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Effect of Reaction Parameters on the Lipase-Catalyzed Kinetic Resolution of (*RS*)-Metoprolol

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Racemic metoprolol is a selective ß1-blocker, which is used in cardiovascular disease treatment. It has been found that (*S*)-metoprolol has a higher affinity to bind the ß-adrenergic receptor compared to (*R*)-metoprolol. Moreover, the regulatory authorities' high market demand and guidelines have increased the preference for single enantiomer drugs. In this work, the lipase-catalyzed kinetic resolution of racemic metoprolol was performed to obtain the desired enantiomer. The type of lipase, acyl donor, and solvent were screened out. This was achieved by *Candida antarctica* B lipase-catalyzed transesterification of racemic metoprolol in hexane and vinyl acetate as the solvent and an acyl donor, which gave maximum conversion of (*S*)-metoprolol (X_S) of 52%, enantiomeric excess of substrate, (ee_s) of 92% and product (ee_P) of 90% with enantiomeric ratio (E) of 62. This method can be considered as green chemistry, which can be applied to produce other enantiopure beta-blockers.

Keywords: Beta-blocker, Chiral drug, Metoprolol, Kinetic resolution, Lipase

INTRODUCTION

Chirality is the property of a system or molecule which is not identical to its mirror image. In contrast, diverse biological, toxicological actions, pharmacological, and metabolism pathways in living beings are exhibited due to the chiral compounds, especially chiral drugs (Kim et al. 2016). The human body consists of abundant homochiral compounds that interact with racemic drugs in an alternating manner; they metabolize the enantiomers via separate paths to generate different pharmacological activities (Nguyen et al. 2006). Beta-blockers are categorized as chiral drugs, or as a heterogeneous group of drugs having dissimilar pharmacologic outlines for their beta-receptor blocking activity, membrane-stabilizing activity (MSA), water solubility or lipid or intrinsic sympathomimetic activity (ISA) (Chrysant et al. 2008).

Racemic metoprolol, which is also

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known as (+,-)1-(isopropyl- amino)-3-[p-(2-methoxyethyl)phenoxy]-2-propanol is a selective ß$_1$-blocker, which has an insignificant membrane-stabilizing agent and has not shown partial antagonist activity (Benfield 1986). Enantiomers have similar chemical and physical properties, but they vary in their optical behaviors, thereby resulting in different bioactivities (Mane, 2016). Therefore, chiral drugs have been enantiomerically and progressively produced in the pharmaceutical industry due to their safety and potential benefits (Gumustas et al. 2018).

Many beta-blockers are still sold as racemates since only pure enantiomers are imperative for dealing with cardiovascular diseases. Due to the demand for pure enantiomers of beta-blockers, most of them are produced via asymmetric synthesis consisting of a multistep reaction. Some of the original materials are very expensive or commercially unavailable.

Recently, for beta-blockers, the lipase-catalyzed transesterification or esterification of racemic propranolol, atenolol, carvedilol, and sotalol has been subjected to the screening of enzymes, solvents, and acyl donors, and then followed by the effects of substrate concentration, reaction temperature, reaction time and others. As a result, these parameters are taken into account; enantiomeric ratio (E), enantiomeric excess of substrates (ee$_s$), and conversion of (S)-enantiomer or (R)-enantiomer. From these findings, a very high ee$_S$, E, and conversion of the enantiomer were achieved for (R)-propanolol (i.e. 96%, 57, and 30%), (S)-atenolol (i.e. 100%, 12, and 100%) and (R)-sotalol (i.e. 84.01%, 11.51, and 49.37%), respectively (Barbosa et al. 2010; Agustian et al., 2016; Swetha et al. 2018).

