (1 : 2.4; 25 mL solution of substrate), and 200 rpm agitation speed, 1000 U PFL.

2.2.5 Molar ratio of substrate : acetyl donor. The effect of substrate to acetyl donor molar ratio on the reaction was investigated by varying the ratio from 1 : 1.2 to 1 : 6.0. The substrate concentration (18.8 mM, 25 mL), enzyme activity (1000 U), temperature (40 °C), and agitation speed (200 rpm) were held constant.

2.2.6 Effect of enzyme loading. The effect of enzyme activity on the reaction of racemic atenolol and vinyl acetate was studied by varying the amount of enzyme from 1000 U to 2500 U. The substrate concentration (18.8 mM, 25 mL), substrate to acetate ratio (1 : 2.4), agitation speed (200 rpm), and temperature (40 °C) were kept constant.

2.3 Analysis of reaction product

2.3.1 UFLC apparatus. Analysis of the atenolol enantiomers was performed using a Shimadzu Ultra Fast Liquid Chromatography (UFLC) LC-20A Prominence, which consists of

two units of LC-20AD dual plunger parallel flow solvent delivery pump, one unit SIL-20ACHT auto-sampler, one unit SPD-20A UV-Vis detector, one unit CTO-20AC column oven, one unit DGU-A3 degasser unit and the CBM-20A system controller. A personal computer is linked to the UFLC using Shimadzu LCsolution Real Time Application software.

2.3.2 Chiral column. A Chiralcel® OD column (250 mm × 4.6 mm) supplied by Daicel Chemical Industries, Ltd. (Japan) was used to perform chiral analysis. As stated in its instruction manual (Daicel Chemical Industries, 2009), this column has a typical operating temperature, pressure and flow rate of 0–40 °C, 5 MPa (maximum) and 1.0 mL min^{-1}, respectively. Its mobile phase is based on a mixture of alkane and alcohol with a limited quantity of basic or acidic modifier compound (Chiral technologies Europe, 2003). This chiral column was connected to a guard column (analytical) KJ0-4282 (Phenomenex) supplied by LT Resources (M) Sdn Bhd.

2.3.3 Sample preparation and analysis. During the enzymatic reactions, at a certain interval time, a 1 mL sample was taken from each reaction flask and placed inside a 1.5 mL vial then kept in a 4 °C refrigerator prior to analysis. All samples were directly injected to the UFLC unit. A modified analytical method developed from the previous methodologies was used.[19–22] 2 μL of samples were injected automatically at a time into the Chiralcel® OD column with a mobile phase consisting of hexane–ethanol–diethylamine (75%–25%–0.1% v/v/v). The UV-Vis detector was set at a wavelength of 276 nm. The UFLC was operated at 35 °C and normal phase. Qualitative and quantitative analyses were conducted on the resulting chromatograms *via* the Shimadzu LCsolution post-run analysis software based on the standard procedure for the instrumentation.

2.4 Calculation of conversions and enantiomeric excess

Conversions of enzymatic reactions were calculated as follows:[23,24]

$$X\ (\%) = \frac{C_0 - C_t}{C_0} \times 100 \quad (1)$$

$$X_S\ (\%) = \frac{C_{S_0} - C_{S_t}}{C_{S_0}} \times 100 \quad (2)$$

$$X_R\ (\%) = \frac{C_{R_0} - C_{R_t}}{C_{R_0}} \times 100 \quad (3)$$

where X is total conversion (%), X_S is the conversion of (*S*)-atenolol (%), X_R is the conversion of (*R*)-atenolol (%), C_0 is the initial amount of racemic atenolol (mM), C_t is the amount of racemic atenolol (mM) at reaction time t, C_{S_0} is the initial amount of (*S*)-atenolol, C_{S_t} is the amount of (*S*)-atenolol at reaction time t, C_{R_0} is the initial amount of (*R*)-atenolol, and C_{R_t} is the amount of (*R*)-atenolol at reaction time t.

The enantiomeric excess of substrate (ee_S) and product (ee_P) and enantiomeric ratio (E) were determined as follows:[25–28]

$$ee_S(\%) = \frac{(C_{R_t} - C_{S_t})}{(C_{R_t} + C_{S_t})} \times 100 \quad (4)$$

$$ee_P(\%) = ee_S \frac{(1 - X)}{X} \times 100 \quad (5)$$

$$E = \frac{\ln\left[(1 - ee_S)\left(\frac{ee_P}{ee_S + ee_P}\right)\right]}{\ln\left[(1 + ee_S)\left(\frac{ee_P}{ee_S + ee_P}\right)\right]} \quad (6)$$

3. Results and discussion

No reaction was observed at 40 °C in the absence of enzyme after 24 h incubation under a constant agitation speed. The reaction produces an ester and acetaldehyde and is considered irreversible. The effects of parameters during the enzymatic transesterification are described.

3.1 Enzyme screening

Table 1 summarizes the conversions and enantioselectivities of the enzymatic transesterification using many enzymes. Most enzymes preferred the (*S*)-atenolol. Lipoprotein *Burkholderia* sp. produced the highest (*S*)-atenolol conversion and infinite enantioselectivity (E) with no change of the (*R*)-atenolol, whilst the PFL gave lower (*S*)-atenolol conversions, 0–25% conversion of the (*R*)-atenolol, and moderate E except the PFL-Sigma. The PFL-Amano was chosen to be studied further.

