A hydrolase with esterase activity expressed from a fosmid gene bank prepared from DNA of a North West Himalayan glacier frozen soil sample

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Abstract

Screening of 20,000 clones of a fosmid gene bank, constructed from DNA extracted from North West Himalaya (NWH) glacier soil sample, using functional approach identified 10 esterase/lipase-producing clones. Of these, a clone designated pFG43 with an insert size of 45 kb which produced the highest concentration of enzyme (467.43 U/mg) was sequenced. Clone pFG43 contained 61 open reading frames (ORF) and of these an ORF of 1155 bp designated ME-003, was found to be closely related to a hydrolase from *Acidobacteria* sps (77% sequence identity and E value = 1e-164) and subsequently identified as a putative cocaine esterase. ORF ME-003 was amplified and sub-cloned using a TA vector system into *E. coli* (DH5 α). The purified recombinant enzyme with a molecular weight of 43 kDa had optimal activity at 40 °C, pH 6 and the highest activity with shorter chain fatty acids than with higher chain length fatty acids. There is insignificant effect of inhibitors on the enzyme activity of ME-003, except PMSF which completely inhibited its activity. ME-003 activity was also inhibited in the presence of copper oxide but remained stable in presence of other metal ions. The enzyme activity was also inhibited in the presence of organic solvents; however, in the presence of 10% isopropanol, 12% of enzymatic activity was retained. Among various detergents, SDS completely inhibited enzymatic activity. The recombinant enzyme also shows enantio-specific activity against the racemic drug intermediates/precursors and exhibited 90% ee against racemic 1-phenyl ethanol and fluoxetine.

Keywords Frozen glacier soil · Metagenomics · Fosmid gene bank · Esterase · Next-generation sequencing

Introduction

Metagenomics is an important technique that has led to the identification of biomes inhabiting the extreme environmental niches. Significantly, this technique has been exploited for the detection of novel biocatalysts and genes encoding enzymes from the microbial samples which are otherwise non-culturable (Leis et al. 2013; Steele et al. 2009). Function-based strategies have been used for the direct detection and isolation of gene products through diverse screening methods, including plate assays, quantitative assays, etc. (Leis et al. 2015). The screening of fosmid gene banks provides the possibility of mining new enzymes with significant sequence diversity from the existing known ones as well as identification of altogether unknown and novel families of enzymes with no similarity in sequence to those previously characterised proteins becomes possible. Previously, the sequence-based metagenomics, e.g., next-generation sequencing (NGS) strategies have been extremely

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successful in identification of novel enzymes, i.e., nitrilases, cellulases, carboxylesterases, lipases and other industrially important enzymes (Beloqui et al. 2006; Liebl et al. 2014; Lorenz and Eck 2005; Robertson et al. 2004; Steele et al. 2009). Esterases (EC.3.1.1.1, carboxyl ester hydrolases) are hydrolytic enzymes that catalyse the hydrolysis of various types of exogenous and endogenous esters, preferably esters composed of short chain of fatty acids into acid and alcohol (Bhavith et al. 2014; Bornscheuer 2002). These industrially important enzymes have major physiological importance being extensively distributed in ecosystem, for example, in bacteria, archaea, and eukaryotes, and their industrial applications include synthesis of novel esters, resolution of chiral carboxylic acids, primary, secondary and tertiary alcohols, resolution of chiral drugs, etc. (Margolin 1993; Montella et al. 2012; Sayer et al. 2015). These enzymes have broad application in the industry, based on the grounds of their versatility, adaptability, stereo-selectivity and their ability to improve synthetic reactions in organic solvents (Bornscheuer 2002). The first ever experimental structure of an esterase enzyme belongs to acetyl-choline esterase (Sussman et al. 1991). Enzymes encoded by extremophilic micro-organisms have unique characteristics, such as enzymatic activity at a broad range of low to high temperatures, pH and different salinity levels (López-López et al. 2014). These enzymes are globally important with reported market of 5 billion USD in 2015 and expected market of 10.7 billion USD in 2024 (World Enzyme to 2017, <https://www.gminights.com/industry-analysis/enzymes-market>). In the present manuscript, the researchers report a novel esterase enzyme from the frozen soil of one of the glaciers of NWH located in Jammu and Kashmir, India through the application of functional metagenomic gene bank construction and NGS analysis.

