Indian J. Applied & Pure Bio. Vol. 31(2), 219-229 (2016).

Process for preparation of (R) and (S)-1-Phenylethanol through enzymatic resolution by *Serratia marcescens* and the characterization of the enantioselective lipase

Madhu Smita and Rohit Sharma*

Department of Microbial Biotechnology,
Panjab University, Chandigarh, India
***Corresponding author- E-mail:** rohit28@pu.ac.in **Phone No.** 91-172-2536161

Abstract

Bioconversion of valuable compounds by enzymes has always been one of the main areas of intense scientific research over the years. In the present study isolation of lipase producing bacteria from edible oil contaminated site, industrial waste soil and water was undertaken. Initial Screening of bacterial isolates was done with *p*-NPP (*p*-Nitrophenyl Palmitate) as lipase substrate. Out of 95 bacterial isolates, 15 isolates were selected on the basis of their lipolytic activity with *p*-NPP. The final selection of the best performing bacterium was done on the basis of its ability to grow on different racemic mixtures and compounds that have a high commercial value (1-Phenylethanol, 2-Pentanol, 2- Octanol, 2-Heptanol). Out of the 15 isolates, RMS 43 was found to be the best to grow on these compounds. Subsequent optimization of different parameters for RMS 43 was carried out to maximize the lipase production. The optimization was done w.r.t. temperature, pH and then the stability of RMS 43 at optimal temperature, pH, with different organic solvents, effect of different modifiers (enhancers, metal ions, surfactants) has been found out. In this study biotransformation of 1-Phenylethanol with the non-commercial and wild type lipase in non-aqueous environment has been investigated. A systematic optimization of the biotransformation reaction parameters such as molar ratio of alcohol:acyl donor, temperature and concentration of water was also performed.

High global demand for lipases makes them the third largest group of commercialized and industrially important enzymes based on the total sales volume only next to proteases and carbohydrases[11]. Lipases (triacylglycerol acylhydrolases, E.C.3.1.1.3) can catalyze various reactions such as esterification, alcoholysis or aminolysis[12]. The application of lipases as catalysts in organic synthesis and as an alternative to harmful chemical catalysts has made them one of the most sought after enzymes in the pharmaceutical, cosmetics,

textile, leather, paper industries as well as in various medical applications such as drugs or biosensors[2]. Among various properties, the high regio-specificity and enantioselectivity of lipases is of special interest to pharma sector for their possible use in chiral switching of drugs. Today in a lot of reactions at one or more than one step, whole cells or isolated lipases are employed on an industrial scale for the chiral resolution *e.g*. In a very recent process established by BASF™ for the kinetic resolution of chiral compound using lipases. A secondary alcohol 1-Phenylethanol, especially (R)-1-Phenylethanol, is a basic chiral building block and synthetic intermediate in various pharmaceutical,fine chemical and agrochemical industries. As 1-Phenylethanol exists as a racemic mixture in its natural form, its resolution into either enantiopure form is highly desirable. The kinetic resolution (KR) of rac-1-phenylethanol by various commercially available enzymes has been attempted by many researchers[14,16,17]. There is a need of microbial enzymes that can display optimal activity at a wide range of temperature, pH and solvents. Some critical issues still to be addressed are substrate solubility, yield or enantioselectivity[7]. Consequently, the demand to identify and characterize new lipases that may have applications in enantioselective biotransformations is steadily increasing. This paper deals with the isolation, characterization of bacteria and extremophilic enzyme produced by it and application of this lipase produced by *Serratia sp.* in the biotransformation of industrial important compounds especially 1-Phenylethanol. The biotransformation converted racemic1-Phenylethanol into 81.78% S-enantiomer.

All the chemicals used were of analytical grade and procured from Merck Specialties Pvt. Ltd, Mumbai; Sigma-Aldrich Pvt. Ltd., Bangalore; Thermo Fischer-Scientific Pvt. Ltd., Mumbai; HiMedia Laboratories Pvt. Ltd., Mumbai.

Sample Collection :

Soil and water samples were collected from various places of industrial waste (edible oil contaminated) and kitchen waste of sweet shops in sterile polyethene bags and bottles.