The conversion can be designed from a study that investigating the effects of essential parameters, such as substrate molarity (Lersbamrungsuk and Srinophakun 2014). Therefore, this study explores the impact of several parameters, such as lipase types, acyl donors, organic solvents on lipase-catalyzed transesterification of racemic metoprolol, which is another group of beta-blocker. Effects of acyl agent concentrations on enantiomeric conversion, ee, and E using the concentration of (*S*)-metoprolol and (*R*)-metoprolol ester were also studied. It will be an eco-friendly practice using the lipases considering that racemate of beta-blockers are available in the market

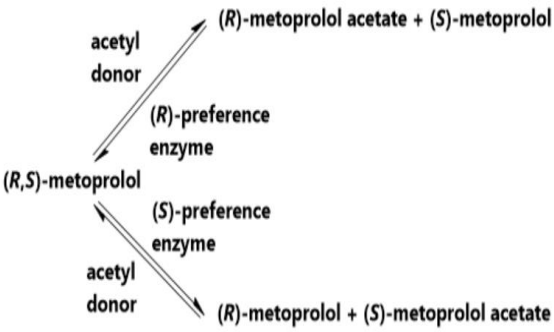

Fig. 1: Reaction scheme of lipase-catalyzed transesterification of (*RS*)-metoprolol.

METHODOLOGY

Chemicals and Reagents

Racemic or (*RS*)-metoprolol was obtained from Maharashtra, India, whereas (*R*)-metoprolol and (*S*)-metoprolol were obtained from Santa-Cruz. All chemicals were used without any pretreatment.

Sample Preparation

Racemic metoprolol (7.52 to 18.80 mM) was dissolved in the solvent and added with vinyl acetate (3.03 to 67 mM). Lipase (1000 to 2000 U) was added to the mixture and then filtered through a 0.45 µm filtering syringe before injecting it into high-performance liquid chromatography (HPLC). The reaction time was set at 18 hrs.

Enantioseparation of (*RS*)-metoprolol

The chromatogram analysis was carried out using an Agilent 1200 HPLC system, which was equipped with an Agilent 1200 high-performance auto-sampler, a quaternary pump, a column oven Agilent 1200, an Agilent UV-VIS DAD detector, and a chemstation data processing system. The mobile phase consisted of hexane: isopropanol: diethylamine (80:20:0.1) v/v/v with the wavelength detection at 274 nm, a flow rate of 0.8 ml/min, and injection volume of 20 µl. The column installed in the system was the Diacel Chiralcel OD column (150 mm, 4.6 mm, 0.5 µm).

Calculations of conversion and enantiomeric excess

Conversions in lipase-catalyzed transesterification are calculated using Eqs. (1) to (3) (Chen et al., 1982; Long et al., 2005a; Long et al., 2005b; Agustian et al., 2016).

$$X\ (\%) = C_o - C_t / C_o \times 100\% \quad (1)$$

$$X_s\ (\%) = C_{so} - C_{st} / C_{so} \times 100\% \quad (2)$$

$$X_R\ (\%) = C_{RO} - C_{RT} / C_{RO} \times 100\% \quad (3)$$

where X is the total conversion (%), X_S is the conversion of (*S*)-metoprolol (%), X_R is the conversion of (*R*)-metoprolol (%), C_0 is the initial concentration of racemic metoprolol (mM), C_t is the concentration of racemic metoprolol (mM) at reaction time t, C_{S0} is the initial concentration of (*S*)-metoprolol, C_{St} is the concentration of (*S*)-metoprolol at reaction time t, C_{R0} is the initial concentration of (R)-metoprolol, and C_{Rt} is the concentration of (*R*)-metoprolol at reaction time t. The enantiomeric excess of the substrate (ee_s), enantiomeric excess of the product (ee_p), and enantiomeric ratio (E) are calculated as shown in Eqs. (4) to (6), respectively (Chen et al. 1982; Guo et al. 1989; Pchelka et al. 2000; Pchelka et al. 2001; Agustian et al. 2016).