Materials and methods

Sample collection

The soil sample was collected from frozen soil of one of the NWH of J&K state, India. The temperature of the frozen soil was -20°C . The other parameters of the glacier were height 4700 m above sea level, longitude and latitude $34^{\circ}9'49''\text{N}$, $75^{\circ}19'49''\text{E}$, respectively and preserved at -20°C till processing.

Construction of fosmid gene bank and screening for esterase/lipase enzyme

Metagenomic DNA was extracted using Ultrapure Ultra-clean Megaprep. soil DNA kit (Mobio Laboratory USA) with some modifications (Verruchi Gupta 2019). The DNA

was precipitated at -20°C overnight with equal volume of 5 M NaCl and double volume of ethanol. The DNA was pelleted by centrifugation at $13,500\times g$ for 30 min at 4°C . The pellet was washed with 70% ethanol twice, air-dried for 5–8 min, and dissolved in $1\times\text{TE}$ and analysed on 0.8% agarose gel in $1\times\text{TAE}$ buffer (Verruchi Gupta 2019). The fosmid gene bank was constructed using CopyControl™ HTP Fosmid Library Production kit (Epicentre, Madison, Wisconsin) with some modifications, and the above DNA was purified using β -agarase method (de Castro et al. 2011; Verruchi Gupta 2019). The fosmid gene bank was screened for genes encoding esterase/lipase enzyme using LB agar containing chloramphenicol (12.5 $\mu\text{g/ml}$) and 1% tributyrin at 37°C for 24–48 h. For secondary screening, LB agar containing chloramphenicol (12.5 $\mu\text{g/ml}$) and 1% olive oil with 1% rhodamine B was used and incubated under same conditions. Esterase activity was assayed using *p*-nitrophenyl butyrate (*p*-NPB) as the substrate (Rasool et al. 2005).

Sequencing and sequence analysis of a fosmid clone with esterase/lipase activity

FosmidMAX™ DNA Purification kit (Epicentre, Madison, Wisconsin) was used for isolation and purification of DNA of fosmid clone pFG43. The sequencing was outsourced to Xcerlis Pvt. Ltd., Ahmadabad, (Gujarat, India) and was undertaken on Illumina platform (MiSeq system). CLC Genomics tool was used for the assembly of NGS data. Prodigal tool (v2.6.3) at metagenomics mode was utilised for gene prediction. NCBI protein blast was done against the protein non-redundant (NR) and refseq database. Top identical proteins were downloaded and aligned using the clustal Omega online tool and visualised through the Mview tool (<https://www.ebi.ac.uk/Tools/msa/mview/>). Multiple sequence alignment was submitted to online phylogeny tool (<http://www.phylogeny.fr>) for generation of phylogenetic tree to visualise the analysed data.

TA cloning

The esterase gene was amplified using primers FE1F 5'TAC GCACTACCGTATCTCGA3' FE2R 5'GGCTAGGGCGCT TGGGACAC3' designed against the esterase gene sequence identified by NGS data. The PCR parameters were 95°C for 5 min, 95°C for 1 min, 55°C for 1 min, 72°C for 3 min, 35 cycles followed by extension at 72°C for 10 min and hold at 4°C . The PCR amplicon of 1.5 kb in size was sub-cloned using TA cloning kit (Invitrogen™ Carlsbad, California, United States). The cloning of esterase gene was confirmed using *SacI* (NEB, 10 U/ μl) and *NotI* (NEB, 10 U/ μl) restriction enzymes which have unique sites in the TA vector.