Isolation of Microbes Producing Lipase :

Soil and water samples from industrial waste were inoculated into broth and streaked on the media containing Peptone-0.5g, Yeast extract- 0.2g, Na_2HPO_4-0.1g, NaCl-0.1g, Oil emulsion-0.25% (v/v) of different oils. Isolation of bacteria was also done on MSM (Minimal Salt Media) containing NH_4Cl-0.01%, Na_2HPO_4-0.2%, KH_2PO_4-0.2%, $MgCl_2.2H_2O$-0.02%. For the isolation of bacteria, different oils (cottonseed oil, mustard oil, olive oil and a combination of both cottonseed and palm oil) were used. Streaking was done from the enriched broth every day to isolate the different bacteria prominent in the media on different days (upto 15 days) at 30°C and 180 rpm. Pure cultures were isolated from it after repetitive streaking of the isolated colonies. Gram staining was performed along with the observation of colony morphology and cell morphology. Tributyrin plate assay was also performed to determine qualitative lipase activity[3]. Isolated cultures were maintained at 4°C as for routine day to day usage. While for long term storage, 20% (v/v) Glycerol stock of the cultures were prepared and kept at -80°C.

Screening of Lipase Producing Microbial Isolate Through Submerged Fermentation:

Nutrient Broth with respective oil (0.25%) (on which the bacteria were isolated) and Calcium Chloride dihydrate (5mM) was used for the shake-flask fermentation of the lipase. The media was inoculated with starter culture of the bacterial isolate (2% v/v) and incubated at 30 ^{o}C with shaking at 180 rpm. Nutrient Broth with bacterial culture at an O.D. of 0.6 (equivalent to 10^4 cells/ml) at 600nm was used for the inoculation into production media.

Lipase Assay :

Lipase activity was measured by calorimetric assay method developed by Winkler and Stuckmann[15] and *p*-Nitrophenyl Palmitate (C-16) was used as a substrate. 200µl Tris-HCl Buffer (pH-7.2, 50mM) was used to dissolve the pellet. The reaction conditions of assay were 30^{o}C for 10 mins and the absorbance was measured at 410 nm. Standard was prepared with the end product *p*-Nitrophenol of different concentration from 2 µg/ml to 20 µg/ml. One unit of enzyme activity (U) is amount of mg of pNP released per min.

Growth of the Bacterial Isolates on Different Concentration of Industrial Important Compounds:

To check the compatibility of the lipase positive cultures with different racemic compounds (1-Phenylethanol, 2-Pentanol, 2-Octanol, 2-Heptanol at their different concentrations (5, 10, 25, 50 mM), different cultures were streaked and observed after 24 hrs to 72hrs for their growth. This was done to check their application to enantiopure the compounds.

Sequencing of 16S rRNA and phylogenetic analysis :

Sequencing of 16S rDNA gene of the isolate RMS43 was performed using Big Dye Terminator version 3.1 cycle sequencing kit and the ABI 3730xl genetic analyzer. The 16S rRNA gene sequence of the isolate was aligned and compared with other genes in the Gen Bank using the NCBI BLASTn program. The sequences from different bacterial isolates showing more than 99% identity were used to construct phylogenetic tree using software suite MEGA 4.1.

Characterization :

Following parameters were optimized for the enhanced lipase activity of *Serratia marcescens* RMS43 using one factor at a time approach (OFAT).

Optimum Temperature and pH and Stability:

Optimum temperature for enzyme activity was obtained by incubating the enzyme from 30^{o}C to 70^{o}C with a gap of 5^{o}C. Activity was checked after incubating the enzyme (20mg crude intracellular enzyme) (crude enzyme is the whole cell enzyme harvested from the shake flask production of the bacteria) at the optimized temperature in 2 ml of 50mM Tris-HCl buffer to know the temperature stability upto 8 hrs. Similarly, optimum pH was noted down from pH 6 to 9 with a gap of 0.6. Hourly samples were withdrawn to note down the pH stability at optimum pH upto 8 hrs.

Effect of Different Additives:

Mannitol, Sucrose, Glycerol, Calcium Chloride dihydrate(5mM conc.) were used as additives to know their effect to enhance the activity of the enzyme and their relative activity was find out w.r.t the control having buffer only.

