International Journal of Biotechnology Research Vol. 3(4), pp. 065-072, June 2015
Available online at http://academeresearchjournals.org/journal/ijbr
ISSN 2328-3505 ©2015 Academe Research Journals

Full Length Research Paper

Purification of an epoxide hydrolase from *A. tubingensis* TF1 and effects of metal ions on its activity towards styrene oxide

Aparajita Duarah[1]*, Soneswar Sarma[2], Archana Yadav[1], Binod Kumar Gogoi[1] and Tarun Chandra Bora[1]

[1]CSIR-North East Institute of Science and Technology, Jorhat-785006, Assam, India.
[2]Department of Biotechnology, Gauhati University, Guwahati-781014, Assam, India.

Accepted 23 March, 2015

The present study aims at investigating the filamentous fungus *Aspergillus tubingensis* TF1 for its ability to produce epoxide hydrolases (EHs) under various reaction parameters. Under optimum media conditions, the highest activity obtained was 6.47 U/mL for phenyloxirane as the substrate. This enzyme was purified up to a purification fold of 4.33 with 0.2 U/mg of specific activity. After SDS-PAGE analysis, a band of approximately 47 kDa appeared when compared with the standard proteins. The effect of metal ions and surfactants on whole cells and the purified enzyme was investigated. The characteristics shown by *A. tubingensis* TF1 EH would be useful for the large scale production of this important enzyme in bioreactors.

Key words: Enantiopure diols, epoxide hydrolase, metal ions, specific activity, surfactants.

INTRODUCTION

Epoxide hydrolases (EHs, E.C. 3.3.2.3) are gaining importance in organic chemistry as these convert epoxides to their corresponding vicinal diols. This is accomplished by the addition of a water molecule and without the requirement of any co-factor, prosthetic groups or metal ions (Archelas and Furstoss, 2001). They belong to the α/β group of hydrolase family. There is growing need for the production of enantiomerically pure epoxides and vicinal diols (Zocher et al., 1999). These compounds are extensively used in pharmaceutical and chemical industries as β-3-adrenergic receptor agonists, antiobesity drugs, N-methyl-D-asparate receptor antagonist, anticancer drugs, central and peripheral dopamine receptors, antidiabetic drugs (Archelas and Furstoss, 2001; Besse and Veschambre, 1994; Genzel et al., 2002) and agrochemicals such as Bower's compound (Archelas, 1993) and hence important building blocks in organic synthesis. Instead of chemical synthesis by hydrolytic chemical resolution with transition metal catalysts (Jacobsen, 2000), organic chemists are inclined towards greener production of these compounds with enzymes. There are reports of commercially available EHs from *Aspergillus niger, Rhodococcus rhodochrous* and human microsomal EHs (Reetz et al., 2004). So, there is greater need to screen and purify EHs from newer sources and organisms.

A number of EHs from microbial sources are already purified and characterized (Jacobs et al., 1991; Nakamura et al., 1994; Mischitz et al., 1995b; Kroutil et al., 1998; Kronenburg et al., 1999; Morisseau et al., 1999; Kotik and Kyslik, 2006). But, owing to rather less number of the EHs showing enough stability and enantioselectivity (Kotik and Kyslik, 2006), search for EHs with excellent enantioselectivity and regio selectivity are increasing. In this study, EHs from *Aspergillus tubingensis* TF1 is taken into consideration. This fungus was isolated from soil sample with low temperature and

*Corresponding author E-mail: aparaitasduarah@rediffmail.com. Tel: 9864269370.

reported elsewhere (Duarah et al., 2013). During our study, we found that the EHs possess enantioconvergent activity which can be exploited to carry out biotransformation reactions converting racemic epoxides to enantiopure compounds. In this present work, we purified EHs from *A. tubingensis* TF1 and studied its enzymatic properties. Before that, the optimum carbon and nitrogen source for optimum cell growth and enzyme expression was taken into consideration which is presented in a separate paper.

MATERIALS AND METHODS

General

All media were purchased from Merck and Himedia. Racemic styrene oxide and (*R*) - and (*S*) - phenylethanediol were from Sigma and Fluka, respectively. DEAE-Sepharose DCL-6B and Sephacryl-200 were obtained from Sigma. *Aspergillus tubingensis* TF1 was grown in shake flasks to obtain sufficient amount of cells and the enzyme activity was determined as previously described (Zocher et al., 1999). Proteins were quantified by the Lowry's method using bovine serum albumin, BSA (Hi-Media) as standard.