Protein purification

For isolation of protein (ME-003), pTA10 clone was grown in LB Broth with ampicillin (100 µg/ml) overnight at 37 °C at 200 rpm till OD 1.8 at 600 nm. The cells were harvested by centrifugation (13,000 ×g for 30 min at 4 °C) and re-suspended in buffer A (50 mM Tris buffer, 200 mM NaCl, pH 8.0). The cell suspension was sonicated using sonicator (Model no Q700-220, QSonica-LLC) in an ice bath for 5 min (30 s on, 1 min off cycle). For purification, the protein was precipitated at 0–40% ammonium sulphate. The precipitated samples were checked for the enzyme activity which was detected only in 40% ammonium sulphate fraction. This enzyme fraction was purified by FPLC (AktaPurifier 10, GE Healthcare Biosciences Ltd. Hongkong) using an Hydrophobic Interaction Column (HiLoad 16/10 Phenyl Sepharose, GE Healthcare Biosciences Ltd. Hongkong) at flow rate of 0.3 ml/min. The fractions showing the enzyme activity were pooled, and dialysed against Buffer C (20 mM sodium phosphate) overnight at 4 °C which was further purified by Ion Exchange Chromatography using Mono Q column (5/50 GL, GE Healthcare Biosciences Ltd. Hongkong) at a flow rate 0.5 ml/min. The different fractions were collected and checked for enzyme activity (Borkar et al. 2009). The purified protein was checked on 12% native PAGE, Zymogram prepared and 12% SDS–PAGE as per protocol (He 2011). The protein concentration was measured with BCA Protein Assay kit (Thermo Fisher Scientific, Massachusetts, United States), using BSA as a standard.

Determination of pH, temperature, substrate specificity and isoelectric point

To determine the pH optimum, 50 mM acetate buffer (pH 5–5.8), potassium phosphate buffer (pH 6.0–7.0), Tris buffer (pH 7.2–9.0), and sodium carbonate-bicarbonate buffer (pH 9.2–10.7) were used. The reaction was performed at 40 °C. The esterase activity was checked by incubating the enzyme at wide range of temperatures, i.e., 4 °C–100 °C with *p*-NPB substrate. Substrate specificity was also studied using different carbon chains of *p*-nitrophenyl ester substrates with variable chain lengths ranging from C2–C16 (Sukul et al. 2018; Zhu et al. 2013). The isoelectric (IEF) point was measured as per standard protocol (Singh et al. 2017).

Effect of metal ions, inhibitors, detergents and organic solvents

The effect of metal ions, inhibitors, detergents and organic solvents on enzymatic activity was determined as per protocol reported by Sukul et al. (2018), Zhu et al. (2013). Various metal ions (NaCl, LiCl, CaCl₂, MgCl₂, FeCl₂, KCl, CuCl₂, and MnSO₄) were added at a final concentration of

1 mM. The effect of inhibitors on ME-003 activity was determined by addition of phenyl methyl sulfonyl fluoride (PMSF), β-mercaptoethanol (β-ME), ethylene diamine tetra acetic acid (EDTA), and dithiothreitol (DTT) at a final concentration of 1 mM. The effect of detergents on ME-003 activity was determined by adding Tween-80, Tween-20, CTAB, TritonX-100, and SDS at 1% concentration. Effects of organic solvents were determined by addition of dimethyl sulfoxide (DMSO), methanol, isopropanol, and acetone at final concentrations of 1 and 10% (v/v). Control was defined as the reaction mixture without adding metal ions, inhibitors, detergents or organic solvents, respectively. All reactions were carried out at 40 °C and pH 6.

Potential application of ME-003 enzyme

Enantiospecificity of ME-003 for the resolution of racemic drug intermediates/precursors (1-phenyl ethanol, flouxetine, and ethambutol) was analysed by following the protocol reported earlier (Chaubey et al. 2012).

Results and discussion

The frozen soil sample was collected from an uninhabited and extremely remote location of the glacier with a view to clone novel genes encoding industrially important enzymes. The fosmid gene bank was constructed using approximately 40–50 kb metagenomic DNA fragments (Fig. 1a). The transformation rate of the recombinant fosmids was 10¹¹ cfu/ml/1 µg of metagenomic DNA (Verruchi Gupta 2019). Analysis of random 80 fosmid clones showed that the average insert size is ~45 kb. Other researchers also report similar insert size using the fosmid vectors (Felczykowska et al. 2014; Liu et al. 2016). Primary screening of 20,000 fosmid clones for lipase/esterase enzyme activity led to the selection of 10 positive clones. However, on secondary screening, 7 positive clones (pFG01, pFG05, pFG09, pFG67, pFG101, pFG43 and pFG99) were selected for further studies (Fig. 1b, c). Out of the seven fosmid clones, pFG43 exhibited the maximum enzymatic activity using *p*-NPB substrate (Table 1) and was used for further analysis. Popovic et al. (2017) reported carboxylesterase activity and validation of the esterase activity of 80 selected genes, belonging to 17 different protein families including unknown and cyclase-like proteins from metagenomic fosmid gene banks from 16 different environments.