Effect of Organic Solvent on Lipase Stability:

The organic solvent stability of the enzyme plays an important role to do non-aqueous enzymology with it. The organic solvents differ from each other in their hydrophobicity or logP value. The changes in activity and stability of the lipases with time on incubation in an organic medium could be correlated with the alteration of the lipases native structure and function[7]. Crude intracellular enzyme (20 mg) was suspended in five different solvents (Methanol, hexane, toluene, petroleum ether and ethyl acetate): 50mM Tris-HCl buffer (1:1 v/v). The reaction samples were collected at an interval of 1hr upto 8 hrs of incubation followed by the final sample collection at 24th hr.

Effect of Metal Ions and Surfactants:

Ca^{2+}, Co^{2+}, Mg^{2+}, Fe^{2+}, K^{+}, NH_4^{+}, Zn^{2+}, Na^{+} and Cu^{2+} were added in their chloride form at 5mM concentration at 30 ^{o}C and 180 rpm. Similarly, different surfactants SDS (anionic), CTAB (cationic), Tween 20, Tween 40, Tween 80 and Triton X-100 (neutral)), Tween 20 (5mM) were added at 5 mM concentration during the time course study of the their effect on *S. marcescens* RMS43 lipase.

Biotransformation reaction: *Lipase-Mediated Transesterification of (R,S)-1-Phenylethanol:*

Crude intracellular lipase in the pellet (RMS 43) was used for the resolution of (R,S)-1-Phenylethanol. Pellet was dissolved in hexane. The substrate (R,S)-1-PE is shown to have good solubility in hexane. The reaction (2 mL) was set up with 25mM substrate, 50 mg harvested cells (enzyme) and 100mM vinyl acetate (acyl donor), Tris-HCl buffer (100µl, pH-7.8). The reaction contents were kept in incubator shaker (180 rpm, 30 ^{o}C). The samples (100 µL) were drawn out from the organic layer of the reaction mixture upto 8 hours at 1 hr interval and then at 24 hour followed by drying on rotavapor. The samples were-dissolved in hexane (100 µL), filtered through 0.22 µm and analyzed using HPLC system (Waters™ 1525) for the formation of product.

Optimization of Transesterification Reaction:

The effect of different concentration of 1-PE, Vinyl acetate, water concentration and temperature on the transesterification of (R,S)-1-PE by RMS43 lipase were evaluated following standard methodology. Different concentration of 1-PE (25mM, 50mM, 100mM and 200mM) and vinyl acetate (100mM, 200mM, 400mM and 800mM) were used for the resolution of 1-Phenylethanol by RMS 43 lipase[6]. Hexane was chosen as solvent on the basis of the more residual activity of the enzyme in it. The substrate: acyl donor ratios tried for the biotransformation reaction were 1:2, 1:4, 1:6 and 1:8. The initial concentration of 1-PE was taken as 25mM for each ratio. Similarly, different concentrations of water (0%, 1.25%, 2.5%, 3.75% and 5% (v/v) (also

act as control)) as 50mM Tris-HCl buffer (pH-7.8) were used with different ratio of substrate and vinyl acetate. In order to determine the optimum temperature for the enantioselectivity of (R,S)-1-PE by the lipase enzyme, a range of temperature (25-55 ^{o}C) was used with 1:4 and 1:6 concentration of 1-PE:VA.

HPLC Analysis :

Analysis of the biotransformed product was done with Normal phase chromatography. The enantiomeric excess was determined on Chiraselect OM (250 × 4.60 mm) column (Merck, Germany) using isocratic elution with n-hexane/2-propanol 90:10 (v/v), flow rate 0.5 ml/min and UV-detection at 215 nm with PDA detector.

Among the 95 isolates evaluated, RMS 43 grew on the highest molarity of the substrates and had a better growth on each compound tested as compared to other bacterial cultures. RMS 43 also show good growth on 10mM of 2-Phenylethanol which has antibiotic properties (Table 1). Its growth is also not significantly retarded in the presence of upto 5mM of MPGM (trans-3-(4'-methoxyphenyl) glycidic acid methyl ester). The isolate RMS 43 was found to be non-motile, Gram negative, aerobic, rod shaped organism. It forms small, bright pinkish red color, circular, moist, opaque, raised colonies with entire margins on Nutrient agar medium.

