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Title: Discovery of a diverse set of esterases from hot spring microbial mat and sea sediment metagenomes

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Abstract

Esterases are an important group of biocatalysts for synthetic organic chemistry. Functional metagenomics allows discovery of novel biocatalysts by providing access to the gene pool of the microbial community of a habitat. Two metagenomic libraries representing the gene pool of sea sediment and hot spring microbial mat were constructed. Functional screening of these libraries resulted in the isolation of total 8 clones with tributyrin hydrolytic activity. Sequence analysis revealed 10 putative lipolytic proteins with 42-99% homology to the protein sequences in the databases, nine of which represented six known esterase families. Four of the encoded proteins represented Family V and amongst others, one each represented the Family VIII, pectin acetylesterase, enterobactin esterase, G-D-S-L family and OsmC domain containing esterase. One unusual lipolytic protein possessed poly-(3-hydroxybutyrate) depolymerase domain fused to lipase/esterase domain. Two phylogenetically related esterases (MLC3 and SLC5) belonging to family V were expressed and purified to homogeneity. The enzymes exhibited environment-adapted temperature optimum and thermostability. MLC3 was able to stereoselectively hydrolyze R-methyl mandelate to produce R-mandelic acid, an important chiral building block, which suggests MLC3 has potential commercial application.

Keywords: Metagenome; Unculturable bacteria; Esterase; Microbial Mat; Sea Sediment.

1 Introduction

A large fraction of environmental bacteria remain unculturable by the routine culturing techniques [1]. Phylogenetic analysis based on 16S rRNA revealed that more than half of the known bacterial phyla are not represented by any cultured members [2]. These unculturable bacteria represent a vast and diverse gene pool for biotechnological applications. Metagenomics aims to explore and exploit the unculturable bacteria from various environmental niches [3]. Function and sequence-based metagenomic approaches have been employed successfully as a powerful tool to identify and isolate biocatalysts from the environmental microbial communities without the need to culture these organisms [4,5].

Many synthetic organic chemistry applications use hydrolases as biocatalysts, and within this class, esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are the dominant biocatalysts [6]. Esterase/lipases are particularly useful for industrial applications as they can act over a broad substrate spectrum, possess high stereoselectivity, do not require co-factors and show exceptional activity in organic solvents [6]. Moreover, these are biodegradable and environmentally-friendly [7]. Lipolytic enzymes were initially classified into VIII families by Arpigny and Jaeger [8]. A large number of new lipolytic genes/enzymes have been identified from different habitats using functional metagenomics [9–11]. Many of these enzymes exhibited unusual properties and represented phylogenetically distinct newer families, increasing the lipolytic enzymes families to XVIII [12].

Heat-active esterase/lipases are important catalysts in biopolymer, pharmaceuticals, and cosmetics' synthesis owing to their thermostability and longer shelf-life [13–15]. Natural hot spring water, sediment, and microbial mats harbor unique bacterial communities that may produce heat-active and thermostable biocatalysts [16–19]. On the other hand, thermolabile esterases are important in food processing [20] and synthesis of heat-sensitive compounds [21,22]. They can also be used for bioremediation of contaminated soils at low temperature [23]. Marine ecosystems are a habitat to various microorganisms that may produce esterases/lipases active at low temperature. They are highly complex and functionally dynamic, encompassing vast microbial and functional diversity [24]. Ocean metagenomics have led to discovery of novel gene variants including that of proteorhodopsin and a variety of enzymes widespread in uncultured marine microbiota [25,26]. Novel esterase/lipase genes have been isolated from sea sediments [27,28] and hot spring soils and sediments [29–31].

The present study was designed to identify new esterases from microbial communities of two distinct habitats, sea sediment and hot spring microbial mat, using functional metagenomics. Screening and sequence analysis revealed 10 putative esterases.

Two phylogenetically related enzymes (MLC3 and SLC5) belonging to family V were expressed, purified and characterized. The enzyme from hot spring microbial mat showed higher temperature optimum and thermostability in comparison to the enzyme isolated from sea sediment sample.

2 Materials and methods

2.1 Sample collection, strains, and culture conditions:

The filamentous microbial mat sample was collected from hot spring (~50°C, pH of 6.8) located in Khir Ganga (Himachal Pradesh, India). The mat was white at the outer surface and slight gray from inside**,** and deposited on stones beneath the hot spring water. The sea sediment sample was collected from the coastal region of Goa (Goa, India). The samples were brought to the laboratory in sterile containers and immediately processed for isolation of metagenomic DNA. *E. coli* DH10B and *E. coli* C41 (DE3) were grown in Luria-Bertani (LB) broth or on LB agar plates at 37ºC. The media were supplemented with ampicillin (100 µg/ml) for maintenance of plasmids.

2.2 DNA manipulation techniques:

The standard procedures for plasmid isolation, restriction enzyme digestion, ligation, and competent cells preparation were used as described by Sambrook and Russell [32].

2.3 Metagenomic DNA isolation and library construction:

Isolation of metagenomic DNA was carried out with minor modification of the protocol described by Zhou and colleagues [33]. Five gram of microbial mat or sea sediment was used for metagenomic DNA isolation. Briefly, microbial mat and sea sediment was suspended in 13.5 ml of extraction buffer [100 mM Tris-HCl (pH 8.0), 100 mM EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1.0% cetyl-trimethyl-ammonium-bromide] and 100 µg/µl of proteinase K. Following incubation at 37 °C for 1 h, with intermittent mixing twice, 1.5 ml of 20% SDS was added and incubated at 65 °C for 2 h with intermittent mixing every 30 min. The supernatant was collected by centrifuging at 7000 *g* for 10 min at room temperature and transferred to a fresh tube. The pellet was resuspended in 5 ml of extraction buffer and 0.5 ml of 20% SDS and incubated at 65 °C for 30 min. The supernatant was collected by centrifuging at 7000 *g* for 10 min at room temperature and added to the supernatant from the first extraction. The previous step was done twice to extract maximum

amount of DNA from the sample. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant, mixed gently, and centrifuged at 8000 *g* for 10 min at room temperature to separate the upper aqueous phase. Metagenomic DNA was precipitated by adding 0.6 V of isopropyl alcohol and washed with 10 ml of 70% ethanol. The pellet was air dried and dissolved in 1 ml of TE buffer, pH 8.0. Metagenomic DNA was further purified by electrophoresis in 0.7% low-melting agarose and high-molecular-weight DNA was eluted from the gel. A metagenomic library was constructed using the procedure described previously [34]. Microbial mat and sea sediment metagenomic DNA was partially digested using Sau3AI. Approximately 250 ng of partially digested DNA fragments in the range of 2-12 kb was ligated into 200 ng of the pUC19 vector which was BamHI digested and dephosphorylated (MBI Fermentas). Ligated product was transformed in *E. coli* DH10B and transformants were selected on LB agar plates supplemented with ampicillin (100 μg/ml). Recombination efficiency was estimated by blue-white screening using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside). Plasmids from randomly selected clones were isolated and digested with EcoRI and HindIII to calculate average insert size of the metagenomic library.