Selection of optimal nutrient media for cell biomass and enzyme production

To find the optimal nutrient medium for enzyme production, different media were used for comparative studies. The media include BM-1 (10.0 g/L glucose, 1.0 g/L yeast extract, 0.5 g/L KCl, 3.0 g/L $NaNO_3$, 0.5 g/L $MgSO_4.7H_2O$, 1.0 g/L KH_2PO_4), MM-A (10.0 g/L glucose, 1.4 g/L yeast extract, 0.2 g/L leucin, 0.5 g/L KCl, 6.0 g/L $NaNO_3$, 0.5 g/L $MgSO_4.7H_2O$, 1.5 g/L KH_2PO_4), NM-90 (30.0 g/L malt extract, 3.0 g/L soy peptone), UMY (10.0 g/L glucose, 3.0 g/L malt extract, 3.0 g/L yeast extract, 5.0 g/L soy peptone) (Melzar et al., 1999), Czapek-Dox media and Medium 19 (1.0 g/L K_2HPO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4.7H_2O$, 0.01 g/L $FeSO_4.7H_2O$, 10.0 g/L fructose and 15.0 g/L peptone) except that corn steep liquor was replaced by peptone (Jin et al., 2004), potato dextrose broth (PDB), and tryptone soya broth (TSB). Ten micro litre of the seed culture was transferred into 50 mL of different sterile medium in 250 mL flask and incubated on a rotary shaker at 200 rpm at 28°C. Aliquots were withdrawn at an interval of 30 min starting from 0 min and centrifuged. The supernatant was kept for further studies at 4°C until it was reused.

Effect of metal ions, detergents and EDTA on cell biomass and EH activity

The influence of a number of monovalent and divalent cations were investigated on the cell growth and EH production. For this, *A. tubingensis* TF1 was grown on 50 mL M19 media in 250 ml Erlenmeyer flasks containing various salts with concentration of 1 mM. Also, the influence of biomass production and enzyme activity was observed with SDS, cetyltrimethylammonium bromide (CTAB, a cationic detergent), Triton-X and Tween-80 (non- ionic surfactants), ethylenediaminetetraacetic acid (EDTA, a chelating agent) at concentrations of 1 mM keeping the other components constant. The study was carried out with triplicates and a control was maintained without the addition of irrelevant metal salts.

Preparation of cell-free extract

A culture of *A. tubingensis* TF1 from 7 L of medium was harvested after 5 days of growth by filtration and suspended in buffer A (10 mM Tris-HCl buffer) containing 1 mM cysteine, 1 mM EDTA and 0.3 mM phenylmethanesulfonyl fluoride (Morisseau et al., 1999). Cells were disintegrated employing a sonicator. After removing the cell debris by centrifugation (9,400×g, 40 min, 4°C) the clear supernatant (400 mL) was used as crude enzyme and for further protein purification. The subsequent steps were followed with buffer A containing 1 mM cysteine, 1 mM EDTA and 0.2 mM phenylmethanesulfonyl fluoride (PMSF) at 4°C to prevent enzyme denaturation.

The crude EHs was precipitated with 80% saturation of ammonium sulphate and allowed to settle overnight at 4°C. The precipitate was collected by centrifugation and dialyzed against buffer A.

Determination of enzyme activity with whole cells and cell free extract

A stock solution of racemic-SO (1 M) was prepared in DMSO prior to each enzymatic reaction. The hydrolysis reaction was initiated by the addition of 5 mM *(±)*-SO stock solution and 1 g whole cells and 70 µl of cell free extract in 10 ml of 10 mM sodium phosphate buffer (pH 7.25) in different glass vials. Incubation was carried out at 30°C at 200 rpm for 45 min.

After the specific time period, aliquots were withdrawn and NBP-colorimetric assay was performed (Zocher et al., 1999). From the reaction buffer containing whole cells and styrene oxide, 100 µl was taken out in test tubes. To this, 10 µl of triethylamine was added to stop the reaction and was followed with the addition of 100 µl triethylene glycol dimethyl ether and 4-(p-nitrobenzyl)-pyridine [NBP] dissolved in methoxyethanol to the reaction buffer. The mixture was incubated at 35-40°C after the addition of the reagents.

The blue pigment developed after the reaction measured at 560 nm against a reference wavelength of 650 nm along with a control containing an enzyme free solution. The decrease in absorption of the solution was found to be inversely proportional to the epoxide hydrolase activity (Duarah et al., 2013).