3.2 Factor of agitation speed

The effect of agitation speed on (*S*)-atenolol conversion is shown in Fig. 2. The speed was studied in the range of 130–260 rpm. Conversions of the (*S*)-atenolol were less than 50% where there were slight differences amongst the speed. Low conversions were found at the lowest agitation speed where the conversion was only 41.44%. The highest agitation speed gave a conversion of 45.95%. High conversions were obtained at the speeds of 200 and 230 rpm, where almost 48% of the (*S*)-enantiomer reacted with the acetyl donor, although the speed of 170 rpm produced a conversion of 46.36%, which was slightly lower than that obtained at 200 rpm. No transesterification of the (*R*)-enantiomer was observed. The speed of 200 rpm was chosen as the optimum value as it gave high conversion of the (*S*)-atenolol, though 230 rpm produced the same conversions. It was in agreement with some previous experimental results. The kinetic resolution of several β-blockers indicated that 200 rpm developed high yields and enantiomeric conversion during the resolution process.[15,25] Higher agitation speeds (>350 rpm) were also applied in the racemic kinetic resolution of racemic tertiary alcohols and cyanohydrins, but speeds of 200–300 rpm were generally used in the transesterification process of racemic alcohols.

Small fluctuations of the substrate enantiomeric excess (ee_S), enantiomeric excess of product (ee_P) and enantiomeric ratio (E) are observed where small differences are considered on the ee_S values. The ee_S produced during the experiments were 22–28% where the highest result was obtained at 230 rpm, though it only differed slightly from the ee_S at 170 and 200 rpm. These ee_S were

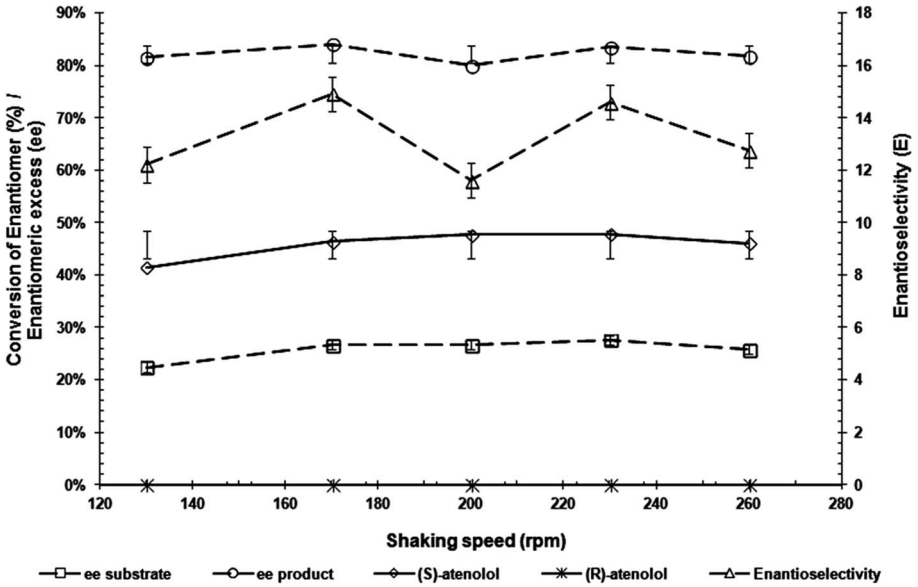

Fig. 2 Effect of agitation speed (18.80 mM racemic atenolol solution, 40 °C, 1000 U PFL-Amano, 1 : 2.4 molar ratio).

low as the (*S*)-atenolol conversions were not high. However, the ee_P values were high. The ee_P values were in the range of 80–84%. The peak result was found at 170 rpm, whilst the result at 230 rpm gave the same ee_P (*i.e.* 84%). The *E* values were in the range of 12–15. The highest and lowest *E* values were given by the agitation speeds of 170 and 200 rpm, respectively.

Fluctuation of *E* throughout the agitation range indicates that differing enantioselectivity of the PFL-Amano towards the racemic atenolol occurs, which means that the ability of this enzyme under the determined reaction conditions to distinguish between enantiomers is not the same. Actually, *E*, which remains constant throughout the reaction periods, is only determined by the environment of the reaction system.[26–28] The resulting *E* values are still low as the reactions with $E < 15$ are usually considered unsuitable.[29] Hence, formulating a new reaction condition is required. From these trials, the agitation speeds of 200–230 rpm produced higher conversions. High values of the enantiomeric excess of substrate (ee_S) were obtained at 170–230 rpm. Better ee_P and *E* were obtained at 170 and 230 rpm.