$$ee_s\ (\%) = C_{Rt} - C_{st} / C_{Rt} + C_{st} \times 100\% \quad (4)$$

$$ee_p\ (\%) = ee_s\ (1 - X) / X \times 100\% \quad (5)$$

$$E = \ln [(1- ee_s)\ (ee_p / ee_s + ee_p)] / \ln [(1+ ee_s)\ (ee_p / ee_s + ee_p)] \times 100\% \quad (6)$$

RESULTS AND DISCUSSION

Effect of Lipase Type

Four types of lipases were examined with an acyl donor (vinyl acetate) was used on the lipase-catalyzed transesterification of (*RS*)-metoprolol in acetonitrile. A comparison study of four lipases at 1000 U showed that the *Candida antarctica* lipase B, (CAL-B) yielded higher E (7.14), ee_s (62%), and ee_P (59%) as well as better X_s (52), compared to other lipases as shown in Fig. 2. The analysis was continued with the same amount of lipases (2mg/ml). Results showed that among all lipases, the CAL-B produced the highest X_S (66%) (Fig. 3). A previous study conducted by Banoth

et al. (2015) reported that *Candida antarctica* cross-linked enzyme aggregate (CAL CLEA) yielded the conversion of the desired compound (C), ee_S, ee_P, and E values at 48.7%, 89.3%, 94.1% and 99.6, which were higher than those produced using *Candida antarctica* lipase, CAL by 44%, 35%, 44%, and 3.5. From Fig. 3, the values of X_S, ee_S, ee_P, and E in this preliminary screening were 66%, 56%, 54%, and 5 using CAL-B.

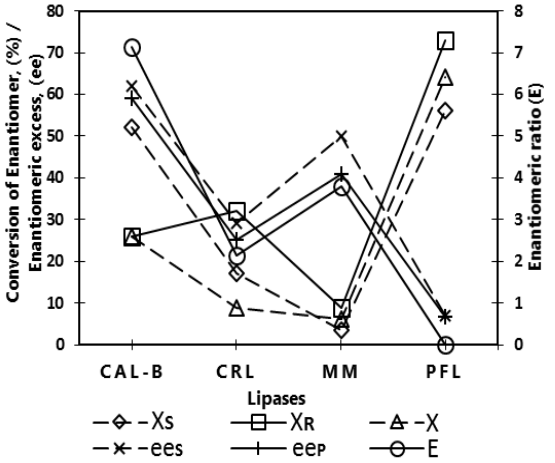

Fig. 2: Lipases (1000 U) in lipase-catalyzed transesterification of racemic metoprolol Conditions: (*RS*)-metoprolol (18.8 mM) was treated with vinyl acetate (67.68 mM) at 40^0C, 1000 U of lipase in 18 hours. CAL-B: *Candida antarctica* lipase B, CRL: *Candida rugosa* lipase, MM: *Mucor miehei*, PFL: *Pseudomonas fluorescens* lipase.

A screening process was performed by Agustian et al. (2016) on *Lipoprotein Burkholderia* sp., *Lipoprotein Pseudomonas* sp., *Pseudomonas fluorescence* lipase Amano, PFL Amano, PFL Fluka and PFL Sigma in the lipase-catalyzed transesterification of (*RS*)-atenolol. It was found that the recommended lipase to be used in the reaction was PFL Sigma with the values of 80.80% $X_{(S)\text{-atenolol}}$, 25.17% $X_{(R)\text{-atenolol}}$, 58% ee_S and 28 E. Even though *Lipoprotein Burkholderia* sp. obtained 100% $X_{(S)\text{-atenolol}}$, the E value was 0. It can be inferred that the types of lipases used in the lipase-catalyzed reaction of racemate influence the values of X, ee, and E. The lipase order shown in Fig. 3 consisted of the X_S values from the lowest to the highest, namely MM (29%), PFL (38%), CRL (40%), and CAL-B (66%). All the E values of the lipases were 2 except for CAL-B (5). In Fig. 3, the values of X_s (66%), ee_s (56%), ee_p (54%), and E (5) were the highest achieved by CAL-B. The comparison was carried out with different types of lipase (CAL-B, MM, PFL, and CRL), and varying amounts of lipase at 1000 U (Fig. 2) and 2 mg/ml (Fig. 3). The values can be improved afterward after examining the effect of acyl donors, solvents, and vinyl acetate concentration.

