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Discovery and characterization of an enantioselective family VIII esterase from effluent treatment plant sludge metagenome

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This study reports on the biochemical characterization of a novel esterase (EstN3) belonging to class C β-lactamases of family VIII esterases from the functional screening of an Effluent Treatment Plant (ETP) sludge metagenome library. The enzyme is 410 amino acids long and does not contain a signal peptide. It showed maximum amino acid sequence similarity with uncharacterized serine hydrolases from *Phenylobacterium* and *Caulobacter* species, suggesting that it is a member of family VIII esterase. EstN3's primary structure contains SxxKs, YSx, KTG, PLGMxDTxF, LxxxPGxxW, and GGxG motifs observed in class C β-lactamases, peptidases, and carboxylesterases of family VIII. This supports its designation as a class C β-lactamase. EstN3 favored shorter-chain p-nitrophenyl esters (C2-C6) based on substrate specificity profiling with p-nitrophenyl esters (C2-18). EstN3 exhibited excellent stereoselectivity in the production of S-mandelic acid under aqueous hydrolytic conditions. We have discovered that EstN3, a family VIII esterase, shows enantioselectivity towards methyl mandelate. The novel esterase was identified from the ETP sludge, indicating that unexplored environments serve as rich reservoirs for the discovery of novel enzymes with unique properties, offering valuable opportunities for advancing biocatalysis and industrial biotechnology.

Keywords Industrial effluent treatment plant sludge metagenome library, S-mandelic acid, Enantioselective esterase, Family VIII esterase, Biocatalysis.

Enantiopure mandelic acid and its derivatives are valuable industrial compounds. They show excellent antimicrobial activity[1] and serve as a building block for the synthesis of a variety of compounds, like antibiotics such as cefamandole[2] and cephalosporin C[3], anti-obesity[4], antithrombotic[5], antitumor[6], and nonsteroidal anti-inflammatory drug (NSAID)[7] synthesis. They are used as chiral resolving agents[8] and for determining the absolute configuration of secondary alcohols[9].

Due to their importance, the preparation of optically pure mandelic acid and its ester derivatives has been of great interest[10–12]. Optically pure mandelic acid can be produced using various physicochemical methods[13,14]; however, these methods have several disadvantages, including reliance on costly and toxic catalysts, the requirement of harsher reaction conditions, energy-intensive operations, the generation of unwanted byproducts, and low purity[13,14].

Biocatalysts are considered a superior alternative to chemical/physicochemical methods for the synthesis of chirally pure compounds. They confer multiple advantages over chemical/physical processes; for instance, enzymatic approaches generally have shorter synthetic routes, and protection of the functional group is not required. They can operate at mild reaction conditions, i.e., at moderate temperature, neutral pH, and atmospheric pressure, thereby minimizing the problem of isomerization, epimerization, rearrangement, decomposition, and other undesired side reactions often associated with traditional chemical reactions[15].

Enzymes from various sources have been investigated for the chiral resolution of mandelic acid[16–18]. The most common approach for obtaining a single enantiomer using lipase/esterase is through enantioselective transesterification in inorganic, organic/aqueous, and ionic liquid or by ester hydrolysis in aqueous media[19]. While transesterification in organic solvents or organic solvent-aqueous mixtures accepts the highest substrate

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concentration, using organic solvents makes the process less environmentally sustainable. The hydrolytic approach generally does not require the use of any organic solvents, thereby providing an environmentally favorable approach, but it suffer from the low substrate or product concentration tolerance of the enzyme[20].

Various microbe-mediated manufacturing methods, including microbial biotransformation, enzymatic catalysis, and strategies for optimizing bioconversion efficiency, are currently being studied[4]. Styrene, biobased L-phenylalanine, and feedstocks such as glycerol and glucose are also used as biocatalytic approaches to yield (R)-mandelic acid[21]. (R)-(-)-mandelic acid was also synthesized via two-step biotransformation using *Saccharomyces cerevisiae* CGMCC No. 3361, resulting in an R-mandelic acid conversion and enantiomeric excess (*ee*) of 99.8% and 100% respectively[21]. Styrene oxide was transformed to R-mandelic acid by endogenous incomplete oxidation and heterologous epoxide hydrolase (SpEH) by a novel cell factory in *Gluconobacter oxydans*[22]. Various phenylalanine derivatives, such as chloro-, fluoro-, and hydroxylated amino acids, have also been used to produce S-mandelic acids with high conversion (21–87%) and enantiomeric excess (ee) (38–97%)[23].

Enzymes are more environmentally friendly as they are produced from renewable resources and are biodegradable. Therefore, several enzymes, such as nitrilase[25], carbonic anhydrase[26], a combination of mandelate dehydrogenase and laccase[27], mandelate racemase[28], and D-lactose oxidase[29], are utilized for biosynthesis. Esterases and lipases, particularly, have been investigated due to their enantioselectivity. They have several biotechnological uses, including the production of biopolymers, medicines, agrochemicals, and taste compounds[24]. They have robust properties, including no requirement for cofactors for activity, high regio- and stereoselectivity, organic solvent tolerance, diverse catalytic activities, such as hydrolysis, esterification, acidolysis, and aminolysis[25,26].

Microorganisms are considered a highly potent source of enzymes used in various industries[27,28]. Discovering enzymes with sought-after properties from microorganisms is a tedious process. Traditionally, the only way to screen an enzyme with desired properties/activities involved cultivating bacteria in the laboratory. This cultivation-based approach was fundamentally limited by the fact that the majority of the microbiome remains unculturable[29], therefore preventing access to their enzyme. Metagenomics has emerged as a powerful alternative to traditional culture-based methods. It is a powerful tool for finding a new type of enzyme from previously uncharacterized sites and finding new genes that could not be found by traditional culture techniques because of a lack of information about the nutritional and growth condition requirement. Over the years, screening of metagenomic libraries has dramatically increased the number of biocatalytically useful enzymes[30].