Phylogenetic analysis of 16S rRNA sequence of isolate RMS 43:

The phylogenetic analysis of the 16S rRNA sequence of RMS 43 shows >99% resemblance with the 16S rRNA sequence of *Serratia marcescens* (Fig.1) indicating it to be apparently closely related with the type strain.

Effect of Temperature and pH :

The evaluation of the enzyme activity at different temperatures shows that the temperature optimum for the enzyme activity is 55 ^{o}C, *i.e.* 2287.5 U/ml (Fig.2). The enzyme displayed higher activity in the temperature range 50-70 ^{o}C. The optimal pH for the activity of RMS 43 lipase was found to be pH 7.8 (Fig. 3).

Thermal and pH Stability :

The determination of the half life of the enzyme showed the enzyme to be quite stable at 55^{o}C and with a half life of about 3 hrs, while at 30^{o}C the half life is about 1 hr (Fig. 4). The stability of enzyme at higher temperature (activity optima) and different pH was determined by incubating the enzyme at pH 7.8 and 7.2 at 55 ^{o}C for different time periods. The residual enzyme activity estimation indicated that the lipase has better stability at pH 7.8 as compared to nearly neutral pH 7.2 (Fig. 5). The enzyme at pH 7.8 retained 85%, 83% and 76% of activity after 1, 2 and 3 hrs of incubation.

Activity Enhancers/ Stabilizers :

The effect of different compounds (Mannitol, Sucrose, Glycerol, and Calcium Chloride dihydrate) on RMS 43 lipase activity was evaluated (Fig. 6) as described. The Calcium Chloride dihydrate and mannitol were found to enhance the lipase activity while Glycerol and Sucrose did not appear to do so.

Effect of Metal Ions and Surfactants :

The effect of different metal ions and surfactants on the activity of lipase was also evaluated. Calcium was found to be the best metal ion to enhance the activity (Fig. 7). Potassium and ammonium ions were the other ions which had positive effect on its activity. Heavy metal ions like Cu^{2+}, Zn^{2+} appeared to have somewhat detrimental effect on the lipase activity. Among the surfactants tested Tween-20 appeared to enhance the activity maximally in comparison to other detergents (Fig. 8).

Solvent Stability :

To ascertain the stability of the lipase, we examined the residual lipase activity after the incubation of lipase in the presence of different solvents. The lipase enzyme retained 78%, 59.65%, 59.65%, 37.05% and 11.15% of its initial activity after incubation for 1 hr in hexane, toluene, control, petroleum ether and ethyl acetate, respectively (Fig. 9).

Biotransformation Reaction of (R, S)-1-Phenylethanol By lipase RMS43 in a Non-Aqueous System:

The resolution of 1-phenylethanol was done by lipase RMS43 pellet in hexane and toluene using vinyl-acetate as an acyl donor (Fig. 10). Acyl donor/alcohol (nucleophile) concentration and their molar ratio are of utmost importance in enzymatic transesterification[4]. Various concentrations of substrates, *i.e.* 25mM, 50mM, 100mM and 200mM were used. 1-PE at25mM concentration was found to be the best. By raising the acyl donor molar concentration with respect to the alcohol concentration, a higher yield of the selective enantiopure compound was obtained.

Microbial lipases have attracted immense attention owing to their biotechnological application in various industries as diverse as fat and oil processing, cosmetics, food processing, chemical synthesis, pharmaceuticals and detergent industry[9]. In our hunt for such microbial lipases that may have biotechnological application, we had screened various suitable habitats for lipase producers and isolated 15 lipase producing organisms based upon their activity towards *p*-NPP. The main rationale of this research was to isolate a bacterium to have a potential to bring about the enantioselective bioconversion of various industrial important compounds, to be used in green chemistry. *Serratia marsecens* RMS 43 is a pinkish-red microorganism. For the commercial value of any enzyme its stability plays a very important role, as the majority of the industrial processes are carried out in relatively harsh conditions. The stability of an enzyme can be in terms of pH, temperature or stability in the organic solvents. The temperature optimum of the enzyme of RMS 43 is 55 ^{o}C. The reported lipases produced by the members of *Serratia* genus belong to family I.3 lipases. The lipases of this family are generally produced by mesophillic or psychrotrophic Gram-negative bacteria. Interestingly, all of them have optimum temperatures for activity in the mesophilic or thermophilic range (35–55 °C), which is considerably higher than the optimal growth temperatures of their source organisms. The optimum pH range for this lipase family is a mildly alkaline pH (pH 7.5–8.5)[1]. The enzyme produced by our isolate *Serratia marsecens* RMS 43 is found to be active in alkaline range