Epoxide hydrolase purification

The dialyzed enzyme solution was applied to a DEAE-Sepharose column (2.0 × 20 cm), equilibrated with buffer A containing 0.13 M KCl. The enzyme was eluted with a linear gradient of 0.1-0.25 M of KCl in buffer A at a flow rate of 0.5 ml/min with a fraction volume of 5 ml. The eluted fractions were assayed for enzyme activity. The fractions showing EH activity were pooled together and concentrated by lyophilisation. This concentrated enzyme solution was applied to a Sephacryl S-200 column (2.0 × 50 cm) previously equilibrated with buffer A adjusted to pH 7.0 containing 10 mM KCl. The fractions were separated at a flow rate of 0.5 ml/min. The active fractions were pooled and concentrated by lyophilisation. The eluted fractions were checked for EH activity and eluent with activity were pooled and concentrated. This solution was stored at 4°C until it was reused.

Epoxide hydrolase assay

Styrene oxide was used as substrate to assay EH activity. Fifty micro litre of the enzyme preparation was added to 5 mL of sodium phosphate buffer (10 mM, pH 7.25) containing 5 mM *(±)*-SO in DMSO. The reaction was incubated at 30°C for 45 min. After the incubation time, 20 ml of ethyl acetate was used to extract the formed diol and unreacted substrate. The amount of diol formed was determined on a Chiralcel OD column in a HPLC. The amount of enzyme that catalyzed the formation of 1 µl diol/min under the above experimental conditions is defined as one EH unit (U). Protein estimation was carried out according to Lowry's method using BSA as standard.

SDS-PAGE for determining molecular weight of purified enzyme

The crude extract and fractions obtained after ion exchange and gel chromatography were loaded onto SDS-polyacrylamide gel. SDS–PAGE was conducted in gels consisting of 10% acrylamide resolving gel and 4% acrylamide stacking gel. The relative molecular weights of the purified protein were determined from the mobility of the standard protein molecular weight markers (Bangalore Genei). Coomassie Brilliant Blue was used to stain the proteins.

Effect of substrate concentration on activity

The saturation kinetics of epoxide hydrolase from the partially purified EH was determined using styrene oxide as the substrate. Crude extract (400 µl) and substrate concentrations ranging from 5 mM to 160 mM (1 M DMSO stock) were reacted in 10 mM sodium phosphate buffer, pH 7.25 for 30 mins at room temperature. At the end of the reaction, NBP colorimetric assay was performed as described previously. The absorbance of non-hydrolysed *R*-SO was recorded in a spectrophotometer at 560 nm (Zocher et al., 1999). The enzyme activity was calculated therefrom. All the experiments were run in triplicates. Respective blanks and controls were kept for each reaction. Kinetic parameters were estimated by plotting 1/ [V_{max}] and 1/ [S] in a Lineweaver-Burk model using GraphPad Prism 6 software (San Diego, California).

Effect of pH and temperature on enzyme activity

The purified EH was tested for its optimum temperature within a temperature range of 15-50°C. The enzyme stability was checked at -20 and 4°C. The effect of pH was obtained by using various pH buffers, of which NaH_2PO_4/citric acid buffer was used for pH 3.0-5.0, sodium phosphate buffer for pH 5.0-8.0, Tris–HCl buffer for 7.0-9.0, glycine/NaOH buffer for pH 9.0-10.0. The standard assay condition was performed with 200 µl enzyme solution in 10 mM sodium phosphate buffer and 5 mM *(±)*-SO prepared as DMSO stock solution. The absorption was measured and the activity was calculated from the values obtained as described in epoxide hydrolase assay.

RESULTS

Optimization of media

The different media investigated had varied effects on cell growth and enzyme production of *A. tubingensis* TF1. The modified Medium 19 showed the maximum EH activity (6.47 U/mL) followed by BM-1 media (4.31 U/mL). The highest cell biomass was observed as 21.6 gm wet weight/L (5.84 gm/dcw) when modified M-19 media was used. PDB and TSB showed similar EH activity. As highest biomass production and enzyme activity was observed in Medium 19, this media was taken as standard media for further optimisation studies.