Numbers of industrially relevant enzymes, such as lipase, esterase, etc., have been discovered from the activated sludge metagenome[31] however, effluent treatment plant sludge is less explored for the isolation of family VIII esterases. This study describes the isolation and biochemical characterization of a new family VIII esterase through functional screening of an ETP sludge metagenome library for the first time.

Materials and methods

Sample collection, bacterial strains, vectors, and culturing conditions

An industrial ETP sludge sample was collected from a pesticide company with a batch production schedule for multiple chlorinated and organophosphorus pesticides. *E. coli* DH10β (Gibco-BRL) was used for metagenomic library construction and sub-cloning host. *E. coli* BL21(DE3) (Invitrogen) was used for EstN3 gene overexpression. Plasmids pUC19 and pET28a (+) were used for metagenomic library construction and protein expression, respectively. Standard Luria Bertani (L.B.) culture and plates were used for *E.coli* cultures, and ampicillin (100 µg/ml) or kanamycin (40 µg/ml) was used for selection.

Metagenomic DNA isolation, library preparation, and functional screening of lipolytic genes

Microbial cells were pelleted by centrifugation (8000 g, 10 min). Metagenomic DNA isolation and library construction were done as described previously[32]. The pellet was resuspended in 20 ml extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1% CTAB) and incubated at 37 °C for 15 min. Lysozyme (5 mg/ml) was added and incubated for 1.0 h, followed by Proteinase K (2.5 mg) and 1% SDS at 65 °C for 1.0 h. The lysate was centrifuged (8000 g, 10 min), and the supernatant was extracted with phenol: chloroform (1:1). DNA was precipitated using 0.6 V isopropanol, pelleted (10,000 g, 20 min), washed with 70% ethanol, air dried, and resuspended in TE buffer. Further purification was done using CTAB precipitation.

For Metagenomic Library Construction, ten micrograms of pond metagenomic DNA were partially digested with Sau3A1, and 2–12 kb fragments were gel-purified. About 200 ng of the digested DNA was ligated into BamHI-digested, dephosphorylated pUC19 vector. The ligation mix was electroporated into *E. coli* DH10B (Micropulser II, Bio-Rad; 2.5 kV, 200 Ω, 25 µF). Transformants were selected on L.B. agar plates containing ampicillin.

Esterase screening was done using a method previously described[33]. Transformants were screened on L.B. ampicillin plates containing 1.0% tributyrin. Clones producing clear halos after 48 h at 37 °C were selected as lipolytic. Positive clones were re-streaked to obtain single colonies and re-confirmed for tributyrin hydrolysis. Re-transformation of the plasmids obtained from positive clones was done to confirm the plasmid-borne nature of the lipolytic activity.

DNA sequence analysis

Primer walking, sequence assembly, and curation of the inserts were performed following the method previously described[34]. Open reading frame (ORF) prediction was done by ORF finder, and the amino acid sequence of each identified ORF was used to find the best hit using the Basic Local Alignment Search Tool (BLAST). Translated protein sequences were queried against the NCBI nr database. Multiple Sequence Alignment (MSA) was done using CLUSTALW[35]. The maximum likelihood method of MEGA version X software (https://www.megasoft

ware.net)[36] was used to construct the phylogenetic tree, employing Poisson Model settings and homogeneous patterning between lineages with 1000 bootstrap replicates. Bootstrap values at nodes were expressed as percentages.

Overexpression and purification of EstN3

Gene-specific primers EstN3F: 5'- CAT*GCTAGC*GACGCCCAGTCGCAGTGGACCG-3' and EstN3R: 5'-GTG*CTCGAG*TCAGTCGATGATCGCGCTGTA-3' were used to amplify *EstN3*. Restriction digestion was done with NheI/XhoI restriction enzymes and cloned into the pET28a (+) vector with the in-frame fusion of the N-terminal Polyhistidine Tag. The recombinant strain was grown in L.B. media with kanamycin (40 µg/ml) at 37 °C until an optical density of 0.5 at 600 nm. Culture was induced with 50 µM Isopropyl β-d-1-thiogalactopyranoside (IPTG) and maintained at 16 °C with shaking at 200 rpm overnight. After incubation, the culture was pelleted down by centrifugation at 8000 rpm for 10 min at 4 °C for harvesting. Harvested cells were resuspended and disrupted by sonication in pH 8.0, 50 mM Tris-HCl containing 300 mM NaCl. Cell debris was removed by centrifugation at 16,000 rpm for 30 min (4 °C). The supernatant was loaded onto a Ni-NTA column, and a five-bed volume of 20 mM imidazole-containing buffer was passed to remove non-specifically bound proteins. The firmly bound protein was eluted using a buffer containing 250 mM imidazole. Desalting was done on PD10 gel filtration columns (GE Healthcare) in 50 mM sodium phosphate buffer at pH 7.0. The proteins' purity was determined using a 10% SDS-PAGE. The protein concentration was determined using the Bradford method[37].

Biochemical characterization of esterase

The p-nitrophenyl (pNP) ester stocks were prepared using an 80:20 (v/v) ratio of acetonitrile to isopropanol. The reactions were initiated with enzyme addition and incubated at 37 °C for 15 min, while control experiments were conducted simultaneously. Absorbance was measured at 405 nm using a BioPhotometer (Eppendorf). Each reaction was performed in triplicate.

Esterase substrate preference (Specificity) was determined by using p-nitrophenyl esters of varying aliphatic chain length, i.e. pNP-acetate (C2), pNP-butyrate (C4), pNP-valerate (C5), pNP-caprylate (C8), pNP-decanoate (C10), pNP-dodecanoate (C12), pNP-myristate (C14), pNP-stearate (C18) as a model substrate. 0.1 mM ester substrate was taken in 0.5 ml of 50 mM sodium phosphate buffer (pH 7.0). The reaction was initiated by adding purified enzymes, and the tubes were incubated at 37 °C for 15 min. Differences between short- and long-chain substrate groups were assessed using the Wilcoxon rank-sum test in R {short-chain: pNP-acetate (C2), pNP-butyrate (C4), pNP-valerate (C5), long-chain: pNP-caprylate (C8), pNP-decanoate (C10), pNP-dodecanoate (C12), pNP-myristate (C14), pNP-stearate (C18)}.