Table

Table 1- Growth of selected bacterial isolates on MSM medium containing different compounds (1-PE, 2-Pentanol, 2-Heptanol , 2- Octanol and 2- PE. Growth of only RMS 43 was checked on MPGM) (++++ = luxuriant growth; +++ = very good growth; ++ = good growth; + = quite growth; - =no growth)

Substrate & conc.	Culture	RMS 19	RMS 20	RMS 43	RMS 75	RMS 77	RMS 78	RMS 84
1-PE (mM)	5	-	-	++++	++	+++	+++	-
	10	-	-	++++	++	++	++	-
	25	-	-	-	-	-	+	-
2-P (mM)	5	++	++	++++	++	++	++	+
	10	++	++	+++	++	++	++	-
	25	-	-	++	+	+	+	-
	50	-	-	++	-	+	+	-
2-H (mM)	5	-	++	++++	-	++	++	+
	10	-	+	+++	-	++	+	+
	25	-	-	++	-	+	-	-
	50	-	-	+	-	+	-	-
2-O (mM)	5	-	++	+++	-	-	++	+
	10	-	-	-	-	-	-	+
	25	-	-	++	-	-	+	+
	50	-	-	-	-	-	+	+
2-PE (mM)	5	-	+	+++	++	++	++	++
	10	-	-	++	-	++	++	+

Fig. 1

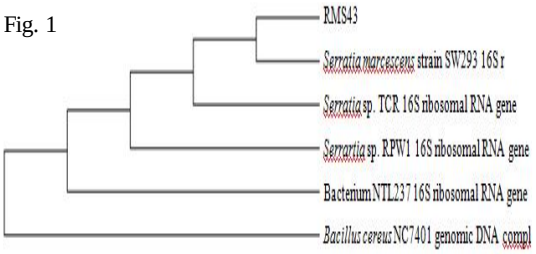

Fig.1-The phylogenetic tree of isolate RMS 43. The 16S rRNA sequence of RMS 43 isolate was used to construct the phylogenetic tree using software MEGA4.1. The isolate displays >99% identity to *Serratia sp*. sequences.

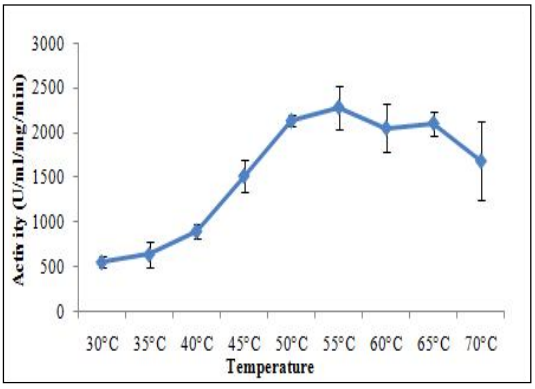

Fig. 2-Temperature optima of RMS43 lipase from 30 ºC to 70 ºC.