3.3 Diversity of substrate concentration

Two substrates are involved in the enzymatic transesterification process *i.e.* racemic atenolol (solid form) and vinyl acetate (liquid form). As a homogenous system should be formed during the reaction, in order to ease the transport of substrates, both substrates must dissolve in THF. The vinyl acetate dissolved easily in THF, but racemic atenolol is not a highly soluble compound. Laboratory tests showed that the maximum solubility of this racemate at 35 °C was 37.60 mM. Trials with concentrations of 46.93 mM and 56.32 mM at 35 °C were not successful as some crystals were still observed visually after some hours' incubation in an orbital shaker at 200 rpm without addition of lipase and acetylating agent. Further observation indicated that the 46.93 mM and 56.32 mM concentrations were dissolved totally at 40 °C, but when they were kept at room temperature, precipitation of solids occurred. Hence, the maximum concentration of racemic atenolol in THF for the experiments was fixed at 37.60 mM in order to prevent sample precipitation before analyses were conducted and to prevent blocking of the UFLC sampling line during the analyses.

The effect of the substrate concentration on the racemic atenolol kinetic resolution at a constant molar ratio of the racemic atenolol to vinyl acetate is shown in Fig. 3. A declining trend on the enantiomeric conversion is observed throughout the racemic atenolol concentrations. The conversion was high at the lowest substrate concentration. It decreased sharply to the lowest conversion at the highest substrate concentration. The decrease of conversion when the racemic atenolol was increased indicates that the inhibition process caused by the substrate(s) could occur during the enzymatic transesterification. At the lowest substrate concentration, the conversion of the (*S*)-atenolol was high *i.e.* 82.72%, however, the (*R*)-atenolol was changed as well (18.48%). As the enzyme activity was constant throughout the trial concentrations (1000 U PFL for 3.75–37.55 mM racemic atenolol), many enzyme active sites were available to react the (*S*)-atenolol with vinyl acetate at the lowest concentration. Hence, not only was much (*S*)-atenolol converted, but also some (*R*)-atenolol enantiomer reacted with vinyl acetate.

At the racemate concentration of 11.26–18.80 mM, conversion of the (*S*)-atenolol drops to 47.69–48.92%, but no reaction on the (*R*)-atenolol was observed. The (*S*)-atenolol conversion continues to decrease to 22.85–27.80% when the racemic atenolol concentration was increased to 30.04–37.55 mM, whilst at the same time 3.73–4.25% of the (*R*)-atenolol was also changed. At 30.04–37.55 mM concentrations, many acetyl–enzyme complexes were formed, and many vinyl acetate molecules still existed in the reaction mixtures as a constant enzyme quantity

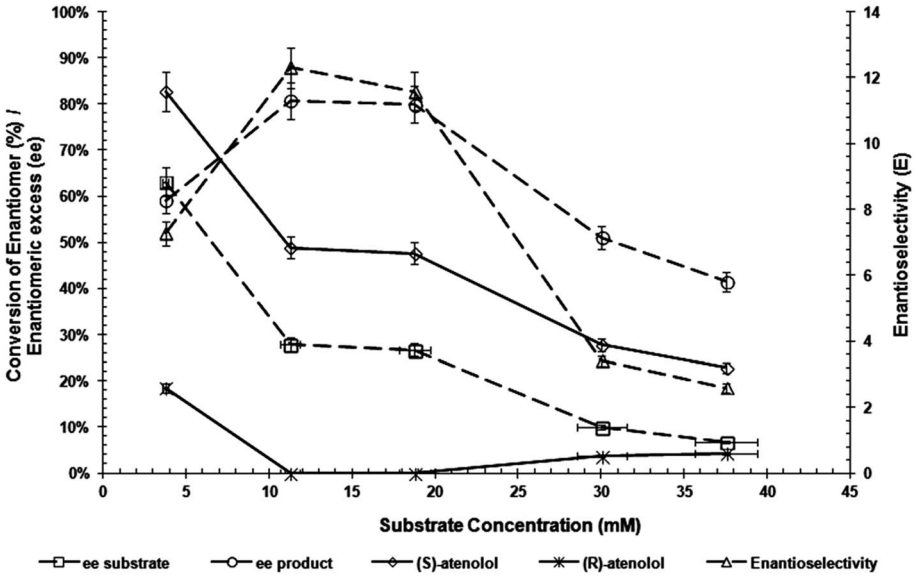

Fig. 3 Effect of racemic atenolol concentration (40 °C, 200 rpm, 1000 U PFL-Amano).

(1000 U) was used. Hence, the reaction environment was enriched with (*R*)- and (*S*)-atenolol and vinyl acetate as constant reaction volume was used during the resolutions. Romero *et al.*[34] demonstrated that the properties of a medium (*e.g.* polarity) were modified when alcohol and acetic anhydride concentrations were increased leading to the reaction that shifted to the more polar substrate(s), and the free amount of substrates in the medium caused enzyme deactivation. Another possible factor is that the amount of enzyme became the limiting substance at high substrate concentration.[31] The reaction time could be a problem as well. Although the applied reaction time was 24 h, it was not sufficient to resolve the racemic atenolol under these concentrations. Hence, increasing the reaction time would probably increase the (*S*)-atenolol conversion.