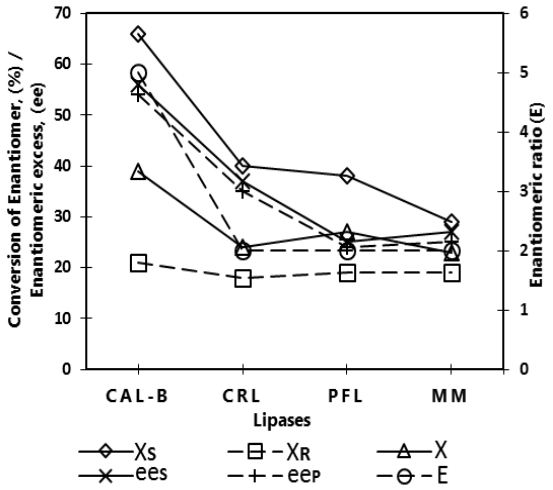

Fig. 3: Lipases (mg/ml) in lipase-catalyzed transesterification of racemic metoprolol. Conditions: (*RS*)-metoprolol (7.52 mM) was treated with vinyl acetate (3.03 mM) at 40^0C, 2 mg/ml of lipase in 18 hours.

The overall E values in CAL-B were 1.21 to 7.14, where CAL-B obtained the

highest value of E (7.14) than other lipases (2 to 3.8). An E value equal to 1 means there is no enantioselectivity. Enantioselectivity in the reaction involves lipase-mediated arises at the deacylation stage with the nucleophilic alcohol attack, thereby resulting in the creation of the first complex of the acyl-enzymes (Paravidino and Hanefeld, 2011).

In the literature, lipase selection depends on the high conversion of the desired compound and enantioselectivity. Thus, CAL-B was selected in this study, due to its highest E and X_s values among all lipases.

Effect of Acyl Donors

The *Candida antarctica* B lipase-catalyzed transesterification of (*RS*)-metoprolol was screened with different acyl donors in acetonitrile (Fig. 4). The selection of the acyl donor is vital for becoming a successful reaction (Dwivedee et al. 2015). Out of the screened three acyl donors, vinyl acetate produced the highest results of ee_S (62), ee_P (59), and E (7.1) compared to the other two acyl donors. In terms of the X_S, among the three acyl donors, the highest was isopropanol (IPA) (58%) followed by ethyl acetate (EA) (53%) and vinyl acetate (VA) (52%). However, the highest E value was VA (7.1), followed by IPA (1.9) and EA (1.7). This result indicated that although IPA and EA have slightly higher X_S value than that of VA, the values of E produced were shallow.

Moreover, although the X_S value was higher for IPA and EA, these acyl donors were not suitable to be used in this reaction as they showed very low ee_S, ee_P, and E. In this study, the X_S value produced by VA was indicated as good due to the highest ee and E values. Many researchers have worked on acyl donors screening in esterification or transesterification processes involving enzymatic reaction, as the solvents consist of an essential part in achieving the reaction. The results from previous screenings of the acyl donors such as acetic anhydride, ethyl acetate, benzyl acetate, vinyl acetate, isopropenyl acetate, and phenylethyl acetate in lipase-catalyzed transesterification of racemate have proven that vinyl acetate produces the highest value of X, ee and E (Banoth et al., 2015). It has been reported that the IPA and EA were used in hydrophobic solvents for the resolutions of (S)- or (R)-enantiomers via kinetic reaction. However, for VA, it is vastly applied due to its effectiveness in hydrophilic or hydrophobic solvents.