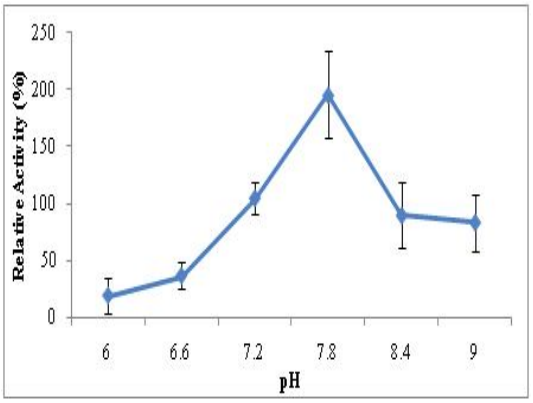

Fig. 3-pH optima of RMS 43 lipase from pH 6.0 to pH 9.0

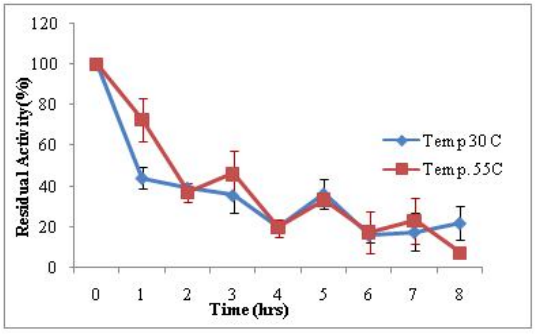

Fig. 4-Temperature stability of RMS 43 at 55 ºC and 30 ºC (control)

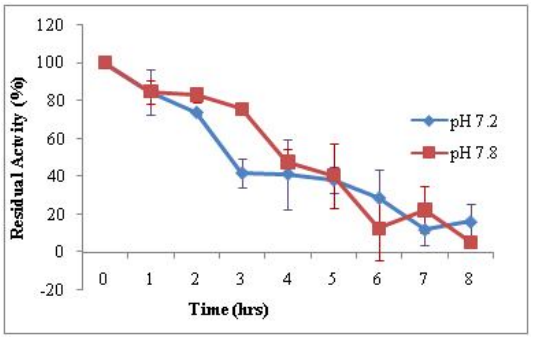

Fig. 5- pH stability of RMS 43 at pH 7.8 and pH 7.2 (control)

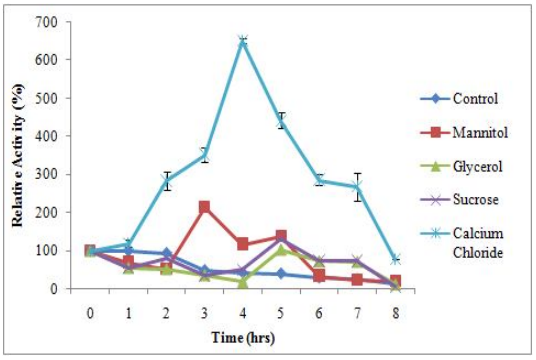

Fig. 6-Mannitol, Glycerol, Sucrose, Calcium Chloride (5mM) were added in the media along with the crude intracellular RMS43 enzyme.

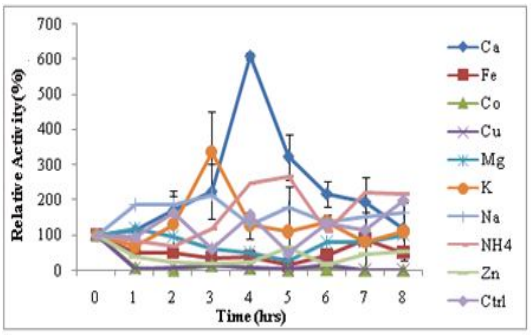

Fig. 7-Effect of different metal ions. Ca^{2+}, Co^{2+}, Mg^{2+}, Fe^{2+}, K^{+}, $NH4^{+}$, Zn^{2+}, Na^{+}, Cu^{2+} were present in 5mM concentration as chloride salts.

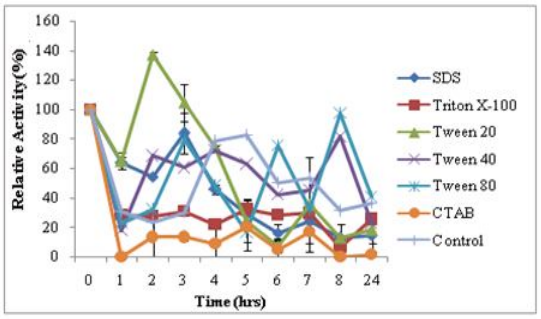

Fig 8-Effect of different surfactants SDS (negatively charged), CTAB (positively charged), Triton X-100, Tween 20, Tween 40 and Tween 80 (neutral) were there at 5mM conc.