The kinetic resolution of the racemic atenolol using immobilised CALB showed that the enantiomeric conversion increased when the racemic atenolol was increased from 3.30 mM to 16.60 mM where constant vinyl acetate concentration (33.30 mM) was used during 120 h.[15] The difference between the present and previous results may be caused by the difference in reaction time and lipase type. Resolutions of racemic propranolol using immobilised CALB[32] produced the same results as the present observation: the highest conversion was found at the lowest racemic propranolol concentration.

The ee_S value was found to decrease when the racemic atenolol concentration was increased. A high ee_S value was observed at the lowest substrate concentration. It decreased to almost 60% when moderate concentrations (11.26–18.80 mM) were applied. High concentrations (30.04–37.55 mM) produced ee_S values less than 10%. However, ee_P and *E* showed the same characteristic, which differed from the eeS results. They reached high results at moderate concentrations (11.26–18.80 mM) where an ee_P of around 80% and *E* of 11.60–12.30 were obtained. Low *E* values were found when high concentrations were used. Chiou *et al.*[29] summarized that ee_P and *E* increased when the substrate concentrations were decreased where the ee_S value did not change. Ong *et al.*[35] stated that a gradual drop in *E* was observed with an increase of the racemic ketoprofen concentration after the *E* value reached a peak at low concentrations. In conclusion, the racemic atenolol concentrations of 11.26–18.77 mM give high ee_P and *E* values, though high conversion and ee_S values are obtained at 3.75 mM.

3.4 Effect of reaction temperature

As rate of reaction is affected by temperature, setting-up reactions at their optimum temperatures will give rise to high conversions of the substrate(s). Commonly, the optimum temperatures are found in the range of 40–60 °C.[33] In these experiments, activities of the PFL during the transesterification process of the racemic atenolol were studied at temperatures of 30–50 °C as the solvent has low boiling point (*i.e.* 66 °C) and the enzyme has an optimum temperature of 55 °C.[34] The effect of temperature on conversion of the (*S*)-atenolol into its ester is shown in Fig. 4. Most of the experimental temperatures produced <50% of (*S*)-atenolol conversion. At temperatures of 30–40 °C, conversions of 46–48% were obtained. High conversion was observed at a temperature of 45 °C, where around 61% of this enantiomer was converted. Low conversion for the (*S*) enantiomer was found at a temperature of 50 °C, where only 30.80% conversion was produced. The resulted conversions throughout the applied temperatures show a profile typical of temperatures *versus* reaction rates. Increasing the reaction temperature means increasing the reaction rate. But, at the optimum temperature, the enzymatic reaction reaches the optimum reaction rate. Increasing the reaction temperature higher than the optimum value causes disruption of the enzyme structure leading to denaturation of the enzyme, hence the reaction rate starts to decrease.[35] The condition of (*R*)-atenolol

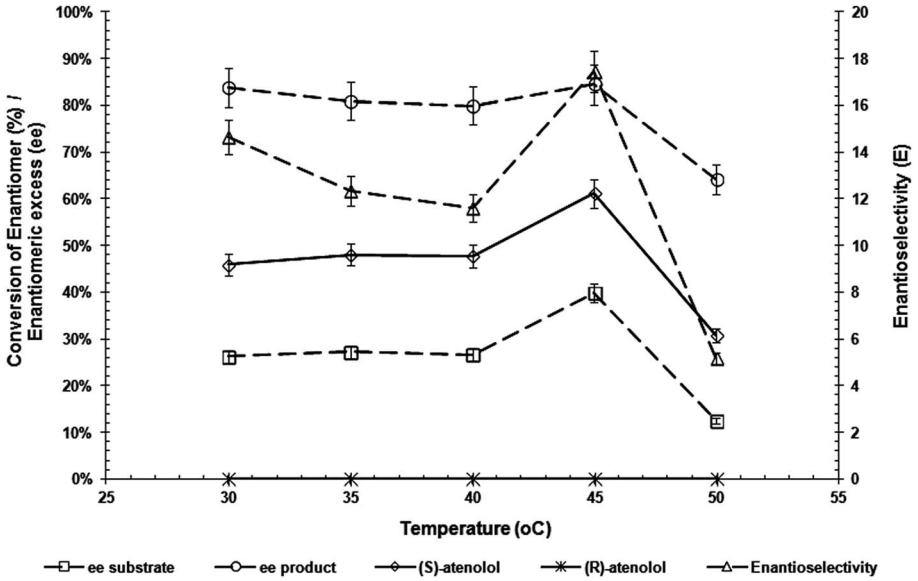

Fig. 4 Effect of temperature (200 rpm, 1 : 2.4 molar ratio, PFL-Amano: 1000 U, 18.80 mM racemic atenolol solution).

in the experiments was also observed (Fig. 4). No conversion of this enantiomer was found at temperatures of 30–50 °C.

High enantiomeric conversions were also developed at temperatures of 37–40 °C. Nunno *et al.*[40] used PFL to resolve a β-blocker precursor with vinyl acetate in hexane in 4 h to achieve 31% conversion of the (*S*)-enantiomer with 93% enantiomeric excess of the ester. Transesterification of racemic mandelonitrile with isopropenyl acetate in dry diisopropyl ether catalysed by PFL gave 57% conversion and 64% enantiomeric excess of product ((*S*)-ester) at 40 °C.[37] Hence, the optimum temperature of 45 °C achieved in this research existed in the range frequently employed in PFL catalysed-reactions.