2.4 Screening for lipolytic clones, sequencing and sequence analysis:

Lipolytic clones were isolated by plating the transformation product on LB ampicillin plates containing 1.0 % tributyrin after incubation at 37 ºC for 2-5 days [34]. Clones showing a zone of clearance around the colony were isolated and purified by single streaking and were again confirmed for their tributyrin hydrolase activity. Plasmids were isolated from the positive clones and retransformed to confirm plasmid-borne nature of the lipolytic activity. The inserts in the unique clones were sequenced by primer walking. The sequences were assembled and curated using Lasergene package (DNA Star, USA). Open reading frames in the assembled sequence for each clone were identified by ORF finder at National Center for Biotechnology Information (NCBI) website and the amino acid sequence of each identified ORF was used to find the closest match by BLAST [35]. Bioinformatics analysis of the putative esterases was carried out by previously described methods [34]. The putative ORFs were queried against the NCBI non-redundant nucleotide database. The translated protein sequences were queried against NCBI non-redundant protein database, Lipase Engineering database[36] and the ESTHER database [37]. Conserved domains were identified and analyzed by multiple sequence alignment using CLUSTALW [38], Conserved Protein database [39] and PROSITE [40]. A phylogenetic tree of lipolytic proteins obtained in the

study and previously known lipase families was made using the maximum likelihood method (MEGA7.0 software) [41] using settings of Poisson Model and homogenous patterning between lineages. To identify the esterase gene responsible for tributyrin hydrolase activity in the clones containing more than one gene with similarity to putative esterases/lipases, the genes were cloned by polymerase chain reaction (PCR) amplification under the arabinose inducible promoter and checked for tributyrin hydrolysis activity by plate assay. The plasmid MLC8 was also subjected to transposon mutagenesis and the mutants with the loss of tributyrin hydrolysis activity were sequenced by transposon-specific primers to identify the site of insertion [34]. Genes MLC8a, MLC8b and SLC1b were cloned in pBAD-myc-his-B vector in SacI/XbaI sites**,** whereas SLC1a was cloned in pBAD-myc-his-C vector in XhoI/EcoRI sites fusing the hexahistidyl (His6) tag at carboxy -terminal. The proteins were expressed under arabinose promoter P_{BAD} and checked for tributyrin hydrolase activity by plate assay.

2.5 Cloning, expression, and purification of esterases:

Esterases encoded by MLC3 and SLC5 were amplified by PCR using gene-specific primers (Supplementary information). The amplified fragment of MLC3 was digested with NcoI/EcoRI and cloned in pET28a vector with in-frame fusion of carboxy-terminal hexahistidyl tag. The PCR product of SLC5 was digested with NheI/SacI and cloned in pET28a to obtain an amino-terminal hexahistidyl tag. The resultant plasmids were used to transform *E. coli* C41 (DE3). The recombinant strains were grown in L. B. broth supplemented with kanamycin (40 µg/mL) until 0.6 OD_{600nm} at 37 °C. The cultures were induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 3 h with constant shaking at 200 rpm at the same temperature. The cells were then harvested at 8000 *g* at 4°C for 10 minutes and disrupted by sonication in 50 mM Tris-HCl (pH=8.0). The cell lysate was centrifuged at 16000 *g* to remove debris. The esterases were purified over Ni-nitriloacetic acid agarose (Invitrogen) and eluted with buffer containing 250 mM imidazole. The eluted fractions containing significant amounts of protein were pooled and desalted using PD10 gel filtration columns (GE Healthcare) using 50 mM sodium phosphate buffer, pH 7.0. The purity of the proteins was checked on a 10% SDS-PAGE.

2.6 Enzyme assays:

Esterase activity was measured by the following procedure. The *p*-nitrophenyl (*p*-NP) ester stocks were prepared in acetonitrile: isopropanol (80: 20 v/v). The enzyme reaction

contained 0.1 mM *p*-nitrophenyl butyrate in 50 mM sodium phosphate buffer (pH 7.0). The reaction was initiated by enzyme addition and incubated at 37 °C for 15 min. The substrate and heat-inactivated enzyme controls assays were performed simultaneously. Absorbance was recorded at 405 nm in a BioPhotometer (Eppendorf). All reactions were carried out in triplicate. Substrate specificity was determined using *p*-nitrophenyl acetate (C_2), *p*-nitrophenyl butyrate (C_4), *p*-nitrophenyl caprylate (C_8) and *p*-nitrophenyl palmitate (C_{16}) in the assay conducted at 37 °C.

The enzyme kinetics was determined by using different concentrations of *p*-nitrophenyl butyrate ranging from 0 – 1.25 mM for MLC3 and 0 – 2.5 mM for SLC5 as substrate in the assay. Michaelis–Menten substrate affinity constant (Km) and maximum velocity for the reaction (Vmax) for both the enzymes were calculated.

The optimum temperature was calculated by performing enzyme assays at temperature range from 10 °C to 70 °C by keeping other assay conditions constant and only varying the temperature of incubation. For thermostability estimation of the enzymes, the assay was carried out after incubating the purified SLC5 and MLC3 at 40 °C, 50 °C and 60 °C for different time intervals. Circular dichroism (CD) spectroscopy was used to perform thermal unfolding experiments and calculate the Tm of MLC3. The CD spectra were acquired using a JASCO J-815 spectropolarimeter in a 0.1 cm path cell at a scanning speed of 100 nm/min. Far UV spectrum measurements were performed in the range of 190 to 250 nm. Thermal unfolding measurements were carried out at 222 nm by increasing the temperature from 20-95 °C at a constant rate of 1 °C/min using 14.4 µM of the protein dissolved in 5 mM sodium phosphate buffer (pH 7). The mean residue ellipticity (θ) was calculated using average molecular mass (32.8 kDa) of the protein. The midpoint of the unfolding denotes the Tm of the protein.

The optimum pH for the activity was determined by conducting assays 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-aminopropanesulfonic acid (CAPS) Buffer for pH 9.0, 9.5, and 10.0). Esterase activity was calculated using *p*-nitrophenol standard curves for each buffer.

The effect of potential inhibitors diethyl pyrocarbonate (DEPC), phenylmethylsulfonyl fluoride (PMSF), mercuric chloride ($HgCl_2$) and 4-hydroxybenzene mercuric acid (pCMB) on esterase activity of MLC3 and SLC5 was observed by incubating the protein with varying concentrations (0.1, 1 and 5 mM) of the inhibitors for 1 h in ice

followed by the esterase assay. Residual activity was calculated considering the activity of the control reaction (containing no inhibitors) as 100%.

Hydrolysis of methyl mandelate to mandelic acid and the enantiospecificity was measured using HPLC. For hydrolysis, 10 mM of methyl mandelate dissolved in 50 mM sodium phosphate buffer, pH 7.0 was incubated at 37 °C in a water bath for 6 hour. Samples were taken at different time intervals. Samples were run on a C-18 column using Acetonitrile: Water (65: 35) at a flow rate of 1 ml/min and the hydrolysis was determined at λmax = 259 nm. Optical purity of the R-mandelic acid was determined using chiral HPLC. The R, S (±) enantiomers' yield of mandelic acid were measured using chiralpak IA (Daicel Chemical**)** at λmax = 228 nm using n-Hexane: Isopropanol: Trifluoroacetic acid (94: 6: 0.2) as the mobile phase at a flow rate of 0.7 ml/min.

2.7 Accession numbers:

The nucleotide sequences obtained from lipolytic clones were deposited in GenBank under accession numbers GU331882-GU331889 and the amino acid sequences were deposited under accession numbers ADM63067-ADM63086.