Sequence analysis of the cloned DNA for identification of the esterase enzyme

The complete sequence of 45 kb insert of the metagenomic DNA in pFG43 clone sequenced by NGS was submitted to

Fig. 1 **a** 0.8% agarose gel showing metagenomic DNA isolated from glacier soil sample of NWH: lane1: uncut λ DNA as control, lane2: metagenomic DNA of glacier soil sample. **b** 1% Tributyrin agar diffusion plate with zone of clearance showing esterase/lipase activity. **c** 1% Olive oil and 1% rhodamine B agar diffusion plate assay with orange fluorescence showing esterase/lipase activity. **d** Phylogenetic tree of novel esterase ME-003 enzyme along with its close homologs

gous hydrolases from the ref. seq. database of BLASTp, the reported protein is grouped in the family of the Coc/NonD family of carboxylesterases. **e** 12% SDS-PAGE showing purified protein ME-003; lane 1: Protein molecular weight ladder, lane 2: purified protein ME-003 of 43 kDa. **f** 12% Native PAGE showing activity of the purified enzyme as a zymogram using α -naphthyl acetate and Fast Blue RR salt as substrates

Table 1 Quantitative estimation of esterase/lipase activity in seven fosmid clones using *p*-NPB as substrate under standard conditions. pFG43 clone showed maximum enzymatic activity

Clone number	Enzymatic activity (IU/ml) ^a
pFG01	0.24
pFG05	0.29
pGF09	0.11
pFG67	0.22
pFG101	0.121
pFG43	0.32
pFG99	0.023

^aOne International Unit (IU) of lipase/esterase activity is defined as the amount of enzyme catalysing the release of 1 μ mol of *p*-nitrophenol per min per ml from *p*-NPB under the standard assay conditions

query coverage with a hypothetical protein DMG80_14820 of *Acidobacteria* bacterium (acc. no. PYX29280.1). Therefore, a BLASTp reference sequence (refseq) database identified the protein reported highest identity of 53%, 40%, 39.5%, 40.6% and 39.5% with a CocE/NonD family hydrolase of *Terrimicrobium saccharophilum* sp. (Acc.no.WP_075078585.1), *Microvirga massiliensis* (acc. no. WP_052763844.1), *Microvirga ossetica* (acc. no. WP_099513428.1), *Mesorhizobium loti* (acc. no. WP_101936522.1) and *Actinomadura macra* (acc. no. WP_084265688.1), respectively. All these hydrolases belong to the esterases. Phylogenetic tree of the top 100 sequences from the BLASTp result was downloaded along with the query novel hydrolase and submitted to the Clustal Omega online tool (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The phylogenetic tree was generated and the novel hydrolase was grouped in the family of CocE/NonD family of carboxylesterases (Fig. 1d). Through the in-depth inspection of the hydrolase sequence a G-X-SY-X-G motif was observed (where X represents any amino acid) which has already been reported in cocaine esterase in the N-terminus of the protein. However, the G-XX-K and G-X-S-X-G motif were not observed in the reported esterase (Bornscheuer 2002).

SRA NCBI (SRA accession: PRJNA517605). The insert has 61 ORFs and the reported novel hydrolase was predicted as ORF no. 6. The protein sequence identified was 385 amino acid long protein which was submitted to GenBank, NCBI (accession no. MK272951). Novel protein was found to have 77% sequence identity over its 100%

Purification and characterisation of enzyme

The molecular weight of purified enzyme as analysed on 12% SDS–PAGE (Fig. 1e) is 43 kDa with pI of 5.3. The maximum enzymatic activity of the purified protein has been detected with *p*-NPB (C4). Its activity towards *p*-nitrophenyl octanoate (C8) reached 60% of the maximum activity, while longer chains of *p*-nitrophenyl esters were catalysed less efficiently (Fig. 2a). The specific activity of the enzyme was 467.43 U/mg using *p*-NPB as the substrate. *p*-NPB assay confirmed that the above enzyme pTA10 is an esterase. Thus, *p*-NPB was used for all subsequent experiments to characterise its catalytic properties. Maximum activity towards this substrate was observed at pH 6 in phosphate buffer (Fig. 2b). The optimal reaction temperature was 40 °C; however, the enzyme retained 66% activity at 60 °C, 40% activity at 80 °C and 15.07% activity at 100 °C (Fig. 2c). The maximum enzymatic activity was observed when the enzyme was incubated for 10 min at 40 °C; thereafter, there is a loss of activity with increase