The enzyme kinetics was studied using different concentrations of pNP-valerate in the 1000–4000 µM concentration range. Kinetic parameters, i.e., K_m and V_{max} for the enzyme, were calculated using Prism 7.0 (GraphPad, San Diego, USA) by fitting the data to a non-linear regression analysis.

Optimum temperature and pH profiling

The optimal temperature for enzyme activity was identified by running an enzyme assay at temperatures ranging from 10 °C to 80 °C while maintaining all other assay parameters constant and only modifying the incubation temperature.

Overlapping pH buffers {50 mM sodium phosphate buffer for pH 6.0, 6.5, and 7.0; 50 mM Tris-Cl Buffer for pH 7.0, 7.5, 8.0, 8.5, and 9.0; and 50 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer for pH 9.0, 9.5, and 10.0} were used to identify the optimum pH. Esterase activity was determined using p-nitrophenol standard curves for each buffer.

Enzyme inhibitors assay

The effect of potential inhibitors phenylmethylsulfonyl fluoride (PMSF), N-ethyl maleimide (NEM), diethylpyrocarbonate (DEPC), 4-hydroxybenzene mercuric acid (pCMB), and mercuric chloride ($HgCl_2$) on esterase activity with three inhibitor concentrations (0.1 mM and 1.0 mM, and 5.0 mM) was investigated by methods previously described[34].

Metal ion and solvent tolerance assay

The effect of metal ions was determined by incubating enzymes in the presence of 1 mM and 5 mM Ca2+, Mg2+, Ni2+, Mn2+, Zn2+, Cu2+, and Fe2 + on ice for 1 h, followed by determination of residual activity. To determine the effect of organic solvents on esterase activity, 10% and 50% (v/v) concentrations of ethanol, isopropanol, acetonitrile, acetone, dimethyl sulfoxide, and dimethylformamide were used. The enzyme activity without additives was defined as 100%.

Methyl mandelate hydrolysis assay

Methyl mandelate hydrolysis was performed at 37 °C in 100 mM sodium phosphate buffer (pH 7.0) following the protocol previously described[34]. Chiral HPLC was used to measure the optical purity of R-mandelic acid. The yield of R, S (±) enantiomers in mandelic acid was measured using Chiralpak IA (Daicel Chemical) at a wavelength maximum (λmax) of 228 nm. The mobile phase was n-hexane: isopropanol: trifluoroacetic acid (94:6:0.2) at a flow rate of 0.7 ml/min. Enantioselectivity is given as the E value, which was determined from the enantiomeric excess of the generated acid and the degree of conversion according to[38].

$$\frac{ln[1-c\,(1+eeP)]}{ln[1-c\,(1-eeP)]} = E$$

Results

Screening of the metagenomic library

Functional screening of the metagenomic library revealed several clones forming a clearance zone around *E. coli* colonies on the L.B.-tributyrin plate (Supplementary Fig. 1). EstN3 was selected for further characterization due to its ability to hydrolyze methyl-mandelate in secondary screening. Sequence analysis of the inserted DNA 1233 bp gene that encodes a 410 amino acid beta-lactamase domain-containing protein. The predicted molecular weight was 45 kDa. Sequence similarity search revealed that EstN3 shows high sequence identity (more than 83%) with serine hydrolases from *Phenylobacterium* and *Caulobacter* species. Among the enzymes with reported biochemical properties, it shows 79.5% amino acid sequence identity with EstG34[39], a compost metagenome feruloyl esterase from an uncultured organism, and 53.23% identity with EstM-N2[40], an arctic soil metagenome-derived esterase from an uncultured bacterium. SignalP-5.0 analysis indicates that EstN3 does not have a signal peptide.

Sequence analysis of the putative esterase gene

The amino acid sequence analysis (Fig. 1) revealed the existence of an SMTK (72–75 residues) consensus sequence, which corresponds to the conserved SxxK motif, which is well-conserved in class-C β-lactamases, family VIII carboxylesterases, and penicillin-binding proteins[31]. In addition, it also contains the YSL (position 194–196), WGG (position 364–366), PLGMKDTAF (position 224–232), LRYQPGAAW (position 184–192), and GGGG (position 274–277) motifs representing the YSx, KTG, PLGMxDTxF, LxxxPGxxW, and GGxG motif, which have been described in other family VIII esterases[41–43]. Phylogenetic analysis of EstN3 with other family VIII members indicates that it clustered in family VIII (Fig. 2).

Overexpression of EstN3

Purified EstN3 was obtained by Nickel-NTA affinity chromatography, after overexpression in *E. coli* BL21(DE3). A distinct band at ~41 kDa was observed for the purified protein on 10% SDS-PAGE. The presence of only one dominant band of EstN3 suggests high purity (Supplementary Fig. 2).

Biochemical properties of EstN3

The substrate specificity of EstN3 was investigated towards pNP esters: pNP-acetate (C2), pNP-butyrate (C4), pNP-valerate (C5), pNP-caprylate (C8), pNP-decanoate (C10), pNP-dodecanoate (C12), pNP-myristate (C14), pNP-stearate (C18) with increasing aliphatic chain length. It displayed the highest hydrolytic activity on the esters with small aliphatic chain length, with 89, 90, and 100% relative activity on pNP-acetate, pNP-butyrate, and pNP-valerate, respectively (Fig. 3A). Its activity decreased significantly towards esters with longer chain length, displaying less than 10% activity with a carbon chain length greater than 6 (*Wilcoxon test*, $p = 6.3 \times 10^{-5}$). The kinetic parameters, K_m and V_{max} of EstN3, were 1524 µM and 386.1 µM/min/mg on pNP valerate, respectively and demonstrated a catalytic efficiency of 0.033 $s^{-1} \cdot \mu M^{-1}$ (Fig. 3B). Temperature and pH profiling assays show that EstN3 displays maximum activity at 45 °C and 8.5 pH (Fig. 3C and D).