In general, low ee_S values were seen. They were found in the range of 13–40%. However, ee_P showed high results *i.e.* 64–84%. Stable ee_S and ee_P values were observed at temperatures of 30–40 °C, where only slight differences between ee_S and ee_P were found. The peak value was observed at 45 °C. Temperatures higher than 45 °C produced lower ee results than at 35–45 °C. Similar results were observed for enantioselectivity, where a high *E* value was obtained at 45 °C after quite stable *E* values at 30–40 °C ($E = 12$–15). Lower *E* results were found at 50 °C (*E*: 3–5). To conclude, high conversion, ee_S and ee_P values are obtained at the temperature of 45 °C, where the *E* is moderate as the value is >15.

3.5 Molar ratio of substrate : acetyl donor

Another important factor in this reaction is the ratio of substrate : acetyl donor. As the substrate for the reaction is (*S*)-atenolol, it must be converted to as high an extent as possible to accumulate at high levels as the target product, but the (*R*)-atenolol existing in the reaction mixture must be managed to undergo as low conversion as possible, or no-conversion, as it will reduce the product enantiomeric excess and enantioselectivity. Since the reaction involves two compounds (the substrate and acetyl donor), their ratio in the enzymatic reaction must be optimized in order to give high conversion. This effort also relates to minimization of reactant costs.[30] The effect of the substrate : acetyl donor ratio is presented in Fig. 5. An increasing trend of the reaction conversion is obtained. In the experiments, vinyl acetate was varied. It was found that only one atenolol enantiomer reacted with the acetyl donor during 24 h *i.e.* (*S*)-atenolol. Conversions of this enantiomer were in the range of 45.24–61.58%, where the highest conversion was obtained at the highest molar ratio. At the lowest molar ratio, the enzymatic reaction gave the lowest enantiomeric conversion (*i.e.* 45.24%) as the reaction mixture lacked a vinyl acetate supply. When the ratios were set at 1 : 2.4–1 : 3.6, 45–51% of the (*S*)-atenolol reacted. Although the conversions increased when the molar ratio was raised from 1 : 4.8 to 1 : 6.0, only a slight conversion difference was observed. The molar ratio of 4.8 was considered to give the highest enantiomeric conversion (*i.e.* 60.89%).

The observed ratio was found following earlier results. Damle *et al.*[16] used a molar ratio of 1–1.2 to convert (*R*)-atenolol to its ester using vinyl acetate or succinic anhydride and lipase PS-D to give 40–42% overall yield and 94% enantiomeric excess of product. Later, Barbosa *et al.*[15] applied a ratio of 1 : 2 to 1 : 10 to convert (*R*)-atenolol. The conversions of 55–75% for the enantiomer were found during 120 h observation, where the substrate molar ratio of 1 : 2 produced the highest conversion. Previously, racemic propranolol was esterified using vinyl acetate with the ratio of 1 : 3 to 1 : 10 at 25 °C.[32] It was found that the highest molar ratio produced the highest conversion of the (*R*)-propranolol. Other experiments used molar ratios of 1 : 1 to 1 : 5.[23,24,42–45]

The ee_S values were generally less than 45%, but high ee_P and *E* values were obtained. The ee_S values tended to increase when the molar ratio was increased. A low ee_S value was obtained at the molar ratio of 1 : 1.2. A small ee_S improvement occurred when the ratios were changed to 1 : 2.4 and 1 : 3.6. However, the

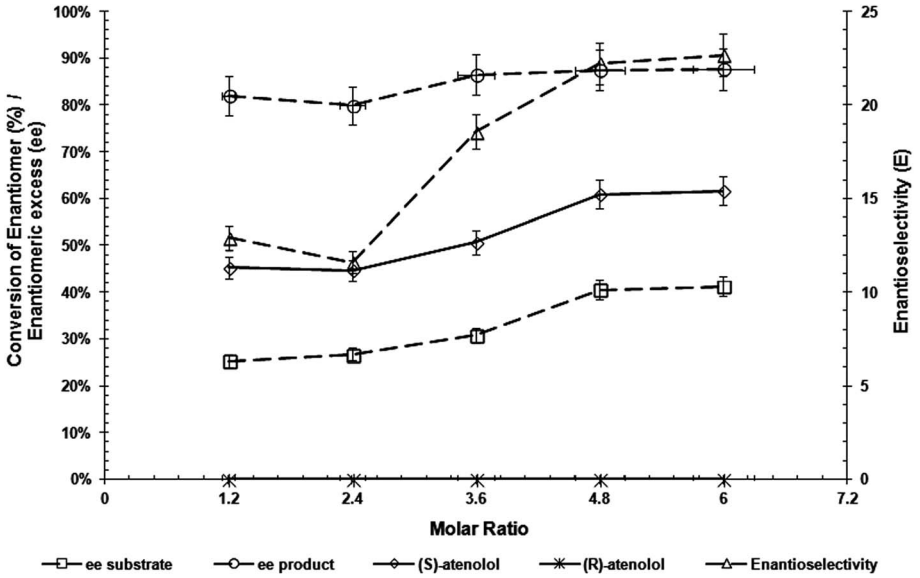

Fig. 5 Effect of substrate molar ratio (18.80 mM racemic atenolol, 40 °C, 200 rpm, 1000 U PFL-Amano).