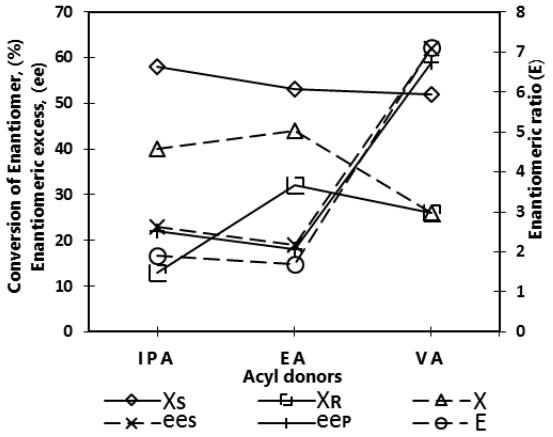

Fig. 4: Effect of acyl donors in lipase-catalyzed transesterification of racemic metoprolol. Conditions: (*RS*)-metoprolol (18.8mM) was added with vinyl acetate (67.68 mM) at 40^0C, 1000 U of *Candida antarctica* lipase B in 18 hours. [IPA: Isopropanol, EA: Ethyl acetate, VA: Vinyl acetate]

Vinyl acetate superiority as an acyl donor in enzymatic reactions is known,

with additional potential to tautomerize in-situ vinyl alcohol to acetaldehyde. This is achieved by its refusal to behave as a competitive substrate, thereby enabling the equilibrium to be shifted to the product (Banoth et al., 2012; 2015). Therefore, vinyl acetate was selected to study the effect of solvents in lipase-catalyzed transesterifi-cation of racemic metoprolol.

Effect of Solvents

Five types of solvents (i.e., diethyl ether, hexane, toluene, dimethyl-formamide, and acetonitrile) were studied with different values of log P in the lipase-catalyzed transesterification of (*RS*)-metoprolol (Fig. 5). In this study, hexane with the log P of 3.5 had greater X_S (73%), ee_S (82%), ee_P (80%), and E (22) compared to other solvents with log P of -0.82 to 2.5. These solvents yielded values in the range of 3% to 62% (X_S), 17% to 65% (ee_S), 16% to 60% (ee_P), and 1 to 7 (E). It is reported in the literature that the biocatalytic rates of the reactions are lower with polar solvents (log P<2) compared to apolar solvents (log P>4) (Banoth et al. 2014; 2015). Therefore, the increasing order of X_S with respect to its solvent was diethyl ether (3%) < toluene (23%) < acetonitrile (56%) < dimethylformamide (62%) < hexane (73%). It suggests that hexane with log P 3.5 is a suitable solvent in lipase-catalyzed transesterification of (*RS*)-metoprolol to obtain high X_S. The previous study in the lipase-catalyzed reaction of racemate in polar and apolar solvents showed that the lowest conversion of the desired compound (C) to the highest was 49.6% (1-4, dioxane with log P -1.1) and 87% (isooctane with log P 4.5) (Banoth et al. 2015).

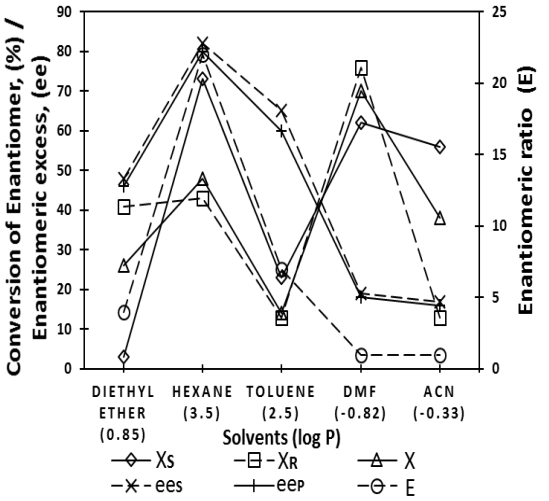

Fig. 5: Effect of solvents in lipase-catalyzed transesterification of racemic metoprolol. Conditions: (*RS*)-metoprolol (18.8mM) was treated with vinyl acetate (67.68mM) at 40^0C, 1000 U of *Candida antarctica* lipase B in 18 hours.