We investigated the effect of known esterase/lipase inhibitors, i.e., PMSF, PCMB, $HgCl_2$, NEM, and DEPC, on EstN3. Except for the NEM, EstN3 was strongly inhibited by the PMSF, $HgCl_2$, and DEPC (Table 1). Esterase activity was not significantly affected by Ca^{2+}, Mg^{2+}, Ni^{2+}, and Mn^{2+}. However, it was significantly inhibited by Cu^{2+} and Fe^{2+} (Table 2). Various organic solvents were tested in 10 and 50% v/v final concentrations for their effect on the EstN3's activity, finding that- 10% (v/v) DMSO, ethanol, and isopropanol had a stimulatory effect on the enzyme activity, and retains more than 80% activity of acetone and acetonitrile and 60% in enzyme lost all its activity in 50% (v/v) solvents (data not shown).

The hydrolytic capacity of the purified esterases of racemic methyl mandelate was checked. EstN3 displayed selectivity for S-methyl mandelate. It produced S-mandelic acid at an enantiomeric excess (e.e.) of 96.26% at 37% conversion (Fig. 4).

Discussion

Among all three life forms, microbes, including bacteria, fungi, yeasts, and actinomycetes, are significant sources of industrially relevant enzymes[44,45]. Earth has an enormous diversity of microorganisms, and a substantial portion of biodiversity remains unexplored, which could harbour millions of different genes/proteins/enzymes possessing different biological and industrial properties[46]. The culture-dependent method has a significant limitation in that the majority of the microbiome is not cultivable[29], therefore preventing access to their enzymes. With the advances in molecular technology, metagenomics emerged as an effective alternative to culture-based methods[47]. Metagenomics is a culture-independent approach that involves extracting and cloning the collective genomic DNA of a microbial community into appropriate vectors. The resulting metagenomic library can then be screened using either sequence-based or activity-based methods to identify genes of interest. Metagenomic studies have successfully identified enzymes with high biotechnological potential[26,30,48].

Metagenomic research has primarily focused on compost, as it contains several valuable enzymes, including endoglucanase, xylanase, and esterase[49,50]. Many esterases have also been isolated from the compost metagenomic library, including EstM2, Est7K, Est8L, Est2K, EstCS3, and Est13L[42,43,51–53], whereas only a few enzymes have been reported from the activated sludge library[54,55].

We have identified an esterase, EstN3, from the functional screening of the metagenomic library from an industrial ETP sludge sample. EstN3 had the highest amino acid sequence identity of 89% with an uncharacterized serine hydrolase from *Phenylobacterium sp.* RIFCSPHIGHO2_01_FULL_69_31. Among the enzymes with reported biochemical properties, it shows 79.5% and 53.23% sequence identity, respectively, with esterase EstG34[39] and EstM-N2[40], which belong to family VIII esterases. Phylogenetic analysis indicates that