ee_S value increased significantly at the ratio of 1 : 6.0 where ee_S was 41%, which was the highest result during the observations; however, it only differed slightly from the ee_S value obtained at the molar ratio of 1 : 4.8, which produced 40% ee_S. The ee_P values were in the range of 80–88%, where the lowest and highest ee_P values were given by molar ratios of 1 : 2.4 and 1 : 6.0, respectively. But, the ee_P value produced at the molar ratio of 1 : 6.0 did not differ highly from the ee_P value obtained at the ratios of 1 : 3.6 and 1 : 4.8. The *E* values were moderate *i.e.* 12–23. Low *E* values were developed by the ratios of 1 : 1.2 (*E*: 13) and 1 : 2.4 (*E*: 12). Although the molar ratios of 1 : 4.8 to 1 : 6.0 gave $E > 22$, only a slight improvement was gained if compared with the result obtained at the ratio of 1 : 3.6 (*E*: 19). High conversion, ee_S and *E* values were obtained at molar ratios of ≥1 : 4.8, however, high ee_P values were observed at molar ratios of ≥1 : 3.6.

3.6 Effect of enzyme loading

As reduction of the substrate(s) during an enzymatic process will depend on the presence of enzyme active sites, the rates of enzymatic reactions will be determined by the quantity of enzyme available in solvent bulk. Generally, the higher the enzyme concentration, the faster would be the reaction rate. But, overloaded enzyme quantity may have a saturated equilibrium based-on the kinetic considerations, and it is not favourable economically.[45] The optimum amount of enzyme is important for shorter reaction times as well as higher yields that make the enzymatic process more cost effective.[31] The effect of enzyme loading is illustrated in Fig. 6. An increasing trend in the (*S*)-atenolol conversion was observed as the PFL quantity was increased. No reaction of the (*R*)-enantiomer was seen. At the initial enzyme activity (1000 U or 41.67% weight of enzyme per weight of supplied racemic atenolol), approximately 48% of the (*S*)-atenolol was changed. When the PFL activity was increased to 1500 U (~62.50% weight of enzyme per weight of supplied racemic atenolol), a high increase was obtained, where a conversion of 71.14% was achieved. An excellent conversion resulted when the PFL loading was two and half times higher than the initial supplied activity. At 2500 U (~104.17% weight of enzyme per weight of supplied racemic atenolol), 100% conversion of the (*S*)-atenolol was achieved. The same result was found when 3000 U (~125% weight of enzyme per weight of supplied racemic atenolol) enzyme activity was used. Hence, higher conversions were found at higher enzyme loadings and *vice versa*. The conversion differences were considered to be caused mainly by the number of PFL active sites available in the mixture. At low enzyme loadings, limited active sites were present for the reaction. The higher the enzyme activity, the more active sites were available (for certain substrate concentration *i.e.* 18.8 mM). The PFL activity of 2000 U gave the maximum conversion for the (*S*)-atenolol enantiomer.

Comparisons with previous enzyme loadings can be made as follows (here the term of weight of enzyme per weight of substrate is applied as no detailed information on the enzyme unit activity from the previous articles are available). Damle *et al.*[16] used 0.00056 g lipase PS-D per g racemic atenolol to obtain 42% overall conversion and 94% ee_P of the (*S*)-ester. A higher enzyme quantity was applied by Barbosa *et al.*[15] where 6.80 g EC-CALB per g racemic atenolol was used to achieve 22.5% conversion and 96% ee. A very high enzyme loading *i.e.* 15.38 g enzyme per g racemic propranolol was supplied to the enzymatic reaction to change 0.05 mM racemic propranolol in 1 mL toluene.[25] The experimental results were quite good where 1.04 g PFL-Amano per g racemic atenolol was employed to give 100% conversion of the (*S*)-atenolol.

The ee_S value was only around 25% when low enzyme loading was used (*i.e.* 1000 U). It reached its peak of 100% after the reaction was supplied with 2500 U, which was equal to 104.17% (weight of enzyme/weight of racemic atenolol) PFL