3 Results

3.1 Features of metagenomic libraries:

Hot spring microbial mat library contained around 168,000 clones with 82.5% of recombinants, with an average insert size of 3.57 kb. In total, about 494 Mb of hot spring microbial mat metagenomic DNA was cloned. The sea sediment library contained ~110,000 clones, with 91.2% frequency of recombinants. Restriction digestion analysis indicated an average insert size of ~4.5 kb and the library contained ~451 Mb of sea sediment microbial community genome.

3.2 Screening for functional lipolytic clones:

Screening of ~39,000 clones of mat metagenomic library on tributyrin resulted in the identification of five clones, designated as MLC1-3, MLC7 and MLC8. The presence of clearance zone in all the clones after single colony re-streaking confirmed them to be true positive clones. Restriction digestion analysis revealed that clone MLC1 and MLC2, MLC7 and MLC8 were siblings (data not shown). All the colonies obtained after retransformation of the plasmids isolated from the stable clones produced tributyrin hydrolysis activity indicating its plasmid-borne nature. Finally, three unique clones (MLC2, MLC3 and MLC8) were used

for further analysis. Clone MLC2 possessed the best hydrolytic activity on tributyrin amongst the three (Fig. 1a). About 27,000 clones of sea sediment library were screened for lipolytic activity. Initially, seven functional clones were isolated and designated as SLC1-SLC7. Clone SLC4 appeared to be a false positive clone upon single colony streaking and rescreening it for tributyrin hydrolysis activity. Analysis of the restriction digestion pattern showed that clones SLC1 and SLC7 had similar digestion pattern, thus they appeared to be sibling clones (data not shown). After confirmation of stable hydrolytic activity post plasmid retransformation, five clones SLC1-3, SLC5-6 were processed for further analysis. The clones expressed variable hydrolytic activity on tributyrin and SLC1 possessed the best activity amongst them (Fig. 1b).

3.3 Sequence analysis and phylogeny:

The insert size of the DNA fragments in the recombinant plasmids varied from 2041-4518 bp and GC % ranged from 58.14-68.39 % (Table 1). The nucleotide sequences of clones MLC2 and SLC3 showed no similarity to the sequences in the database indicating that these metagenomic fragments may belong to yet uncharacterized microorganisms. BlastN analysis of other clones revealed 72-97% identity at various coverage levels to sequenced microbes as summarized in Supplementary Table S1. BlastP analysis revealed that the encoded proteins are 42-99% identical at the amino acids level with the sequences in the protein database (Table 1).

The Clone MLC2 (GU331882) had an open reading frame encoding a putative pectin acetylesterase. A putative ribosome binding site (GGGAG) was identified upstream of the start site and a signal peptide was predicted at the N-terminal region. This encoded protein contained truncated pectin acetylesterase domain and an alpha/beta hydrolase superfamily domain. This protein had two G-x-S-x-G motifs, at positions 178-182 (G-C-S-A-G) corresponding to the active site and 206-210 (G-D-S-A-G). It belongs to Carbohydrate Esterase family 12.

Sequence analysis of MLC3 (GU331883) resulted in the identification of a 280 amino acids protein having homology with a hypothetical protein of *Halothiobacillus sp.* XI15. It had complete domains for alpha/beta hydrolase fold, diene lactone hydrolase family, predicted hydrolases/acyltransferases and lysophospholipase. Conserved motif G-x-S-x-G was also predicted by multiple sequence alignment. This lipolytic gene belonged to Family V of bacterial lipolytic proteins (Fig. 2).

MLC8 (GU331884) revealed the presence of two ORFs similar to lipolytic genes in different frames (Table 1). ORF-A was 42% identical to GDSL-like Lipase/Acylhydrolase of *Chloroflexi* bacterium OLB13. It contained domains for G-D-S-L family and S-G-N-H subfamily of lipolytic enzymes which was grouped in Family II (Fig. 2). Enzymes of this subfamily usually contain a distinct G-D-S-L sequence motif (Arpigny and Jaeger, 1999) instead of the conventional G-x-S-x-G lipase/esterase motif and conserved residues Ser, Gly, Asn and His in four conserved blocks I, II, III, and V. ORF-B was similar to a putative esterase and contained domains for enterochelin/enterobactin esterase, which are reported to act on the ester bonds of enterobactin [42]. Ferric enterobactin esterases also possess the conventional G-x-S-x-G motif, are newly discovered and less characterized lipolytic enzymes, which form their own separate family (Fig. 2). Transposon insertions in GDSL-like lipase/acylhydrolase (ORF-A) and acyl-CoA dehydrogenase resulted in loss of activity of the transposon mutants. No transposon insertion was found in the putative esterase gene (ORF-B) out of the 18 negative mutants sequenced, indicating that the tributyrin hydrolase activity was from ORF-A. It also indicates that the GDSL-like lipase/acylhydrolase and acyl-CoA dehydrogenase are part of an operon (Supplementary Fig. S1). The ORFs were further cloned and expressed from an arabinose induced promoter. Expression of ORF-A resulted in tributyrin hydrolysis activity but no activity was observed in clone expressing the ORF-B confirming that tributyrin is not a substrate for the putative esterase encoded by ORF-B (Supplementary Fig. S2).

The clone SLC1 (GU331885) contained two lipolytic genes. ORF-A with the best match with Est16 and ORF-B had the best match with alpha/beta hydrolase fold. PROSITE search revealed the presence of an arginine-rich region (PS50323) at the carboxyl terminus at the protein encoded by ORF-B. Both the encoded proteins harbored complete conserved domains for alpha/beta hydrolase proteins, lysophospholipase and predicted hydrolases/acyltransferases. Multiple sequence alignments revealed the presence of conserved G-x-S-x-G motif for lipolytic proteins. Both the encoded proteins belonged to Family V of bacterial lipolytic proteins (Fig. 2). ORF-A and ORF-B were individually cloned and both the genes possess tributyrin hydrolase activity (Supplementary Fig. S2).

SLC2 (GU331886) matched with serine hydrolase and harbored complete domains for β-lactamase class C (AmpC) and other penicillin binding proteins. PROSITE search led to the identification of prokaryotic membrane lipoprotein (PS51257) at the N-terminal region, which is synthesized along with the precursor signal peptide. It belonged to Family VIII of lipolytic proteins (Arpigny and Jaeger, 1999) as illustrated in the tree (Fig. 2). The members

of this family are reported to have lipolytic rather than β-lactamase activity. The enzymes of this family usually possess the S-x-x-K motif near the C-terminal region rather than the conventional G-x-S-x-G motif.

In clone SLC3 (GU331887), an open reading frame of 1809 bp encoding 602 amino acids protein was identified for hydrolytic activity. An alternate start codon CTG was predicted 447 bp upstream of the ATG start codon (encoding a 751 amino acid protein). The putative ribosome-binding site (GAGGA) was predicted nine base pairs upstream of CTG and was predicted to be the actual start site of the gene. It had an average molecular mass of 82.6 kDa. The encoded protein harbored the complete domains for homoserine acetyltransferase (MET2), alpha/beta hydrolase fold PRK06765 at the amino terminus and poly-(3-hydroxybutyrate) depolymerase (LpqC) and truncated lipase/esterase domains at carboxy-terminus (Fig. 3a). It best matched with a hypothetical protein of marine Gamma Proteobacterium HTCC2148 with 54% identity. The encoded protein contained the typical G-x-S-x-G motifs (at position 64-68, G-A-S-M-G) at the carboxyl terminal in the lipase/esterase region and (at position 423-427, G-N-S-N-G) in the poly-(3-hydroxybutyrate) depolymerase region at the amino terminus.