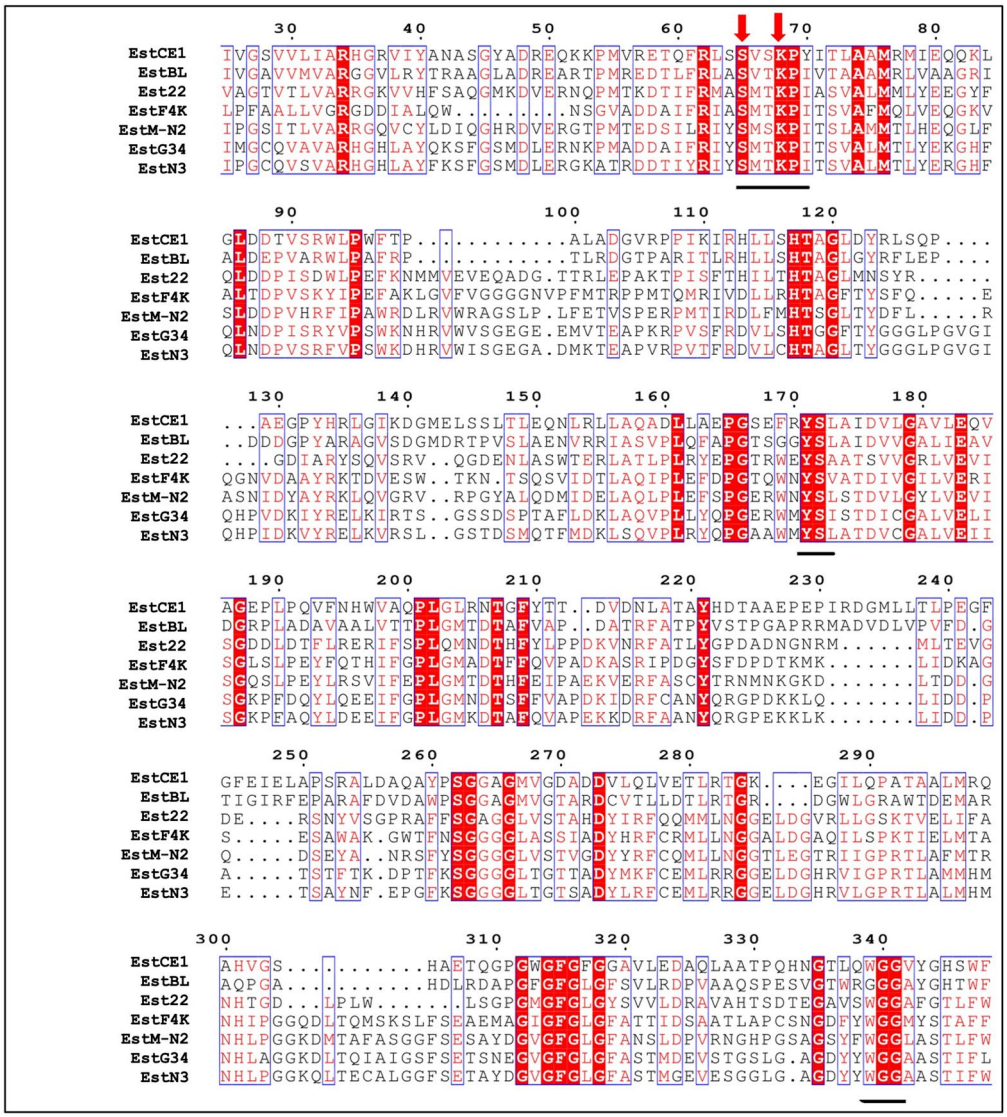

Fig. 1. Multiple sequence alignment of EstN3 and selected family VIII.2 esterases (Selected Region only). Multiple sequence alignment was performed with Clustal Omega (1.2.4) and visualized with ESPript 3.0. Conserved residues are shaded in red (0.7 Similarity threshold). The catalytic Ser and Lys are marked with an arrow. The number at the beginning represents the position of the amino acid in the sequence. The abbreviations and accession numbers of proteins used in this analysis are as follows: EstCE1 [uncultured bacterium pCosCE1] (AAY90130.1), EstBL [*Burkholderia cepacia*] (AAX78516.1), Est22 [uncultured bacterium] (AGT17593.1), EstF4K [uncultured microorganism] (AEH57832.1), EstM-N2 [uncultured bacterium] (AEA07655.1), EstG34 [uncultured microorganism] (AIL90340.1).

it belongs to the esterase/lipase family VIII. It contains the amino acid motifs SxxK, YSx, WxG, PLGMxDTxF, LxxxPGxxW, and GGxG, reported in other family VIII esterases[41–43]. EstN3 lacks the traditional esterase/lipase GxSxG pattern, which contains the catalytic serine residue. Instead, the serine residue of the SxxK motif has been proposed as the catalytic nucleophile[56], and the lysine residue has been suggested to take part in the acylation step[42]. The YSx motif's tyrosine residue is believed to play a role in substrate recognition and control of substrate specificity[41]. Another motif, i.e., KTG, of class C β-lactamases, is replaced with the WGG motif in EstN3. While the motif is not strictly conserved in family VIII, the glycine residue within it appears to be more consistently conserved[57], and esterases containing this motif have been reported to display higher acyltransferase activity[58].

EstN3 was most active on short-chain esters, with maximum activity on the pNP-valerate (Fig. 3A), similar to other family VIII members[40,59]. It displayed less than 10% activity on ester with chains longer than C5. This strong preference for a smaller chain substrate indicates that EstN3 is a true esterase rather than a lipase. EstN3 shows the highest activity at pH 8.5 and 45 °C (Fig. 3C, D). It retains more than 50% activity in the 7.0 to 9.0 pH range and 45% at pH 10. EstN3 shows a broad temperature activity range, displaying 60% maximum activity at 20 °C and 51% at 60 °C. These biochemical properties indicate that EstN3 is a mesophilic and slightly alkaliphilic enzyme. EstN3's ability to hydrolyze short-chain esters under milder temperatures and pH could be helpful in

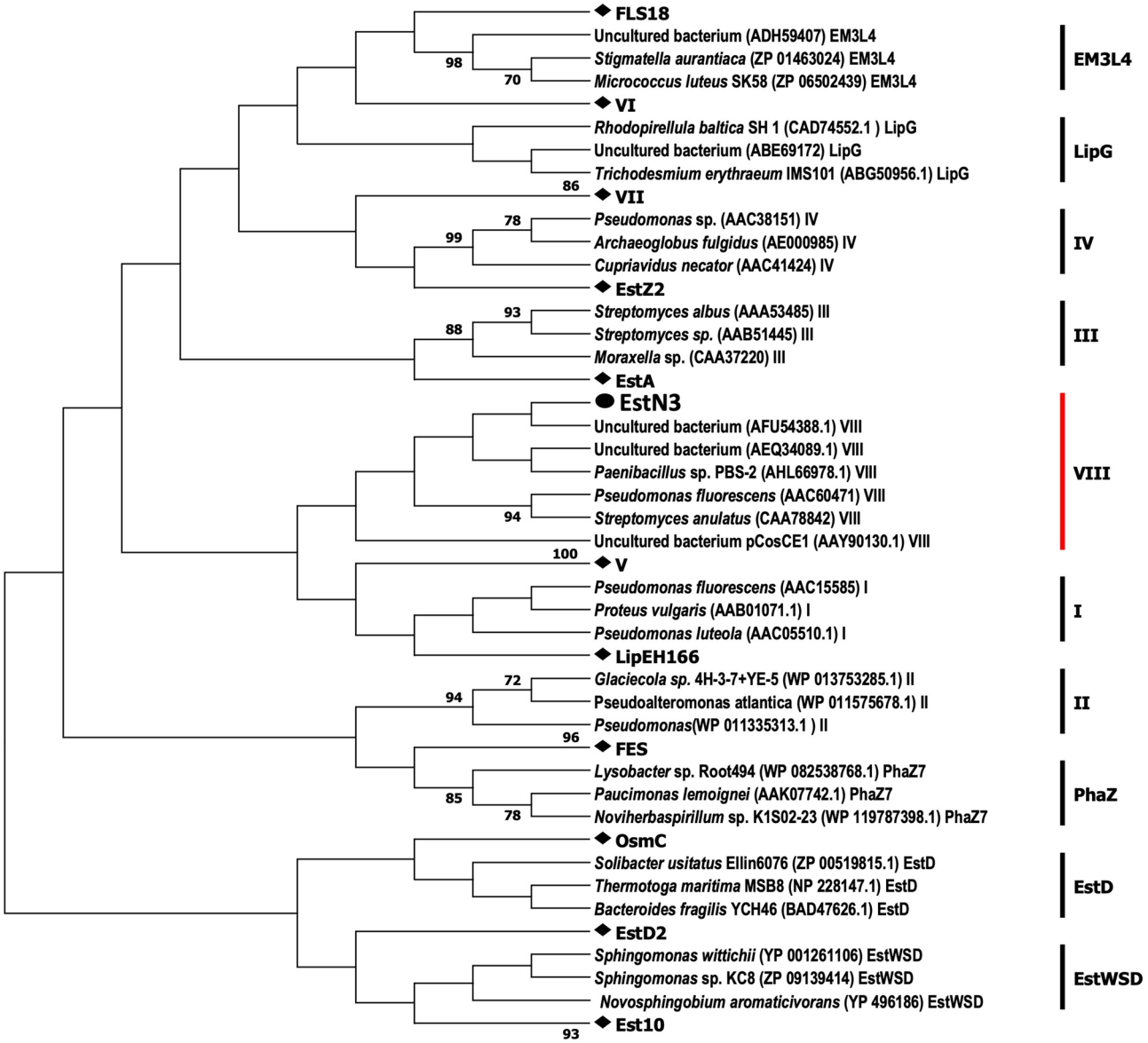

Fig. 2. Phylogenetic analysis of EstN3. The phylogenetic tree was generated using the maximum-likelihood method (MEGAX software). The protein sequences for related lipolytic enzymes were retrieved from GenBank. The numbers at the nodes indicate the bootstrap percentages of 100 replicates.