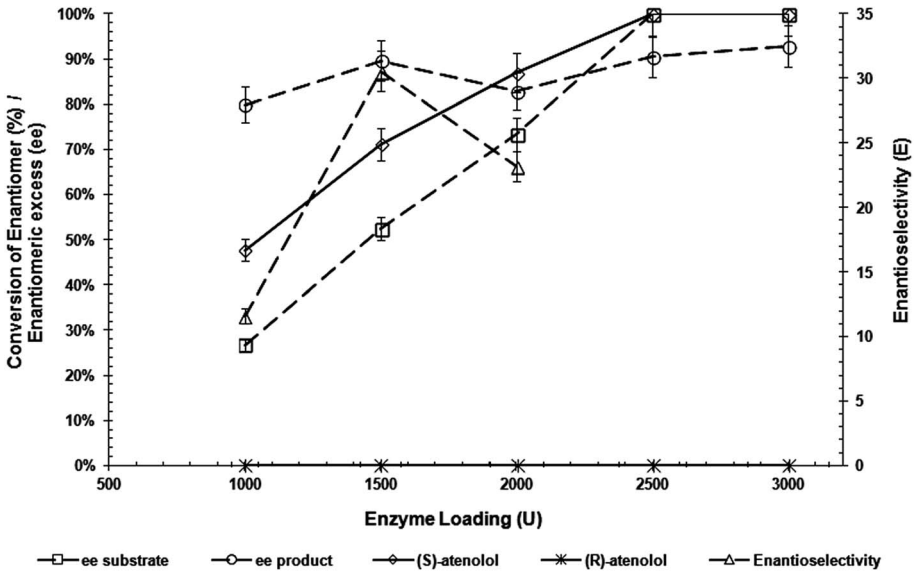

Fig. 6 Effect of enzyme loading (18.80 mM racemic atenolol solution, 2.4 molar ratio, 40 °C, 200 rpm, 24 h).

loading. The same result was observed when the enzymatic reaction used 3000 U PFL activity. High ee_P values were obtained during the observations. The ee_P values were in the range of 78–93%. As in the previous part, the ee_P value increased when the enzyme activity was increased. The enzyme activity of 2500 U, which provided 100% conversion of the (*S*)-atenolol, gave an ee_P of 90%, whilst the 3000 U PFL-Amano activity developed a slightly higher ee_P than the 2000 U PFL-Amano activity. The enantioselectivity values were high as well. At the enzyme activities of 1000–2000 U, the *E* values were 12–31. Moreover, at the higher enzyme activities, infinite *E* values were found as the conversions of the (*S*)-atenolol were 100%. As described above, at the PFL-Amano activity of >2000 U, 100% conversion of the (*S*)-atenolol, 100% ee_S, more than 90% ee_P and infinite *E* values are obtained.

4. Conclusion

The free enzyme-based kinetic resolution of racemic atenolol *via* a transesterification reaction in a batch system produced some prospective enzymes, which mostly preferred the (*S*)-atenolol. Though free lipoprotein *Burkholderia* sp. gave the highest conversion and enantioselectivity, free *Pseudomonas fluorescens* lipase (Amano) was chosen. One-factor-at-a-time observation on this process using free PFL (Amano) obtained a temperature of 45 °C, agitation speeds of 170–230 rpm, enzyme activity of ≥2500 U, racemic atenolol concentrations of 11.26–18.80 mM and substrate molar ratio of 1 : 6.0 as conditions that gave good conversion, enantiomeric excess and enantioselectivity. At these operating conditions, the conversions of 45–100%, ee_S values of 25–100%, ee_P values of 80–93% and *E* of 12–∞ were obtained. At high enzyme loadings and substrate molar ratios, high conversion, ee_S, ee_P and *E* values were found. Low conversions and ee_S values were found at low agitation speeds and *vice versa*. However, ee_P and *E* tended to fluctuate within the speeds. Reductions of conversion and ee_S values were obtained when the substrate concentrations were increased, but, at low concentration values, not only the (*S*)-atenolol reacted, but also the (*R*)-atenolol. ee_P and *E* reached high results at medium substrate concentrations. The resulting conversions showed typical profiles for temperature *versus* reaction rate. Increasing the reaction temperature increased the reaction rate, but, at the optimum temperature, the enzymatic reaction reaches the optimum rate. High conversion, ee_S and ee_P values were obtained at 45 °C.

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References

1 B. Anroop, B. Ghosh, V. Parcha, A. Kumar and J. Khanam, *J. Drug Delivery Sci. Technol.*, 2005, **15**(2), 187.
2 S. R. Mehta, B. M. Bhawal, V. H. Deshpande and M. K. Gurjar, *US Pat.* 6,982,349B1, 2006.
3 J. Agustian, A. H. Kamaruddin and S. Bhatia, *Process Biochem.*, 2010, **45**(10), 1587.
4 R. B. Kawthekar and G. J. KiM, *Helv. Chim. Acta*, 2008, **91**, 317.
5 D. S. Bose and A. V. Narsaiah, *Bioorg. Med. Chem.*, 2005, **13**, 627.
6 R. B. Kawthekar, W. T. Bi and G. J. Kim, *Appl. Organomet. Chem.*, 2008, **22**, 583.