The clone SLC5 (GU331888) contained an alpha/beta hydrolase protein. The encoded protein harbored complete conserved domains for hydrolases/acyltransferases (MhpC), alpha/beta hydrolase proteins and lysophospholipase (PldB). Multiple sequence alignment revealed the presence of the G-x-S-x-G motif and other conserved residues participating in the catalytic triad and represented Family V of lipolytic proteins (Fig.2).

In clone SLC6 (GU331889)**,** an ORF encoding for an OsmC (osmotically induced protein C) like family protein was identified. Close hits resulted in matches with OsmC (osmotically induced protein C) like family protein and putative hydrolases, and revealed the presence of domains characteristic for lipase/esterase at amino terminal and complete OsmC related domains (TIGR03549) at the carboxyl terminal (Fig. 3b). Homology searches also reveal that this type of protein is widely distributed over the microbial taxa. OsmC like proteins belong to a family of bacterial proteins related to detoxification of organic hydroperoxides [43]. Conserved domains related to lipolytic enzymes G-x-S-x-G and OsmC (two cysteine residues in the C-terminal region) were also deduced from multiple sequence alignments. This protein belongs to a recently described carboxyl-esterase family having a fused OsmC domain [44].

We attempted to clone and express all the ten putative esterase genes by heterologous expression in *E. coli* in order to characterize the enzymes further. Genes SLC1a, SLC1b,

MLC8a and MLC8b were expressed using arabinose promoter and genes MLC3, SLC3 and SLC5 were expressed using T7 promoter with hexahistidine-tags. The esterase genes from MLC2, SLC2 and SLC6 failed to express in any of the two systems. Substrate preference assay using different *p*-nitrophenyl esters and crude enzymes (cell free extract) revealed that all the enzymes prefer short chain esters (C2 and C4) and did not show significant activity for higher chain length substrates (Supplementary Figs. S3-S8). This indicates that all the identified enzymes are esterases.

3.4 Characterization of esterases from MLC3 and SLC5:

Hot spring microbial mat derived enzymes are expected to be thermostable and work at a higher temperature, while the enzymes from sea sediment were expected to be less thermostable and work at a lower temperature. Two phylogenetically related enzymes belonging to family V with an amino acids identity of 46% were identified from these different environments i.e. MLC3 from hot spring microbial mat metagenome and SLC5 from sea sediment metagenome. These two proteins were selected for further characterization and to confirm their adaptation to the specific environment.

MLC3 and SLC5 were purified using immobilized metal affinity chromatography and had molecular weights of 32.8 kDa and 31.2 kDa, respectively (Fig. 4a, 4b). Recombinant esterases from MLC3 and SLC5 could catalyze the hydrolysis of *p*-nitrophenyl esters of short chain length with maximum activity for butyrate and did not catalyze long chain substrates (Fig. 4c**).** The kinetic parameters for MLC3 and SLC5 differed widely, with MLC3 having a Km of 134 µM and Vmax of 14.55 µmol/min/mg (Fig 4d) whereas SLC5 has a Km of 196.5 µM and Vmax of 2.393 µmol/min/mg (Fig. 4e). As expected, MLC3 had temperature optima at 50°C**,** whereas SLC5 had a temperature optimum of 37 °C (Fig. 5a). MLC3 had a specific activity of 0.76 µmoles.min^{-1}.mg^{-1}**,** whereas SLC5 had a specific activity of 0.34 µmoles.min^{-1}.mg^{-1} measured at the temperatures of their optimum activity with *p*-nitrophenyl butyrate as a substrate. MLC3 showed higher thermostability in comparison to SLC5 and retained 96% of the activity at 40 °C and 73% at 50 °C after 1 h but lost complete activity in 15 min at 60 °C, whereas SLC5 retained only 34% of the initial activity at 40 °C and completely inactivated in 15 min at 50 °C (Fig 5b). MLC3 retained 50% of its activity even after five hours of incubation at 50 °C (Fig 5c). CD experiments also indicate the stability of the enzyme at 50 °C. It has a Tm of 67.4 and starts to unfold above 70 °C (Fig 5d). Both the enzymes were active at alkaline pH with maximal activity at pH 8.5 (Fig. 6). All the tested inhibitors reduced the activity of MLC3 and SLC5.

MLC3 could hydrolyze 46.75 % of methyl mandelate to mandelic acid in six hour (Fig. 7). It is enantioselective towards production of R-mandelic acid with enantiomeric excess of 71.25 and enantioselectivity of 11, whereas SLC5 did not hydrolyze methyl mandelate under the test conditions.

4 Discussion

In this work, we identified10 putative esterases by screening two metagenomic libraries representing a hot spring microbial mat and a coastal sea sediment from India. The nucleotide sequence analysis of the clones revealed that only two sea sediment clones matched with significant query coverage and identity to two marine bacteria, while the other clones showed low query coverage and identity between 72 to 79%. Two clones showed no significant similarity to any known sequence in the database. These results indicate that the metagenomic inserts of the clones were originated from yet uncharacterized organisms of these samples. Nine out of ten putative esterases could be assigned to six characterized lipase/esterase families based on sequence homology and phylogenetic affiliation. Interestingly, two putative esterases possessed two complete domains fused in a single protein and may have bifunctional activity. Bifunctional proteins have been identified in the past in a metagenomic study on antibiotic resistance proteins [45] indicating the presence of such fused proteins in nature but the significance of such fusion is not clear. The esterase from clone SLC3 contained an amino-terminal homoserine acetyltransferase domain fused to poly-3-hydroxybutyrate (PHB)-depolymerase domain at the carboxyl-terminal. Only one similar protein was identified in the database**,** which also belonged to a marine gamma proteobacterium. Esterases with the LpqC domain are reported in some marine enzymes of EM3L4 and FLS18 family indicating that these proteins may have some function in marine bacteria [46]. The α/β-hydrolase-fold domain was fused to an OsmC superfamily domain at the carboxyl-terminal in the esterase from clone SLC6. Similar fused proteins are present in a diverse group of bacteria and esterases fused with the OsmC domain from *Pseudoalteromonas arctica* and other bacteria are recently classified into a new family of lipolytic enzymes [44]. A truncated esterase domain devoid of OsmC showed higher thermostability indicating the role of OsmC-domain in thermolability of the protein [47]. Fusion of OsmC-domain from this protein to xylanase also resulted in the decrease in the thermostability of the fused protein in comparison to wild-type enzyme confirming the role of OsmC-domain in introducing thermolability in the fused protein [48].

Characterization of two phylogenetically related proteins from the two distinct habitats with 46% amino acid identity revealed distinct temperature optima and thermostability, which is in line with our assumption and previous reports that enzymes from different habitats will show adaptation to the temperature of the site of their origin [49,50]. Both the enzymes showed strong inhibition with all the tested enzyme inhibitors i.e., PMSF, DEPC, pCMB, and $HgCl_2$ Most esterases possess serine, histidine and aspartic acid residues in the catalytic triad. Inhibition with PMSF and DEPC suggested that the active site serine and histidine residues are easily accessible in both the enzymes [27,51]. Inhibition by $HgCl_2$ and pCMB indicated at the importance of cysteine residue/s in or near the active site of the two esterases [47].