food processing and synthesizing flavoring compounds. The broad temperature activity range of EstN3 could be beneficial for various applications. Enzymes with high activity at lower temperatures could help to decrease the cost of operation in an industrial setting by carrying out the reaction at a lower temperature, reducing the energy consumption, and have potential applications in bioremediation of soil, detergent formulation, and in the synthesis of thermolabile compounds[60].

Compared to other family-VIII enzymes, kinetic and physicochemical profile of EstN3, a Km of 1524 µM for pNP-valerate, catalytic efficiency of 0.033 ($s^{-1}\cdot\mu M^{-1}$) and optima at pH 8.5 and 45 °C indicates moderate substrate affinity but a comparable alkaline preference and mesophilic behaviour. However, in contrast, some family-VIII members from cold environments show much lower temperature optima (EstM-N2), while compost-derived alkaline esterases (Est7K) display similar pH optima, suggesting EstN3 is particularly suited for aqueous hydrolytic resolution at moderate temperatures and alkaline conditions[40,43].

EstN3 has conserved SxxK (SMTK) and YSx motifs, which together with strong inhibition by PMSF and DEPC support a serine-based nucleophile with an adjacent basic residue contributing to acylation, while potent loss of activity with thiol-directed reagents ($HgCl_2$, PCMB) and inhibition by Cu^{2+}/Fe^{2+} point to the functional importance of cysteine or other metal-labile side chains near the active site. These results reflects patterns reported for other family-VIII esterases (for example EstQE and Est7K) and provides a structural rationale for preference of short-chain substrates by EstN3. These observations therefore strengthen the assignment of