Effect of metal ions and detergents on cell biomass and EH activity

The cell growth was monitored and measured in media added with 1 mM additives comprising metal ions and non-ionic detergents. When EH activity was assayed under the above mentioned conditions in the presence of various cations, the highest activity is obtained with K^+ ions (4.97 U/mL) whereas Mg^{2+} and Zn^{2+} ions have similar effects on cell growth as well as enzyme production. When Fe^{2+} ions were used at 0.1% concentration, the activity was greater than that at a concentration of 0.2%. For Mn^{2+} and Ca^{2+}, it can be deduced that enzyme production decreased but at the same time, cell growth is unaffected. The media when supplemented with CTAB, showed that there was no

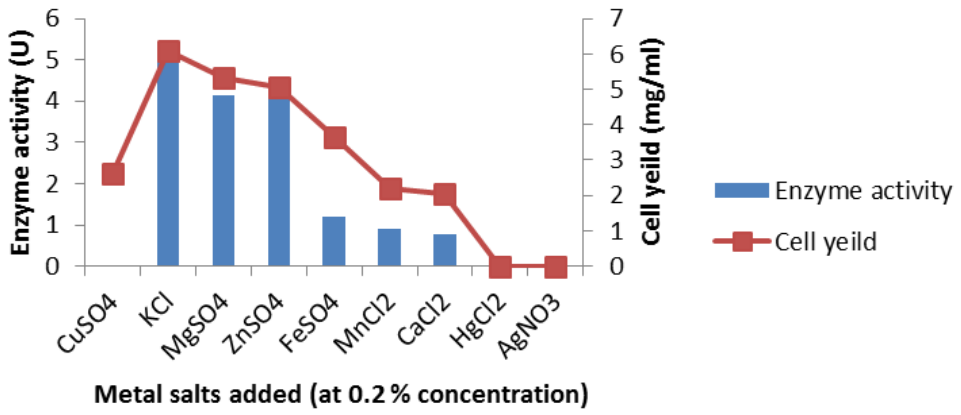

Figure 1. Graph depicting effect of various salts on cell growth and enzyme production by A. tubingensis TF1. Values are expressed as mean of triplicates performed independently. The reaction was performed at 30ºC for 45 mins in 10 mM sodium phosphate buffer, pH 7.25.

Table 1. Purification of EH from *A. tubingensis* TF1.

Steps in purification	Total activity (U/ml)	Yield (%)	Total protein (mg)	Specific activity (U/mg)	Purification factor
Crude extract	6.71	100	145.38	0.0461	1
Ammonium sulphate	4.211	62.75	46.31	0.09	1.95
DEAE-Sepharose	0.857	12.77	5.24	0.163	3.53
Sephacryl-200	0.18	2.68	0.91	0.2	4.33

apparent growth of the fungus. With Triton-X and EDTA, cell growth was insignificant (Figure 1). On addition of different surfactants to the growth media, both cell growth and enzyme activity declined. The media when supplemented with CTAB, there was no apparent growth of the fungus. With Triton-X and SDS, cell growth was negligible. Among the surfactants tested, Tween-20 supported biomass production. Enzyme activity for Triton-X, Tween 20 and SDS was 5%, 18% and 6%, respectively compared to the enzyme activity when no surfactants were added in the media (Fig. 2) Cell growth in all the media added with various additives is less compared to the control without any additives. Addition of glycerol, phenylmethanesulfonyl fluoride (PMSF) and cysteine when added to the growth media did not affected the biomass production as well as the enzyme activity to a great extent. (Fig. 3).

Purification of EHs

The epoxide hydrolase from *A. tubingensis* TF1 was purified upto 4.33-fold with a four-step procedure. The results are summarized in Table 1. Mycelia were harvested in the growing phase. Cell disruption was obtained by administering the mycelia in buffer A in a sonicator as it produced reproducible results. Specific activity was checked and found to be 0.461 U/mg in the crude extract. The crude extract was subjected to 80% $(NH_4)_2SO_4$ precipitation and membrane dialysis. The membrane dialysed fraction was applied onto a DEAE-Sepharose column. A linear salt gradient was applied for eluting the enzyme. The EH activity was eluted in the final concentrations of salt at 0.23 M KCl concentration (Figure 4). A single band was obtained after SDS-PAGE and Coomassie Brilliant Blue staining of the enzyme after anion-exchange step. Gel filteration chromatography on Sephacryl-200 resulted in a faint corresponding protein band leading to a loss in enzyme activity when checked. The molecular weight of the purified enzyme was revealed to be approximately 47 kDa (Figure 5) as observed from the SDS-PAGE analysis and estimated from the mobility of standard proteins.

Effect of pH and temperature

The effect of pH and temperature on the purified and pooled fraction of *A. tubingensis* TF1 was analysed. The enzyme was active in the range of 5.0-9.0 and the maximum activity was observed at pH 7.5. *A. tubingensis* TF1 EH was stable at all tested pH with 27% and 18% of the initial activity at pH 4 - 5.5 and above pH 8 respectively which changed to total deactivation at pH 9.5 and above. The optimum temperature of the purified fraction was at 30°C.