MLC3 hydrolyzed industrially important substrate methyl mandelate with stereoselectivity for R-enantiomer. The enantiomeric excess and enantioselectivity of the purified enzyme can be further improved by modifying the reaction conditions or enzyme by cross linking/immobilization as have been done previously for esterases [52].
Overall sequence, phylogenetic and enzymatic features indicate that the identified esterases from metagenomic libraries have potential to be part of the toolbox of synthetic organic chemists.

Authors' Contributions

RR and RS planned the experiments. RR, GS, MKY performed the experiments. RR, GS, MKY and RS analyzed and interpreted the data. RR, GS, and RS wrote the manuscript. All authors reviewed the final manuscript before submission.

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Compliance with Ethical Standards

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This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure legends

Fig 1 Lipolytic activity of clones isolated from metagenomic libraries. Activity was observed after 72 h of incubation at 37 °C on 1 % tributyrin containing plates. (a) hot spring metagenomic library clones, Label 1: *E. coli* DH10B/pMLC2, 2: *E. coli* DH10B/pMLC3, 3: *E. coli* DH10B/pMLC8 and C; *E. coli* DH10B/pUC19 (control) and (b) sea sediment library clones, Label 1-3: *E. coli* DH10B/pSLC1- 3, 4-5: *E. coli* DH10B/pSLC5-6 and C: *E. coli* DH10B/pUC19 (control).

Fig 2 Phylogenetic analysis of lipolytic proteins in this study and representative members of previously identified families. The phylogenetic tree was generated using the maximum-likelihood method (MEGA7.0 software). The protein sequences for previously known lipolytic enzymes were retrieved from GenBank. The numbers at the nodes indicate the bootstrap percentages of 1000 replicates.

Fig 3 Schematic representation of the conserved domains of esterase genes. (a) SLC3 and (b) SLC6.

Fig 4 Purification and characterization of esterases MLC3 and SLC5. (a) SDS-PAGE analysis of purified SLC5; lane 1: marker, lane 2: purified SLC5. (b) SDS-PAGE analysis of purified MLC3; lane 1: marker, lane 2: purified MLC3. (c) Substrate preference of enzymes towards various *p*-nitrophenyl esters. Relative activity on *p*-nitrophenyl esters was measured. The maximum activity, i.e., 0.51 µmoles.min^{-1}.mg^{-1} and 0.34 µmoles.min^{-1}.mg^{-1} obtained for *p*-nitrophenyl butyrate was taken as 100% for MLC3 and SLC5**,** respectively. Michaelis-Menten plot at 37 °C, pH 7.0 for 15 min with *p*-Nitrophenyl butyrate of (d) of MLC3 and (e) SLC5.

Fig 5 Effect of temperature on the activity of the recombinant esterases. (a) Optimum temperature of the recombinant esterases MLC3 (●) and SLC5 (▼). (b) Thermostability of the recombinant esterases. Residual relative activity of MLC3 after incubation at 40 °C (●), 50 °C (○) and of SLC5 after incubation at 40 °C (▼), and 50 °C (∇) was measured. **(c)** Thermostability of MLC3 (●) at 50 °C was tested for extended time. After incubation for 1-12 h, residual activity was measured by standard enzyme activity assay. (d) Circular dichroism assay of MLC3 conducted at pH 7 at ramp rate of 1 °C/min.

Fig 6 Effect of pH on the activity of the recombinant esterases. Relative activity of MLC3 (●) and SLC5 (▼) was measured in different pH buffers as described in material and methods with *p*-nitrophenyl butyrate as substrate.

Fig 7 Conversion of methyl mandelate to mandelic acid. The reaction mixtures contained 10 mM methyl mandelate in 50 mM sodium phosphate buffer, pH.7.0 in a final volume of 5 mL. The reaction mixtures were incubated at 37 °C in a water bath. The concentrations methyl mandelate (●) and mandelic acid (○) were determined by HPLC at different time intervals.

Table 1. Properties of the esterases identified from the two metagenomic libraries.

Clone				**Best Match**				
Name (Accession No.)	**Insert size (bp)**	**GC %**	**ORF (aa)**	**Homologous protein**	**Accession No.**	**Organism**	***e* value**	**Identity (%)**
MLC2 (ADM63067.1)	3540	66.61	360	Pectin acetylesterase	KXK48940.1	*Chloroflexi* bacterium OLB13	8e-90	162/370 (44%)
MLC3 (ADM63071.1)	3881	67.15	280	Hypothetical protein	WP_058574994.1	*Halothiobacillus* sp. XI15	2e-85	132/266 (50%)
MLC8[a] (ADM63072.1) (ADM63075.1)	4518	63.13	ORF-A 227	GDSL-like Lipase/Acylhydrolase	KXK48714.1	*Chloroflexi* bacterium OLB13	1e-59	94/226 (42%)
			ORF-B 360	Esterase	KXK25087.1	*Chloroflexi* bacterium OLB15	6e-106	168/346 (49%)
SLC1[a] (ADM63076.1) (ADM63077.1)	2041	63.55	ORF-A 302	Est16	AKN78217.1	Uncultured bacterium	0.0	258/302 (85%)
			ORF-B 407	Alpha/beta hydrolase fold protein	WP_012110585.1	*Parvibaculum lavamentivorans*	2e-154	218/290 (75%)
SLC2 (ADM63079.1)	3312	58.24	395	Serine hydrolase	WP_007164393.1	*Erythrobacter* sp. NAP1	0.0	286/392 (73%)
SLC3 (ADM63080.1)	3887	58.14	751[b]	Hypothetical protein	WP_050756406.1	Marine gamma proteobacterium HTCC2148	0.0	372/692 (54%)
SLC5 (ADM63083.1)	2683	68.39	278	Alpha/beta hydrolase	WP_054556817.1	*Cobetia* sp. UCD-24C	0.0	275/276 (99%)
SLC6 (ADM63085.1)	3580	58.97	469	Osmotically inducible protein C	WP_019622077.1	*Amphritea japonica*	1e-174	243/401 (61%)

[a] Clone has two lipolytic open reading frames
[b] Start codon is CTG

Table 2. Effect of different inhibitors on the esterase activity of MLC3 and SLC5.

Inhibitor	**Concentration (mM)**	**Residual Activity (%)**	
		MLC3	SLC5
Control		100 ±0.5	100 ±0.5
PCMB[a]	0.1	88.8 ±10.3	20.8 ±0.5
	1.0	30.0 ±0.4	3.5 ±1.2
$HgCl_2$[b]	0.1	1.8 ±0.4	23.9 ±3.2
	1.0	2.0 ±0.2	12.9 ±0.7
	5.0	0.5 ±2.0	11.7 ±0.3
DEPC[c]	0.1	4.5 ±1.8	12.0 ±0.5
	1.0	4.7 ±1.7	8.9 ±1.2
	5.0	5.6 ±0.40	5.6 ±0.2
PMSF[d]	0.1	2.3 ±1.6	19.5 ±2.7
	1.0	2.8 ±2.3	19.3 ±0.7
	5.0	3.1 ±0.3	11.0 ±0.4

[a] p-chloromercuriobenzoate
[b] mercuric chloride
[c] diethyl pyrocarbonate
[d] phenylmethylsulfonyl fluoride