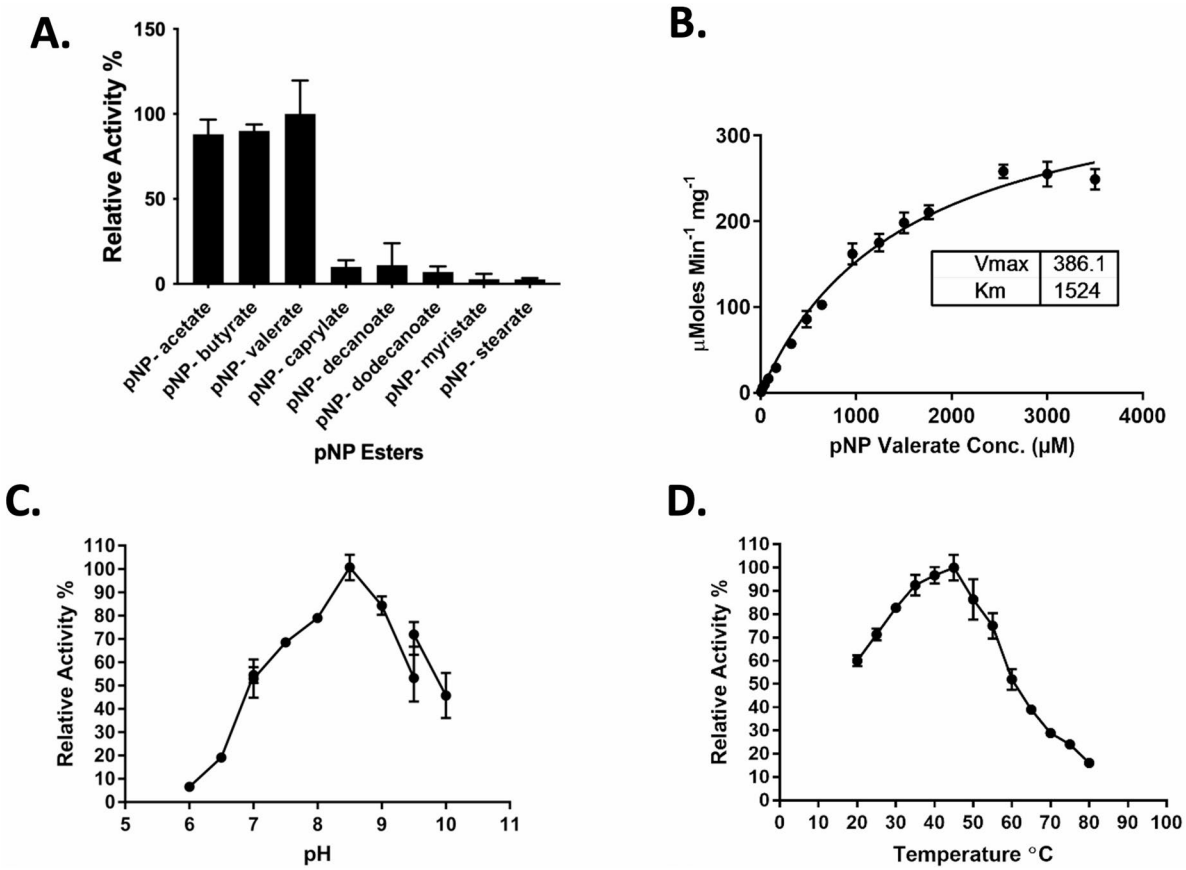

Fig. 3. Biochemical assay of EstN3 (**A**) Substrate preference of EstN3 towards various p-nitrophenyl esters: Reaction without the enzyme was taken as a control. Absorbance was recorded at 405 nm in a BioPhotometer (Eppendorf). All reactions were carried out in triplicate. The highest activity (3.88 +/- 0.1 µMole min^{-1} mg^{-1}) was taken as 100%. (**B**) Kinetic Parameter of EstN3: For kinetic parameters (K_m and k_{cat}), enzyme activity was determined over a range of pNP-valerate concentrations (0–3500 µM), at 37 °C, pH 7.0, for 15 min. Kinetic parameters were calculated by non-linear regression analysis of raw data fit (to the Michaelis-Menten functions) using GraphPad Prism software (version 7.00 for Windows). (**C**) Effect of pH on the activity of EstN3: Assays were carried out in different pH buffers (50 mM sodium phosphate buffer for pH 6.0, 6.5, and 7.0; 50 mM Tris-Cl Buffer for pH 7.0, 7.5, 8.0, 8.5, 9.0- and 50-mM N-cyclohexyl-3-aminopropane sulfonic acid (CAPS) Buffer for pH 9.5, and 10.0). Esterase activity was calculated using p-nitrophenol standard curves for each buffer. All the tests were performed at 37 °C using pNP-valerate as the substrate. All reactions were carried out in triplicate, and the highest activity (96.79 +/- 2.4 µMole min^{-1} mg^{-1}) was taken as 100%. (**D**) Effect of temperature on the activity of EstN3. The enzyme activity was measured using pNP-valerate as a substrate at temperatures ranging from 10 to 80 °C and pH 7.0. All reactions were carried out in triplicate. The error bars represent the standard deviation of the means. The highest activity (74.83 +/- 0.25 µMole min^{-1} mg^{-1}) was taken as 100%.

EstN3 to the class-C β-lactamase-type (family VIII) catalytic framework and show how motif architecture and chemical sensitivity together explain esterase activity[43,58,61].

Esterases have demonstrated extraordinary stability in organic and organic-aqueous solutions[62,63]. Organic solvents can affect the activity, stability, regiospecificity, and stereoselectivity of the enzymes, allowing multiple applications of the same enzyme[64–66]. We investigated the solvent tolerance of EstN3 in a range of organic solvents. At 10% (v/v) concentration, its activity was increased by 10% in the presence of ethanol, 5% in isopropanol, and 18% in the presence of DMSO. It exhibits mild sensitivity to acetone, acetonitrile, and DMF, retaining 87%, 92%, and 60% activity in the respective solvents; however, it loses all of its activity at a 50% (v/v) concentration (Table 3).

Purification and enrichment of the particular enantiomer can be accomplished with enantioselective esterases[41]. Enantiopure methyl mandelate and mandelic acid are crucial chiral building blocks in the pharma and cosmetic industries. We tested the chiral resolution capability of the EstN3 on the racemic methyl mandelate. It displayed excellent enantioselectivity towards S-methyl mandelate. With 37% conversion, it showed a 96.26% *ee* and E value of 93 (Fig. 4). By selectively hydrolysing the (S)-form enantiomer, EstN3 can be utilised to purify the racemic mixtures and enrich the (S)-form enantiomer.

Inhibitor	Concentration (mM)	Residual Activity %
Control	0	100 ± 5
PCMB[a]	0.1	4.49 ± 0.39
	1	5.77 ± 0.49
	5	6.45 ± 0.29
$HgCl_2$[b]	0.1	3.66 ± 0.036
	1	3.86 ± 0.048
	5	3.38 ± 0.084
DEPC[c]	0.1	9.82 ± 1.16
	1	2.76 ± 0.096
	5	2.31 ± 0.70
PMSF[d]	0.1	5.42 ± 1.0
	1	3.38 ± 0.24
	5	2.29 ± 0.048
NEM[e]	0.1	101.07 ± 10.96
	1	82.65 ± 5.81
	5	76.68 ± 16.06

Table 1. Effect of different inhibitors on EstN3's activity: assays were carried out by incubating the protein with three inhibitor concentrations (0.1 mM, 1 mM, and 5 mM). Activity without an inhibitor was taken as 100%. All reactions were carried out in triplicate. The residual activity percentage is presented as a mean value with standard deviation. [a]:p-chloromercuriobenzoate, [b]: mercuric chloride, [c]: diethyl pyrocarbonate, [d]: phenylmethylsulfonyl fluoride, [e]: N-ethylmaleimide.

Cation	Relative activity %	
	1 mM	5 mM
Ca^{2+}	85.63 ± 1.13	89.42 ± 3.43
Mg^{2+}	79.09 ± 1.43	93.65 ± 11.68
Zn^{2+}	81.49 ± 3.85	40.15 ± 10.15
Ni^{2+}	71.79 ± 0.36	67.85 ± v3.32
Mn^{2+}	77.39 ± 1.33	74.08 ± 2.26
Cu^{2+}	10.08 ± 1.71	1.10 ± 0.65
Fe^{2+}	5.37 ± 3.44	ND

Table 2. Effect of various divalent cations on EstN3's activity: cation tolerance was evaluated by measuring esterase activity at 1.0 and 5.0 mM cation concentration. Divalent cations on EstN3's Activity. All reactions were carried out in triplicate. The relative activity percentage is presented as a mean value with standard deviation. Reaction without enzymes was taken as control.

Based on the literature search, we discovered that EstN3 is the only member of family VIII esterase reported to have enantioselectivity towards methyl mandelate. A further literature search revealed that lipase-based resolution is primarily based on a transesterification reaction, whereas esterase-based resolution usually involves estereolytic activity. A lipase from *Pseudomonas stutzeri* LC2-8 demonstrated enantio-selective hydrolytic activity on methyl mandelate by selectively acylating S-mandelic acid in diisopropyl ether using vinyl acetate as an acyl donor[67]. It had the highest reported substrate tolerance of 180 mM. Apart from those two lipases, most lipases and esterase-catalyzed enantiomeric resolution either have low selectivity (Table S1) or resolution as carried out at lower substrate concentration than EstN3. If we compare enantiomeric resolution based on hydrolysis in aqueous media, EstN3 has the highest working substrate concentration tested (Table S1).