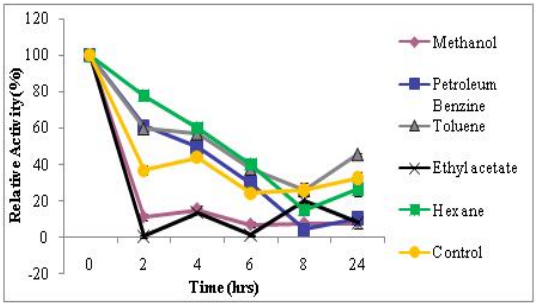

Fig. 9-Solvent stability experiment of RMS 43 lipase show good stability in hexane and Toluene followed by Petroleum ether than control, *i.e.*, buffer.

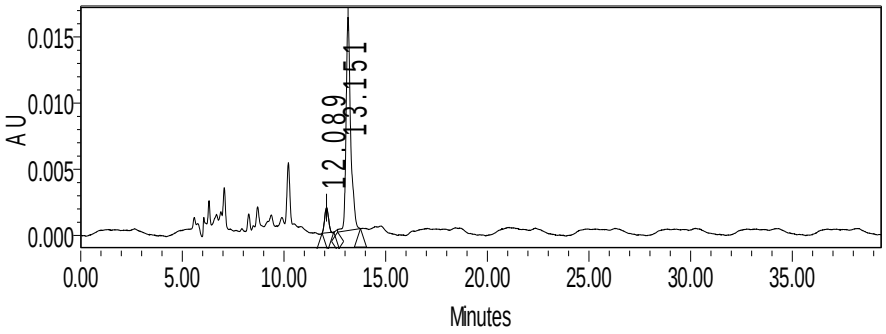

Fig. 10- HPLC profile chromatograph of Racemic 1-PE transformed into S-form with 81.8% enantiopurity with Chiraselect OM™ column and Hexane:IPA::0.45:0.05 as solvent. The residual substrate, (S)-1-phenylethanol, remained with the % of 81.78. From the standard, it has shown that peak at the retention time 13.151 is of S-enantiomer of 1-Phenylethanol. The lipase RMS43 was found to have high activity of esterification towards (R)-1-phenylethanol.

	Name	Retention Time	Area	% Area	Height
1	R	12.089	25249	9.11	1902
2	S	13.151	251875	90.89	16132

with its pH optimum at about pH 7.8. The enzyme produced by the isolate is found to be stable at high temperature. Its thermal stability is further increased in the basic pH range, extending its potential commercial usage[13]. The activity of enzyme can be further enhanced by addition of some modifiers. Co-factors are generally not required for lipase activity. However, divalent cations such as Calcium often stimulates enzyme activity by combining with the released fatty acids as observed in the case of lipase isolated in current study. Surfactants like Tween-20 are known to increase the lipase activity by acting as an emulsifier-providing a better interface for lipase action. The Tween-20 enhanced the

activity of the lipase isolated. Solvent stability of the lipases in organic solvents augments their versatility for non-aqueous enzymology. Methanol (log P= -0.76) and Ethyl acetate (log P=0.68) has been considered comparatively more polar than Hexane (log P=3.9), Toluene (log P=2.8) and Petroleum Ether (log P=3.2). RMS 43 lipase is very stable in non-polar solvents; maximum in hexane (40% after 6 hrs) and then in toluene (37.5% after 6 hrs) followed by Petroleum ether (30% after 6 hrs) than Control (24.5% after 6 hrs). It may be due to the rigidity of the enzyme pocket in these solvents[10].

The use of activated acid acyl donors such as vinyl ester in the enantioselectivity reaction by RMS 43 has an advantage, since the vinyl alcohol released in the degradation of the vinyl ester tautomerizes to acetaldehyde, which cannot act as substrate for the enzyme[8]. Since the reaction is reversible, an increase in the acyl donor concentration is expected to result in higher product yields and shift the chemical equilibrium towards the synthesis. In Fig. 10 the first peak at retention time 12.089 is of R-enantiomer while the other one at 13.151 is for S-enantiomer. The decision between the peak of R and S was done after running the HPLC with pure S-enatiomer. It is demonstrated that S-enantiomer is 81.78% more than the R-enantiomer. Thus, the enzyme from *Serratia marsecens* RMS 43 isolate has good potential in resolving (RS)-1-Phenylethanol.

Conflict of Interest: The authors declare that they have no conflict of interest.

The financial assistance from DST-PURSE, is gratefully acknowledged.

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