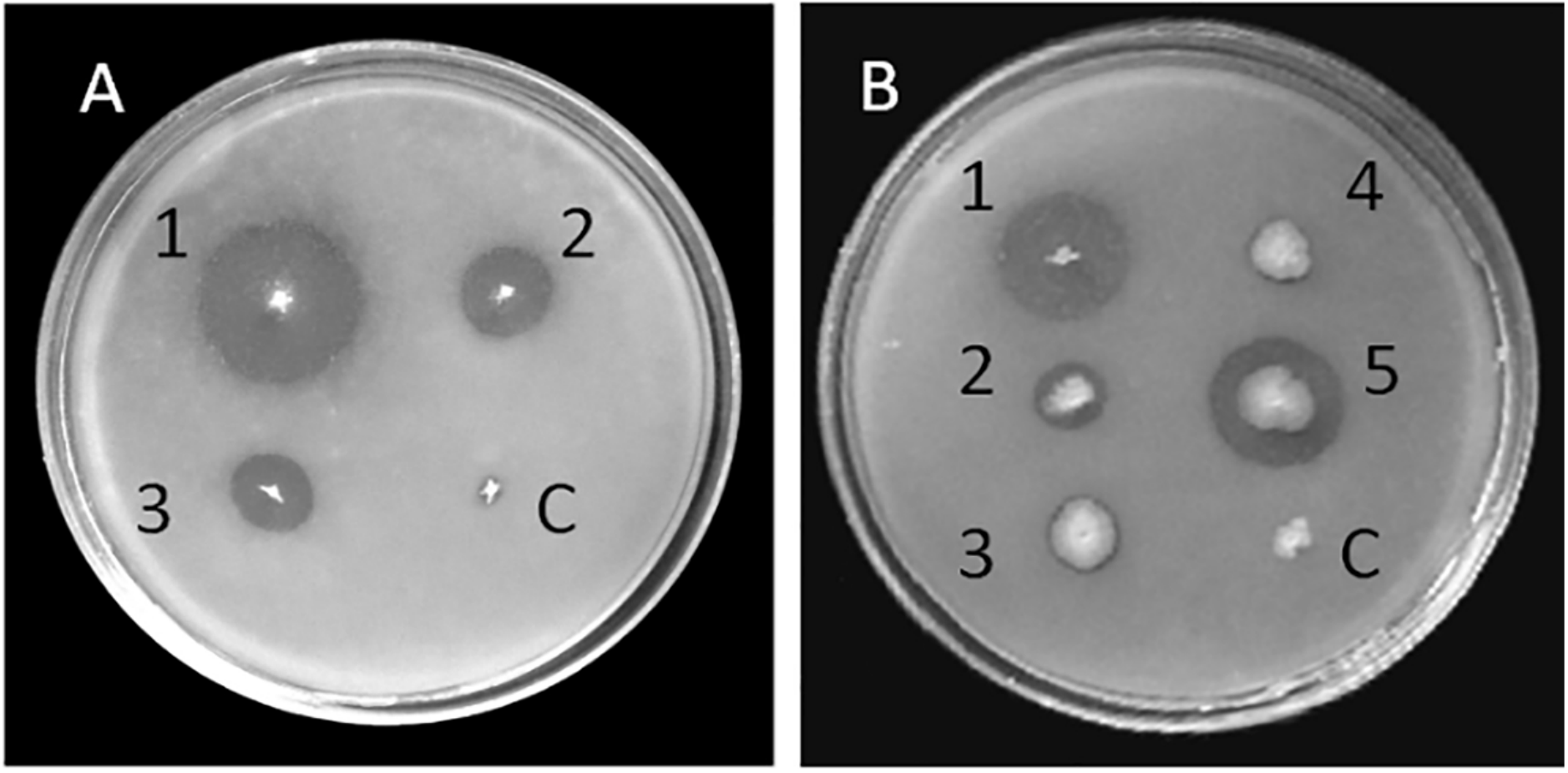
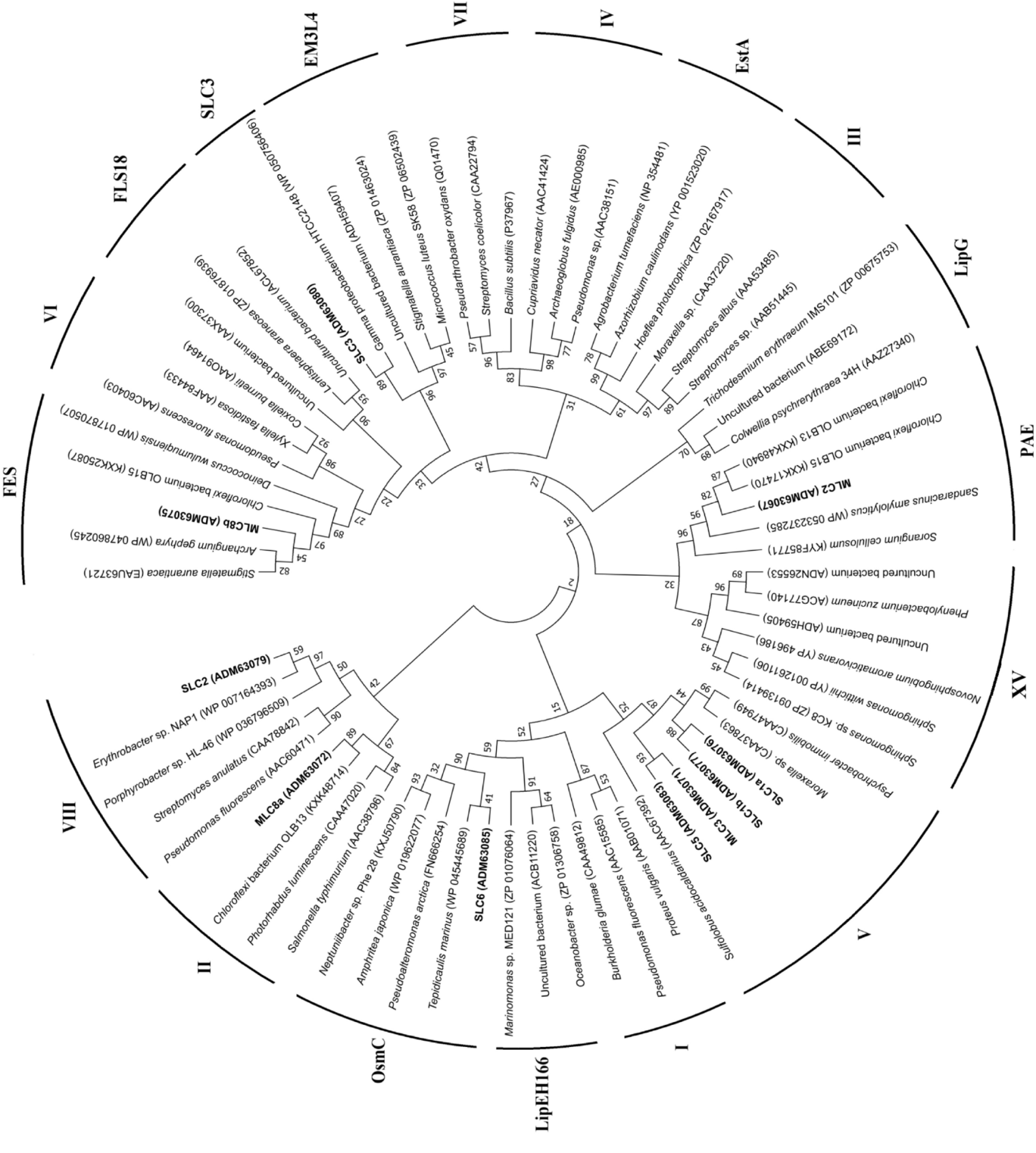