7 R. B. Kawthekar, W. T. Bi and G. J. Kim, *Bull. Korean Chem. Soc.*, 2008, **29**(2), 313.
8 B. P. Dwivedee, S. Ghosh, J. Bhaumik, L. Banoth and U. C. Banerjee, *RSC Adv.*, 2015, **5**, 15850.
9 K. Kitaori, Y. Takehira, Y. Furukawa, H. Yoshimoto and J. Otera, *Chem. Pharm. Bull.*, 1997, **45**(2), 412.
10 Y. Takehira, N. Saragai and K. Kitaori, *US Pat.* 5,130,482A, 1992.
11 Y. Takehira, N. Saragai and K. Kitaori, *US Pat.* 5,223,646A, 1993.
12 R. Bhushan and M. Aora, *Biomed. Chromatogr.*, 2003, **17**, 226.
13 R. Bhushan and S. Tanwar, *Biomed. Chromatogr.*, 2008, **22**, 1028.
14 S. V. Damle, P. N. Patil and M. M. Salunkhe, *Bioorg. Med. Chem.*, 2000, **8**, 2067.
15 O. Barbosa, C. Ortiz, R. Torres and R. Fernandez-Lafuente, *J. Mol. Catal. B: Enzym.*, 2011, **71**, 124.
16 S. V. Damle, P. N. Patil and M. M. Salunkhe, *Synth. Commun.*, 1999, **29**, 3855.
17 H. Zhang, H. Shao, A. Youmei and Z. Zhang, *Chromatographia*, 2008, **68**, 653.
18 A. Ghanem and H. Y. Aboul-Enein, *Tetrahedron: Asymmetry*, 2004, **15**, 3331.
19 J. Ekelund, A. V. Arkens, K. Bronnum-Hansen, K. Fich, L. Olsen and P. V. Petersen, *J. Chromatogr. A*, 1995, **708**, 253.
20 M. I. Santoro, H. S. Cho and E. R. Kedor-Hackmann, *Drug Dev. Ind. Pharm.*, 2000, **26**(10), 1107.
21 A. K. Singh, E. R. Kedor-Hackmann and M. I. R. M. Santoro, *J. AOAC Int.*, 2001, **84**(6), 1724.
22 H. Mikuldas, I. Cepanec, A. Sporec, M. Litvic and V. Vinkovic, *J. Sep. Sci.*, 2005, **28**, 251.
23 W. S. Long, A. H. Kamaruddin and S. Bhatia, *Chem. Eng. Sci.*, 2005, **60**, 4957.
24 W. S. Long, A. H. Kamaruddin and S. Bhatia, *J. Membr. Sci.*, 2005b, **247**, 185.
25 C. S. Chen, Y. Fujimoto, G. Girdaukas and C. J. Sih, *J. Am. Chem. Soc.*, 1982, **104**, 1294.
26 Z. W. Guo and C. J. Sih, *J. Am. Chem. Soc.*, 1989, **111**, 6836.
27 B. K. Pchelka, A. Loupy, J. Plenkiewics, A. Petit and L. Blanco, *Tetrahedron: Asymmetry*, 2001, **12**, 2109.
28 B. K. Pchelka, A. Loupy, J. Plenkiewicz and L. Blanco, *Tetrahedron: Asymmetry*, 2000, **11**, 2719.
29 T. W. Chiou, C. C. Chang, C. T. Lai and D. F. Tai, *Bioorg. Med. Chem. Lett.*, 1997, **7**(4), 433.
30 R. E. Gawley, *J. Org. Chem.*, 2006, **71**, 2411.
31 L. E. Janes and R. J. Kazlauskas, *J. Org. Chem.*, 1997, **62**, 4560.
32 M. J. Zmijewski, G. Sullivan, R. Persichetti and J. Lalonde, *Tetrahedron: Asymmetry*, 1997, **8**(8), 1153.
33 D. Rotticci, PhD thesis, Royal Institute of Technology, 2000.
34 M. D. Romero, L. Calvo, C. Alba and A. Daneshfar, *J. Biotechnol.*, 2007, **127**, 269.
35 A. L. Ong, A. H. Kamaruddin, S. Bhatia, W. S. Long, S. T. Lim and R. Kumari, *Enzyme Microb. Technol.*, 2006, **39**, 924.
36 O. Barbosa, C. Ariza, C. Ortiz and R. Torres, *New Biotechnol.*, 2010, **27**(6), 844.
37 W. Aehle, *Enzymes in Industry*, Wiley-VCH Verlag GmbH, Weinheim, 2004.
38 Sigma-Aldrich (M), *Pseudomonas fluorescens lipase and lipoprotein lipase*, Sigma-Aldrich, publishing web http://www.sigmaaldrich.com/catalog/Search?interface=All&term=pseudomonas fluorescens lipase, accessed 20-10-2012.
39 S. Stoker, *General Organic and Biological Chemistry*, Brooks/Cole, Belmont, 5th edn, 2010.
40 L. D. Nunno, C. Franchini, A. Scilimati, M. S. Sinicropi and P. Tortorella, *Tetrahedron: Asymmetry*, 2000, **11**, 1571.
41 M. Inagaki, J. Hiratake, A. Nishioka and J. Oda, *Bull. Inst. Chem. Res., Kyoto Univ.*, 1989, **67**(3), 132.
42 A. Ghanem and V. Schurig, *Tetrahedron: Asymmetry*, 2003, **14**, 57.
43 M. Kawasaki, M. Goto, S. Kawabata, T. Kodama and T. Kometani, *Tetrahedron Lett.*, 1999, **40**, 5223.
44 M. Kawasaki, M. Goto, S. Kawabata, T. Kodama and T. Kometani, *Tetrahedron: Asymmetry*, 2001, **12**, 585.
45 D. Zhou, X. Xu, H. Mu, C. E. Hoy and J. Adler-Nielsen, *J. Food Lipids*, 2000, **7**, 263.