In comparison, this study yielded lower X_S of 63% in dimethylformamide with log P of -0.82 and higher X_S of 73% in hexane with a log P of 3.5. Hexane produced the highest X_S among other solvents (diethyl ether, toluene, acetonitrile and dimethylformamide). The behavior of the *Candida antarctica* lipase B conversion in hexane is related to its capacity in transesterification reactions. Another study on lipase-catalyzed betulinic acid and diethylamine in the mixture of chloroform to hexane (9:1) v/v has yielded 64.9% of betulinic acid amide (Yusof et al., 2016). In this study, the enantiomeric purity (ee_S) was affected by the value of solvent log P. At log P<2 (i.e., -0.82, -0.33, and 0.85), the ee_S values were 17 to 48%, whereas at log P>2 (i.e., 2.5 and 3.5), the ee_S values were 65% and

82%. These results showed that the ee_S values increased along with the increase of solvent log P.

This pattern was similar to the ee_P with slightly lower values obtained. From the literature, studies on the lipase-catalyzed reaction of racemate in organic solvents with various values of log P have been conducted. It was found that toluene with 2.5 (log P) produced the best ee_S (97.4%) and ee_P (95.5%) results (Soni et al., 2017). In comparison with this study, ee_S and ee_P were 82% and 80% in hexane. The previous study used *Pseudomonas fluorescens* lipase and toluene as solvent, whereas this study used *Candida antarctica* lipase B and hexane as solvent. The E values remained as 1 at -0.85 and -0.33 (log P).

Meanwhile, at 0.85, 2.5, and 3.5 (log P), the E values increased to 4, 7, and 22, respectively. Organic solvents are proven by chemists to have high lipase stability whereby an efficient conformation with high stability is associated with a greater likelihood of catalysis. It then reflects higher E and ee as the organic system achieves a good activity in terms of biocatalysis system due to the unique interfacial activation of lipases (Kumar et al., 2016). Moreover, a few studies conducted revealed that solvent with the highest E value is recommended for its best performance in the analysis. If the value is more than 100, it indicates excellent enantioselectivity. It was further reported that some variations influence the E value in the solvent physicochemical properties. In reactions involving esterification, lipase's enantioselectivity in hydrophobic solvents, particularly CAL-B, has been reported to increase (Barbosa et al. 2010). Several findings have also shown that the E value is high when the lipases are used in non-polar solvents, such as toluene or hexane (Pchelka et al. 2000; Wielechowska and Plenkiewicz 2005). Therefore, hexane was chosen as a solvent for the lipase-catalyzed transesterification of (*RS*)-metoprolol using CAL-B to study the effects of acyl agent concentration in obtaining the maximum E, ee_S, and ee_P.

Effect of Acyl Agent Concentration

The resolution was carried out using three concentrations of vinyl acetate (i.e., 38, 55, and 67 mM) with 1 mg/ml (2000 U) of *Candida antarctica* lipase B in hexane. The study was conducted to understand the effect of vinyl acetate concentrations on X, ee, and E values. The maximum E (62), ee_p (90%), and ee_s (92%) with 52% of X_S were achieved at 67 mM of vinyl acetate (Fig. 6).