Figure 1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VIII
XV
PAE
FES
FLS18
SLC3
EM3L4
EstA
LipG
OsmC
LipEH166
Stigmatella aurantiaca (EAU63721)
Archangium gephyra (WP 047860245)
MLC8b (ADM63075)
Chloroflexi bacterium OLB15 (KXK25087)
Pseudococcus wulumuqiensis (WP 017870507)
Xylella fastidiosa (AAF84433)
Coxiella burnetii (AAO91464)
Uncultured bacterium (AAX37300)
Lentisphaera araneosa (ZP 01876939)
Uncultured bacterium (ACL67852)
SLC3 (ADM63080)
Gamma proteobacterium HTCC2148 (WP 050756406)
Uncultured bacterium (ADH59407)
Stigmatella aurantiaca (ZD 01469024)
Micrococcus luteus SK58 (ZP 06502439)
Pseudarthrobacter oxydans (Q01470)
Streptomyces coelicolor (CAA22794)
Bacillus subtilis (P37967)
Cupriavidus necator (AAC41424)
Archaeoglobus fulgidus (AE000985)
Pseudomonas sp.(AAC38151)
Agrobacterium tumefaciens (NP 354481)
Azorhizobium caulinodans (YP 001523020)
Hoeflea phototrophica (ZP 02167917)
Moraxella sp. (CAA37220)
Streptomyces albus (AAA53485)
Streptomyces sp. (AAB51445)
Trichodesmium erythraeum IMS101 (ZP 00675753)
Uncultured bacterium (ABE69172)
Colwellia psychrerythraea 34H (AAZ27340)
Chloroflexi bacterium OLB13 (KXK48940)
Chloroflexi bacterium OLB15 (KXK17470)
MLC2 (ADM63067)
Sandaracinus amylolyticus (WP 053237285)
Sorangium cellulosum (KYF85771)
Uncultured bacterium (ADN26553)
Phenylobacterium zucineum (ACG77140)
Uncultured bacterium (ADH59405)
Sphingomonas wittichii (YP 496186)
Novosphingobium aromaticivorans (YP 001261106)
Sphingomonas sp. KC8 (ZP 09139414)
Psychrobacter immobilis (CAA47949)
Moraxella sp. (CAA37863)
SLC1a (ADM63071)
SLC1b (ADM63076)
MLC3 (ADM63083)
SLC5 (ADM63077)
Sulfolobus acidocaldarius (AAC67392)
Proteus vulgaris (AAB01071)
Pseudomonas fluorescens (AAC15585)
Burkholderia glumae (CAA49812)
Oceanobacter sp. (ZP 01306758)
Uncultured bacterium (ACB11220)
Marinomonas sp. MED121 (ZP 01076064)
SLC6 (ADM63085)
Tepidicaulis marinus (WP 045445689)
Pseudoalteromonas arctica (FN666254)
Amphritea japonica (WP 019622077)
Neptunibacter sp. Phe 28 (KXJ50790)
Salmonella typhimurium (AAC38796)
Photorhabdus luminescens (CAA47020)
Chloroflexi bacterium OLB13 (KXK48714)
MLC8a (ADM63072)
Pseudomonas fluorescens (AAC60471)
Streptomyces anulatus (CAA78842)
Porphyrobacter sp. HL-46 (WP 036796509)
Erythrobacter sp. NAP1 (WP 007164393)
SLC2 (ADM63079)

Figure 2

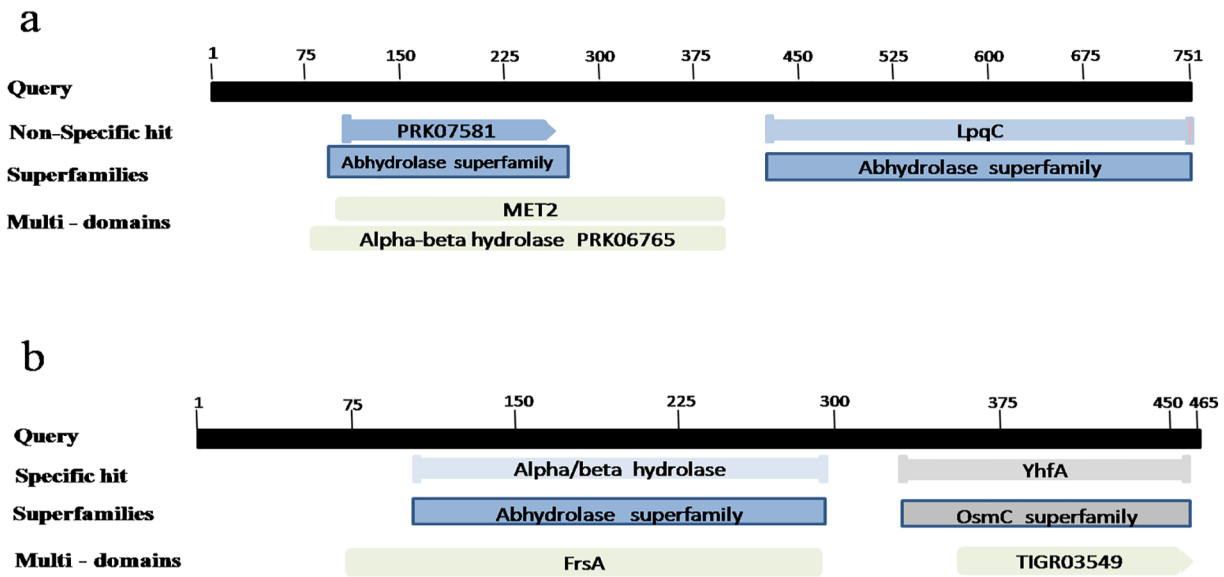
a
1
75
150
225
300
375
450
525
600
675
751
Query
Non-Specific hit
PRK07581
LpqC
Superfamilies
Abhydrolase superfamily
Abhydrolase superfamily
Multi - domains
MET2
Alpha-beta hydrolase PRK06765
b
1
75
150
225
300
375
450
465
Query
Specific hit
Alpha/beta hydrolase
YhfA
Superfamilies
Abhydrolase superfamily
OsmC superfamily
Multi - domains
FrsA
TIGR03549