in incubation time (Fig. 2d). ME-003 had almost no significant effect on its activity in presence of β -mercaptoethanol, EDTA and DTT; however, use of PMSF exhibited nearly complete loss of activity of enzyme. The activity of the enzyme remained stable in the presence of a number of metal ions, though its activity was inhibited in the presence of copper oxide. The activity of enzyme was inhibited in the presence of majority of organic solvents tested, though 12% activity was retained in 10% isopropanol. Among various detergents, SDS completely inhibited its enzymatic activity. Petrovskaya et al. (2016) reported an esterase (molecular wt. 36 kDa, pI 5.72) with optimum activity at 45 °C under a little alkaline condition. However, (Zhang et al. 2017) reported an esterase with optimum activity at 35 °C, pH 10 and pI 9.09. Zarafeta et al. (2016) reported an esterase which could hydrolyse medium chain fatty acids at temperature range of 25–60 °C and pH 7–8. Similar results have been reported on esterase characterisation by Sukul et al. (2018) and Zhu et al. (2013).

Fig. 2 Characterization of ME-003 enzyme with different substrates, pH and temperatures. **a** Graph showing enzymatic activity of ME-003, with different substrates ranging from C2 to C16. The maximum activity was found with *p*-NPB (C4); **b** graph showing stability of enzyme at different pH ranging from 5 to 11. The optimal enzymatic activity was found at pH 6; **c** graph showing stability

of enzyme at different temperatures 4–100 °C using *p*-NPB as substrate. The maximum enzymatic activity was recorded at 40 °C; **d** graph showing the stability of enzyme at 40 °C with different incubation time, the maximum activity was recorded at incubation time of 10 min

Fig. 3 Kinetic resolution of enzyme with various drug intermediates/precursors. **a** Hydrolysis of chiral auxiliary 1-phenyl ethanol results in conversion of 35% and enantiomeric excess (ee) of 90%; **b** resolution of racemic fluoxetine intermediate results in 25% conversion with 90% ee; **c** hydrolytic resolution of ethambutol drug intermediate results in conversion of 15% with 70% ee

Kinetic resolution of drug intermediates

ME-003 exhibited enantiospecificity against the racemic drug precursors/intermediate, while R (+) enantiomer was hydrolysed concentrating the S (−) enantiomer (ee90%) in 1-phenyl ethanol (Fig. 3a), fluoxetine drug intermediates (Fig. 3b), S (+) enantiomer was hydrolysed concentrating the R (−) enantiomer (ee70%) in ethambutol drug intermediate (Fig. 3c). Lipases/esterase showing enantioselectivity have also been reported earlier (Chaubey et al. 2012; Rasool et al. 2005).

Conclusion

In the present work, the authors report a novel hydrolase gene with esterase activity cloned via metagenomic route from the frozen soil sample of extremely remote and uninhabited glacier—a unique niche of NWH, J & K. The gene was amplified by PCR and sub-cloned in *E. coli* (DH5α) employing TA vector. The enzyme isolated from TA clone was purified and characterised in detail. It consists of 385 amino acids with a molecular weight of 43 kDa. The enzyme showed the highest activity with *p*-NPB (C4), which decreased with substrates having longer acyl chains longer than 12 carbons concluding that it is an esterase. The optimum activity of the enzyme was observed at 40 °C, pH 6 (pI 5.3); however, it retained

66% activity at 60 °C, 40% activity at 80 °C and 15.07% activity at 100 °C. Metal ions, detergents, inhibitors and organic solvents also affected the enzyme activity. The enzyme is enantio-specific against a number of racemic drug intermediates/precursors which concludes that the enzyme has potential in drug industry.

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Author contributions VV planned the research; VG executed the experiments including cloning, screening, characterization and purification; VG analysed the NGS data; IS undertook the bioinformatics studies of the NGS data; PK helped in isoelectric focusing work; and SR supervised the experimental work. All the authors contributed in MS preparation.

Compliance with ethical standards

Conflict of interest The author(s) declare that they have no conflict of interest.

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