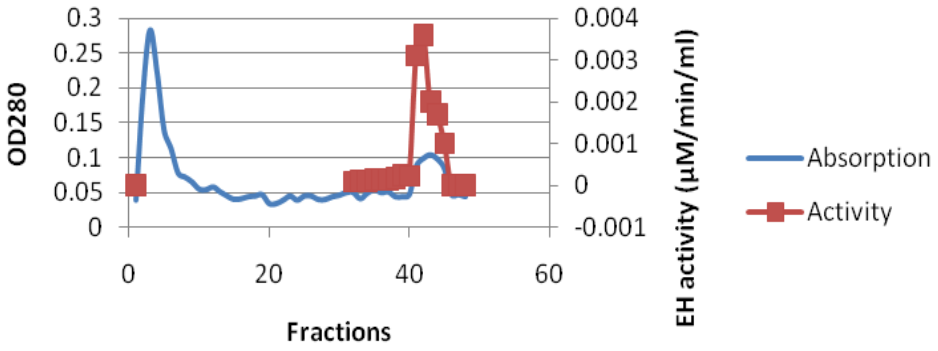

Figure 2. Graph depicting effect of various surfactants or detergents on cell growth and enzyme production by A. tubingensis TF1. Values are expressed as mean of triplicates performed independently.

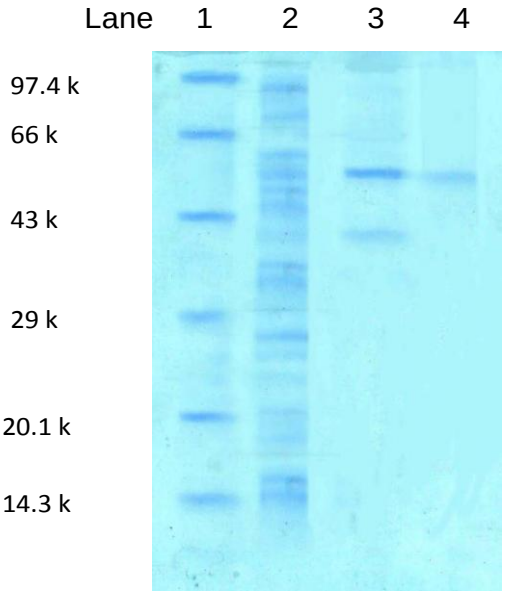

Figure 3. Graph depicting effect of various additives on cell growth and enzyme production by A. tubingensis TF1. Values are expressed as mean of triplicates performed independently.

Effect of additives on enzyme activity

The effects of various metal ions, detergents and organic solvents on EH activity were examined with styrene oxide 5 mM as the substrate (Table 2). The purified protein concentration was maintained at 0.5 mg/mL with varied final concentrations of the additives. Among the various metal ions tested for enzyme stability, maximum activity concentration and the initial reaction rate is described by the Michaelis–Menten Equation:

$$V = \frac{V_{max} \times S}{K_m + S}$$

was observed with K^+ (91.24%). The enzyme activity was inactivated by Cu^{2+}, Hg^{2+} and Ag^{2+}.

Determination of kinetic parameters with crude extract of *A. tubingensis* TF1

The relationship between the initial substrate

Initially, the reaction rate was directly proportional to the substrate concentration. When the substrate concentration was increased beyond 80 mM, the reaction rate became independent of substrate concentration

reaching a maximum of 3.06 mM/min/mL at 160 mM. The data were fitted in a non-linear regression model with a regression coefficient of 0.9722 and the Michaelis constant (K_m) was evaluated to be 56.7±15.23 mM when the maximum initial velocity (V_{max}) is 4.42±0.570 mM/min (Figure 6).

DISCUSSION

The media composition and concentration of each component greatly influence the growth and enzyme production in a particular species. The media optimisation reactions for *A. tubingensis* TF1 led to maximum biomass and enzyme production with modified Medium 19 among the tested media. This media contained fructose and peptone as the carbon and nitrogen source, respectively. Jin et al. (2004) used this media for studying the biohydrolysis of styrene oxide and its derivatives. Melzer et al. (2008) got the highest EH activity when BM-1 media was used along with trace 0.1 g/L trace elements. Previous workers have reported maximum cell growth in media containing glucose (Kotik and Kyslik, 2006; Nellaiah et al., 1996; Jin et al., 2012).