There are advantages of using hydrolytic reaction in the aqueous system, as some of the organic solvents that are used in lipase/esterase-based resolution, such as diisopropyl ether (DIPE), cyclohexane, are expensive, difficult to recover, and difficult to handle at an industrial scale, as they require special safety precautions[68].

Family VIII esterases are one of the least explored esterases for their biotechnological applications. Only a handful of family VIII enzymes have been investigated for their chiral selectivity. Est8L, a family VIII esterase, showed enantioselectivity for the (S)-methyl-3-hydroxy-2-methylpropionate[52]. Est7K, another member of family VIII esterase, was isolated from a compost metagenomic library. It has particular S-enantiomer selectivity towards (S)-methyl-3-hydroxy-2-methylpropionate[43]. However, no family VIII esterase has been found to have enantioselectivity towards mandelic acid; therefore, EstN3 offers an excellent example of the biotechnological potential of this family. EstN3 tends to be highly valuable in the pharmaceutical and fine chemical industries due to its capacity to specifically identify S-mandelic acid. Derivatives of enantiopure mandelic acid are utilized

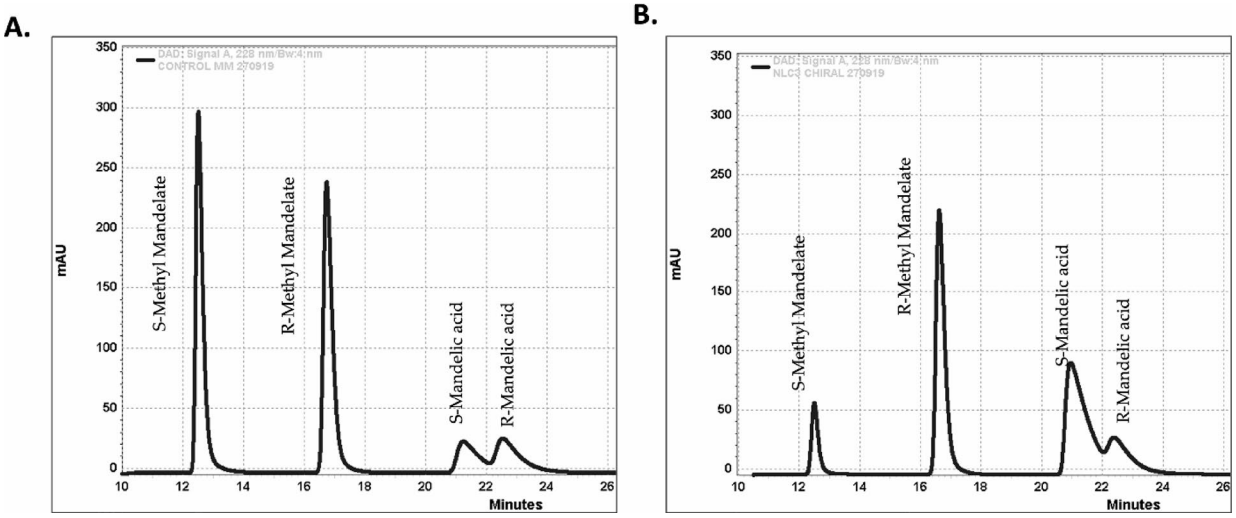

Fig. 4. Chiral HPLC chromatograms: **(A)** Methyl mandelate control reaction chromatogram. **(B)** Methyl mandelate with EstN3 chromatogram. The reaction was performed in a 5 mL volume in a pH 7.0, 50 mM sodium phosphate buffer at 37 °C. Samples were analyzed by HPLC using a Chiralpak IA (Daicel Chemical). Absorbance was measured at λ_{max} = 228 nm, and samples were eluted using n-Hexane: Isopropanol: Trifluoroacetic acid (94: 6: 0.2) as the mobile phase at a flow rate of 0.7 ml/min.

Solvent (10% {v/v})	Relative activity %
Acetonitrile	87.10 ± 1.71
Acetone	92.19 ± 5.13
DMF	60.08 ± 8.13
DMSO	118.12 ± 1.98
Ethanol	109.98 ± 2.66
Isopropanol	105.96 ± 2.24

Table 3. Effect of various organic solvents on EstN3's activity: tolerance was evaluated by measuring esterase activity in 10% (V/V) organic solvents. Activity without any metal or organic solvent was taken as 100%. All reactions were carried out in triplicate. The relative activity percentage is presented as a mean value with standard deviation.

in the manufacturing of cardiovascular agents, anti-inflammatory medications, and antibiotics. As EstN3 is produced from an ETP sludge metagenome library, it offers compared to chemical catalysts.

The finding supports waste-to-resource approaches in biotechnology by highlighting the unrealized potential of wastewater settings such as ETP sludge for the mining of novel biocatalysts.

Conclusion

The gene EstN3 was found and investigated in this work using the ETP sludge metagenomic library. EstN3, encoded by a gene of 1233 bp, is a new member of the family VIII esterase, most active at alkaline pH and at a moderate temperature. It also demonstrated tolerance for divalent cations and retained more than 60% of its activity in various organic solvents.

In addition to evaluating EstN3's enzymatic properties, our study found that EstN3 has good enantioselectivity towards S-mandelic acid. This is a first investigation on the stereoselectivity of S-mandelic acid by a class VIII esterase expressed by the gene chosen from the industrial ETP sludge metagenomic library.

Our examination of the novel family VIII esterase EstN3, which exhibits a wide range and effective stereoselectivity, addresses the deficiency of research on functional genes encoding esterases related to the enantioselectivity of mandelic acid and offers substantial support for the advancement of family VIII esterases as biocatalysts.

Data availability

The *estN3* gene sequence can be retrieved using accession number MT991076 in GenBank.

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Author contributions

Manish Kumar Yadav: Acquisition, analysis, or interpretation of data, Validation, Writing – original draft. Ravi Ranjan: Acquisition, analysis, or interpretation of data. Pratima Verma: Data curation, Writing – review & edit-

ing. Rakesh Sharma: Conception, Supervision, Funding acquisition, and Writing – review & editing. All authors reviewed the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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