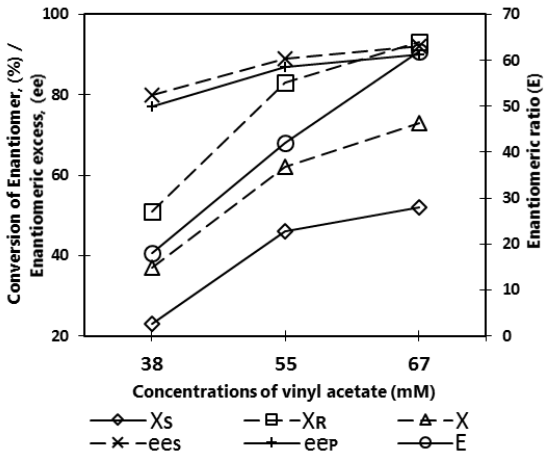

Fig. 6: Effect of vinyl acetate concentrations in lipase-catalyzed transesterification of racemic metoprolol. Conditions: (*RS*)-metoprolol (18.8 mM) was treated with acyl donor (38 to 67 mM) at 40^0C, 1mg of *Candida antarctica* lipase B (2000 U) in 18 hours.

In a previous study on lipase-catalyzed transesterification of (*RS*)-atenolol, using

of vinyl acetate concentrations from the ratio of 1.2 to 6.0 had increased the $X_{(S)\text{-atenolol}}$ as vinyl acetate concentration is increased with the value produced above 40 to 50% (Agustian et al. 2016). It is in agreement with this study as the X_S value increased from 23 to 52% in the range of 38 to 67 mM of vinyl acetate concentration.

The highest X_S (52%) was produced from increasing the concentration of vinyl acetate in this study. A similar study on the effect of vinyl acetate concentration in lipase-catalyzed esterification of propanolol showed an equal performance when the X value increased with the increase in vinyl acetate concentrations. According to Barbosa et al. (2010) and Van et al. (2004), a production of good X in the lipase-catalyzed reaction of racemate suggests there is no inhibition on the lipase by the vinyl acetate within the range of concentrations studied.

At 38 mM, 55 mM and 67 mM (VA), the ee_S significantly increased with 80%, 89%, and 92%. Lower ee_S was recorded at lower vinyl acetate concentration. A study carried out by Agustian et al. (2016) reported ee_S values of 40% and 41% when the concentrations of vinyl acetate increased from 90.24 mM (1:4.8) to 112.8 mM (1:6.0) at fix concentration of atenolol (18.8 mM) using *Pseudomonas fluorescence* lipase. The trend for ee_S in this study was similar to ee_P. The ee_S of 92% and ee_P of 90% obtained were the highest results recorded during the observation.

The E (62) value in this study was found to be moderate (i.e., 20-100) (Swetha et al., 2018) when the reaction was catalyzed by free *Candida antarctica* lipase B and the value was slightly higher than the E value obtained by Barbosa et al. (2010), which recorded a value of 57 in *Candida antarctica* B lipase-catalyzed esterification of (*RS*)-propanolol.

The researchers have thus concluded the enol esters (i.e., commonly vinyl acetate) have higher efficiency in organic solvents in esterification due to their high reactivity in serine residues from the catalytic site of lipases (Banoth et al. 2015; Barbosa et al. 2010; Dwivedee et al. 2015; Agustian et al. 2016). Moreover, these enol esters provide irreversible lipase-catalyzed esterification or transesterification of racemate reaction caused by keto-enol tautomerization due to the vinyl alcohol formed from the lipase serine acetylation (Barbosa et al. 2010; Dwivedee et al. 2015; Agustian et al. 2016).

CONCLUSIONS

Lipase-catalyzed transesterification of racemic metoprolol was achieved in the range of X_S and ee_s, respectively, 3% to 73% and 17% to 92%. *Candida antarctica* lipase B has more favorable energetic conformation within the parameters, thereby bringing up the possibility for catalysis to take place and thus generating a moderate selectivity (E = 62). X_S and ee_S for (*S*)-metoprolol were obtained from the *Candida antarctica* B lipase-catalyzed transesterification of (*RS*)-metoprolol in a reaction medium applied with selected parameters (i.e., type of lipase, acyl donor, solvent, and vinyl acetate concentration). This is an example of green chemistry with a simple and direct route to produce enantiopure metoprolol. This application can thus be applied in producing other enantiopure beta-blockers.

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