One of the most influencing parameters for the growth and enzyme production for fungal cells is utilisation of metal ions. Of the various mono- and divalent cations tested for their effect on the cell biomass or production of EH, maximum cell growth and EH production resulted with K^+ followed by Mg^{2+} and Zn^{2+}. However, Fe^{3+}, Mn^{2+} and Ca^{2+} slightly induced EH production, but Ag^+, Cu^{2+}, and Hg^{2+} totally inhibited EH production. *Rhodosporidium toruloides* UOFS Y-0471 EH was inhibited by Group 2 metal (Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}) and transition metal ions (Co^{2+}, Hg^{2+} and Ag^{2+}) (Botes et al., 1999). JHEH was also inactivated by Hg^{2+} (Debernard et al., 1998). The influence of detergents on cell growth and EH activity was investigated. The surfactants have a permeabilizing effect on the cell by creating pores in the cell membrane and extract out the proteins. CTAB significantly inhibited cell growth whereas Tween-20 supported both cell and enzyme production. It infers that for biomass production, addition of surfactants or chelating agents for binding metal ions is not necessary. Instead, addition of monovalent salts leads to considerable increase in both biomass and enzyme production.

The EH from *A. tubingensis* TF1 was partially purified using four purification steps. Protein losses is minimised due to less number of purification steps. Cysteine, EDTA and PMSF were added to the media in all the subsequent steps as it helped in stabilizing the enzyme (Morisseau et al., 1999). As reported by Botes et al. (1999), EH do not bind to cation exchangers, hence an anion exchanger was used for retention of *A. tubingensis* TF1 EH. Gel filteration on Sephacryl-200 resulted in a loss in enzyme activity when checked and a faint corresponding protein band appeared. EH from *A. niger* LCP 521 (Morisseau et al., 1999) and *A. niger* SQ-6 (Liu et al., 2007) were purified to homogeneity with 4% and 42.3% yields respectively. Purification to homogeneity was not obtained in *A. tubingensis* TF1 epoxide hydrolase producing a relatively low yield. While purification, enzyme activity was considerably lost in the following steps and in the final gel filtration chromatography step, the enzyme was found to be unstable as seen from the SDS-PAGE band. The enantioconvergent hydrolysis of styrene oxide was determined using whole cells/crude extract of the fungal cells and the final purified fraction which was established to be similar.

A clear purified band was obtained after the ion exchange chromatography step. A protein size of approximately 47 kDa was shown in the SDS-PAGE gel without a reducing agent after Coomassie Blue staining indicating its similarities to *A. niger* LCP521 EH. The elution profile of the purified EH in size exclusion chromatography with that of the standard proteins was not compared keeping the scope for further research.

At very low and high temperatures, the activity was less which indicates that the enzyme performs slowly due to low kinetic energy of EH at lower temperature, whereas at high temperatures, the enzyme gradually denatures. The enzyme was found to be stable at -20 and 4°C for 6 months. Enzyme stability maintained at these temperatures suggested its adaptation in a wide range of physiological conditions. The pH and temperature stability of *A. tubingensis* TF1 EH is similar to that of *A. niger* LCP521 (Morisseau et al., 1999). Glycerol stabilises the enzyme activity when present in the reaction media. The EH from *Nocardia* EH1 is ineffective to glycerol (Kroutil et al., 1998). The surfactants have a permeabilizing effect on the cell by creating pores in the cell membrane and extracting out the proteins. On studying the effects of various additives on enzyme activity, it was found that surfactants increased the EH activity than without the addition of any additive. The *A. tubingensis* TF1 EH shows considerable similarities with *Nocardia* EH1 (Kroutil et al., 1998) where enzyme stabilisation is achieved by addition of non-ionic detergents. In both the microorganisms, enzyme activity increased on addition of surfactants indicating unfolding of globular proteins. Among the various metal ions tested for enzyme stability, maximum activity was observed with KCl. Heavy metal ions Cu^{2+}, Hg^{2+} and Ag^{2+} when present deactivated epoxide hydrolase activity because of their interference with the -SH- moiety in the active site. Activity was increased on addition of Mg^{2+} ions and CTAB. This study revealed that CTAB is detrimental to whole cells whereas for purified EH, it enhanced enzyme activity. The investigated EH has a wide range of temperature adaptability maintaining its hydrolysing activity at 55°C. The enzyme was active in the range of 5.0-8.0 and the maximum activity was observed at pH 7.5. Above and below this range, no activity could be detected. These results are comparable to those obtained when whole cells of *R. glutinis* were taken for

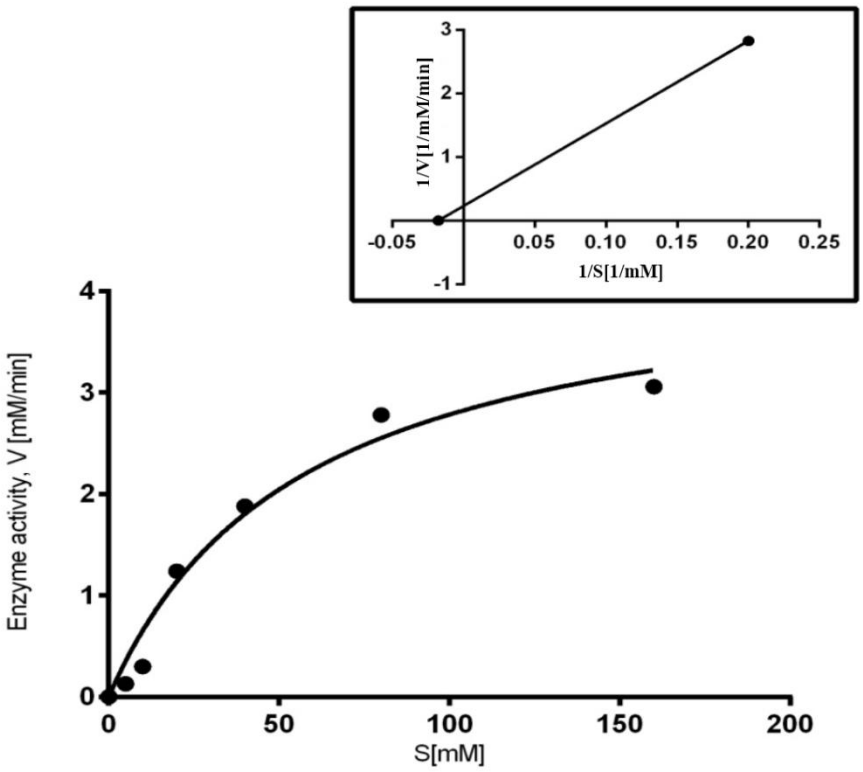

Figure 4. DEAE-Sepharose DCL-6B chromatography of A. tubingensis TF1 EH. Absorbancies at 280 nm were monitored continually and aliquotes (100 µl) of the fractions were analysed for enzyme activity. Active fractions were combined and concentrated. No EH activity was detected at fractions eluted before fraction 32. The majority of the purified EH eluted in fractions 39 to 42.

the catalytic resolution of styrene oxide (Yeates et al., 2007). As seen from the graph (Figure 4), the *A. tubingensis* TF1 EHase has a higher K_m, making it possible that an increase in the substrate concentration will not saturate the enzyme. Instead, as the substrate concentration varies, the enzyme activity will also vary so that product formation will depend on substrate availability.

The study indicated that the fungal epoxide hydrolase isolated from *A. tubingensis* would be an efficient biocatalyst and offer a new paradigm of research for green manufacturing of industrially relevant chemical synthons. The present research on purifying the epoxide hydrolase proves to be cost effective with minimum purification steps so as to minimize the lost in enzyme activity. For industrial applications, factors in scale-up bioprocess reactions have to be observed which may be different than in shake flasks condition.

ACKNOWLEDGEMENTS

The authors thank Council of Scientific and Industrial Research (CSIR) for providing the financial support and Dr. R. S. Rammaiah, Director, CSIR-NEIST, Assam, India for providing the facilities to carry out the work.

REFERENCES

Archelas A, Delbecque JP, Furstoss R (1993). Microbial Transformations 30. Enantioselective hydrolysis of racemic epoxides: the synthesis of enantiopure insect juvenile hormone analogs (Bower's compound) Tetrahedron Asymmetry 4: 2445-2446.

Archelas A, Furstoss R (2001). Synthetic applications of epoxide hydrolases. Curr. Opin. Chem. Biol., 5:112-119.

Besse P, Veschambre EN (1994). Chemical and Biological Synthesis of Chiral Epoxides. Tetrahedron 50: 8885-8927.

Botes AL, Litthauer D, van Tonder A, van Dyk MS (1999). Physico-chemical properties of the epoxide hydrolase from *Rhodosporidium toruloides.* Biotechnol. Lett. 21:1137–1144.

Debernard S, Morisseau C, Severson TF, Feng L, Wojtasek H, Prestwich GD, Hammock BD (1998). Expression and characterization of the recombinant juvenile-hormone epoxide hydrolase (JHEH) from *Manduca sexta*. Insect Biochem. Mol. Biol. 28:409–419.

Duarah A, Goswami A, Bora TC, Talukdar M, Gogoi BK. Enantioconvergent biohydrolysis of racemic styrene oxide to R-phenyl-1, 2-ethanediol by a newly isolated filamentous fungus Aspergillus tubingensis TF1. Appl Biochem Biotechnol 2013; 170:1965-1973.

Genzel Y, Archelas A, Broxterman QB, Schulze B, Furstoss R (2002). Microbiological transformation: 50. Selection of epoxide hydrolase for enzymatic resolution of 2-, 3-, or 4-pyridyloxirane. J. Mol. Catal. B: Enzym. 16:217-222.

Jacobs MHJ, Van den Wijngaard AJ, Patenga M, Janssen DB (1991). Characterization of epoxide hydrolase from an epichlorohydrin-degrading
Pseudomonas sp. Eur. J. Biochem. 202: 1217–1222.

Jacobsen EN (2000). Asymmetric catalysis of epoxide ring-opening reactions. Acc. Chem. Res. 33:421-431.

Jin H, Li Zu-yi, Dong Xiao-wei (2004). Enantioselective Hydrolysis of Various Substituted Styrene Oxides with *Aspergillus niger*-CGMCC 0496. Org. Biomol. Chem. 2:408-414.

Jin HX, Hu ZC, Zheng YG (2012). Enantioselective hydrolysis of epichlorohydrin using whole *Aspergillus niger* ZJB-09173 cells in organic solvents. J. Biosci. 37(4):695-702.

Kotik M and Kyslik P (2006). Purification and characterisation of a novel enantioselective epoxide hydrolase from *Aspergillus niger* M200. Biochim. Biophys. Acta. 1760(2): 245-252.

Kronenburg NAE, Mutter M, Visser H, de Bont JAM, Weijers CAGM (1999). Purification of an epoxide hydrolase from *Rhodotorula glutinis*. Biotechnol. Lett. 21:519-524.

Kroutil W, Genzel Y, Pietzsch M, Syldatk C, Faber K (1998). Purification and characterization of a highly selective epoxide hydrolase from *Nocardia* sp. EH1. J. Biotechnol. 61:143–150.

Liu Y, Wu S, Wang J, Yang Liu, Sun (2007). Cloning, expression, purification and characterization of a novel epoxide hydrolase from *Aspergillus niger* SQ-6. Prot. Exp. Puri. 53:239–246.

Melzer G, Junne S, Wohlgemuth R, Hempel DC, Gotz P (2008) Production of epoxide hydrolases in batch fermentations of *Botryosphaeria rhodina*. J. Ind. Microbiol. Biotechnol. 35:485–493.

Mischitz M, Faber K, Willetts A (1995). Isolation of a highly enantioselective epoxide hydrolase from *Rhodococcus* sp. NCIMB11216. Biotechnol. Lett. 17:893–898.

Morisseau C, Archelas A, Guitton C, Faucher D, Furstoss R, Baratti JC (1999). Purification and characterization of a highly enantioselective epoxide hydrolase from *Aspergillus niger*. Eur. J. Biochem. 263(2):386-395.

Nakamura T, Nagasawa T, Yu F, Watanabe I, Yamada H (1994). Purification and Characterization of Two Epoxide Hydrolases from *Corynebacterium* sp. Strain N-1074. Appl. Environ. Microbiol. 60 (12):4630-4633.

Nellaiah H, Morisseau C, Archelas A, Furstoss R, Baratti JC (1996). Enantioselective hydrolysis of p-nitrostyrene oxide by an epoxide hydrolase preparation from *Aspergillus niger.* Biotechnol. Bioeng. 49:70–77.

Reetz MT, Torre C, Eipper A, Lohmer R, Hermes M, Brunner B, Maichele A, Bocola M, Arand M, Cronin A, Genzel Y, Archelas A, Furstoss R (2004). Enhancing the Enantioselectivity of an Epoxide Hydrolase by Directed Evolution. Org. Lett. 6(2):177-180.

Yeates CA, Smit MS, Botes AL, Breytenbach JC, Krieg HM (2007) Optimisation of the biocatalytic resolution of styrene oxide by whole cells of *Rhodotorula glutinis*. Enz. Microb. Technol. 40:221-227.

Zocher F, Enzelberger MM, Bornscheuer UT, Hauer B, Schmid RD. A Colorimetric Assay Suitable for Screening Epoxide Hydrolase Activity (1999). Anal. Chim. Acta. 391: 345-351.