Figure 3

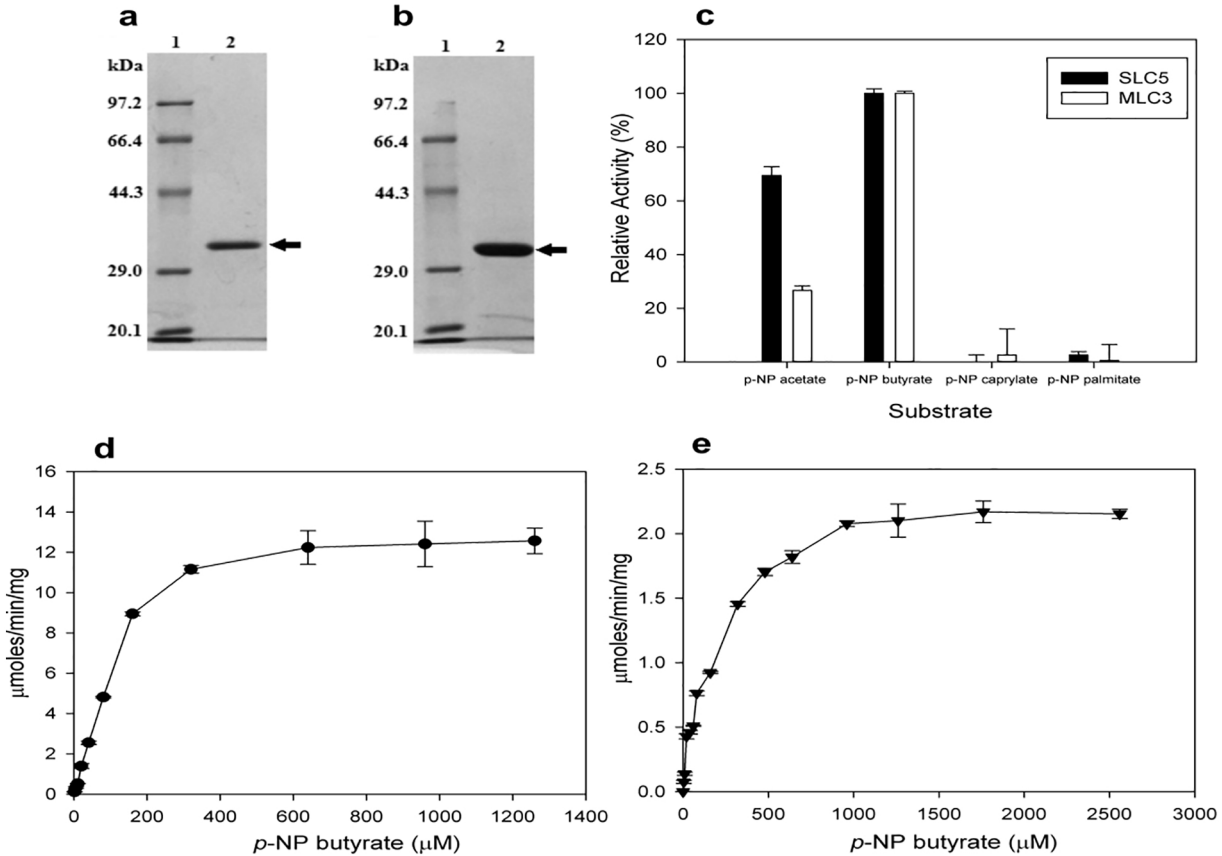
a
1
2
kDa
97.2
66.4
44.3
29.0
20.1
b
1
2
kDa
97.2
66.4
44.3
29.0
20.1
c
SLC5
MLC3
Relative Activity (%)
120
100
80
60
40
20
0
p-NP acetate
p-NP butyrate
p-NP caprylate
p-NP palmitate
Substrate
d
µmoles/min/mg
16
14
12
10
8
6
4
2
0
0
200
400
600
800
1000
1200
1400
p-NP butyrate (µM)
e
µmoles/min/mg
2.5
2.0
1.5
1.0
0.5
0.0
0
500
1000
1500
2000
2500
3000
p-NP butyrate (µM)

Figure 4

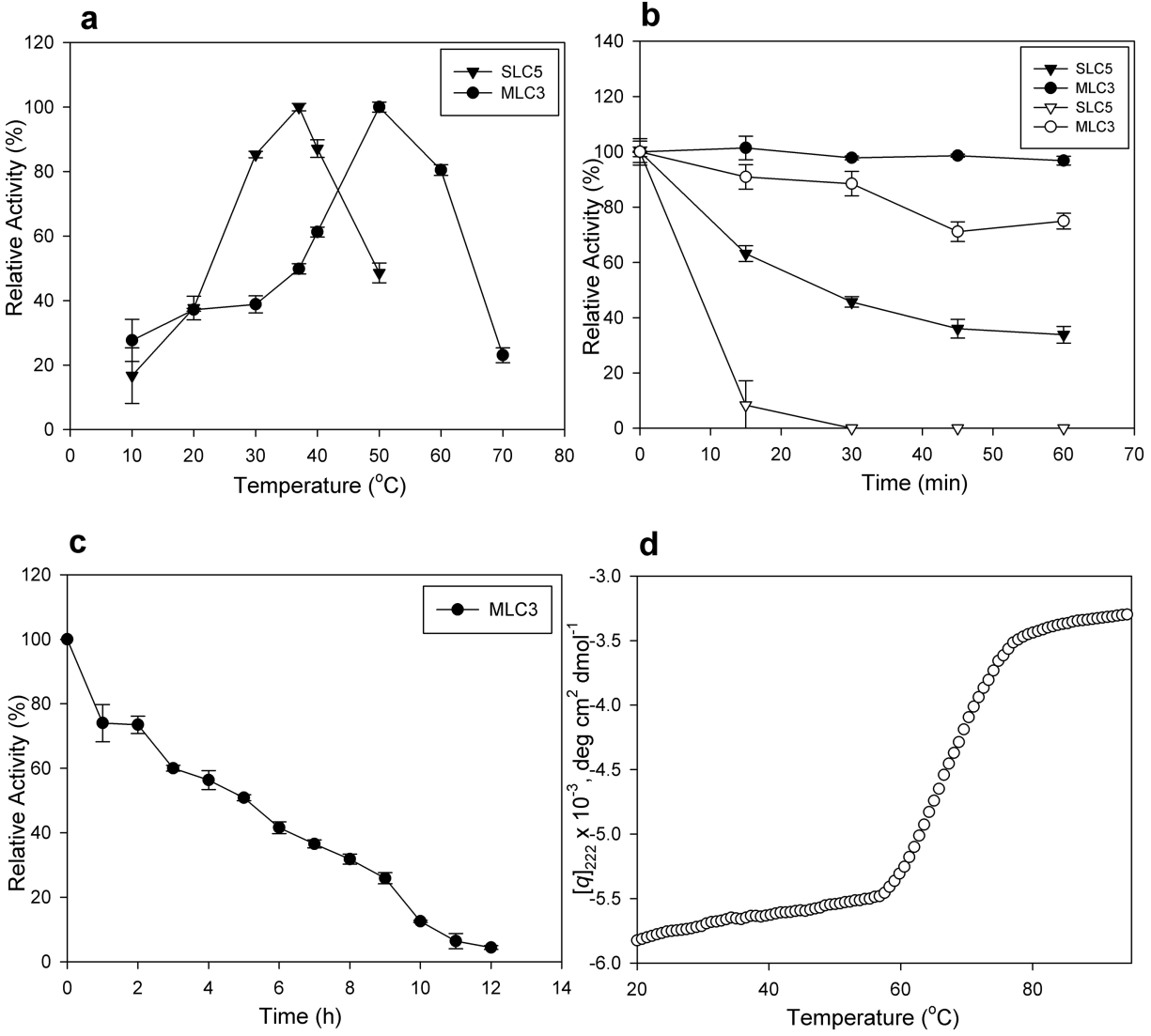
a
Relative Activity (%)
Temperature (°C)
SLC5
MLC3
b
Relative Activity (%)
Time (min)
SLC5
MLC3
SLC5
MLC3
c
Relative Activity (%)
Time (h)
MLC3
d
[q]222 x 10-3, deg cm2 dmol-1
Temperature (°C)

Figure 5

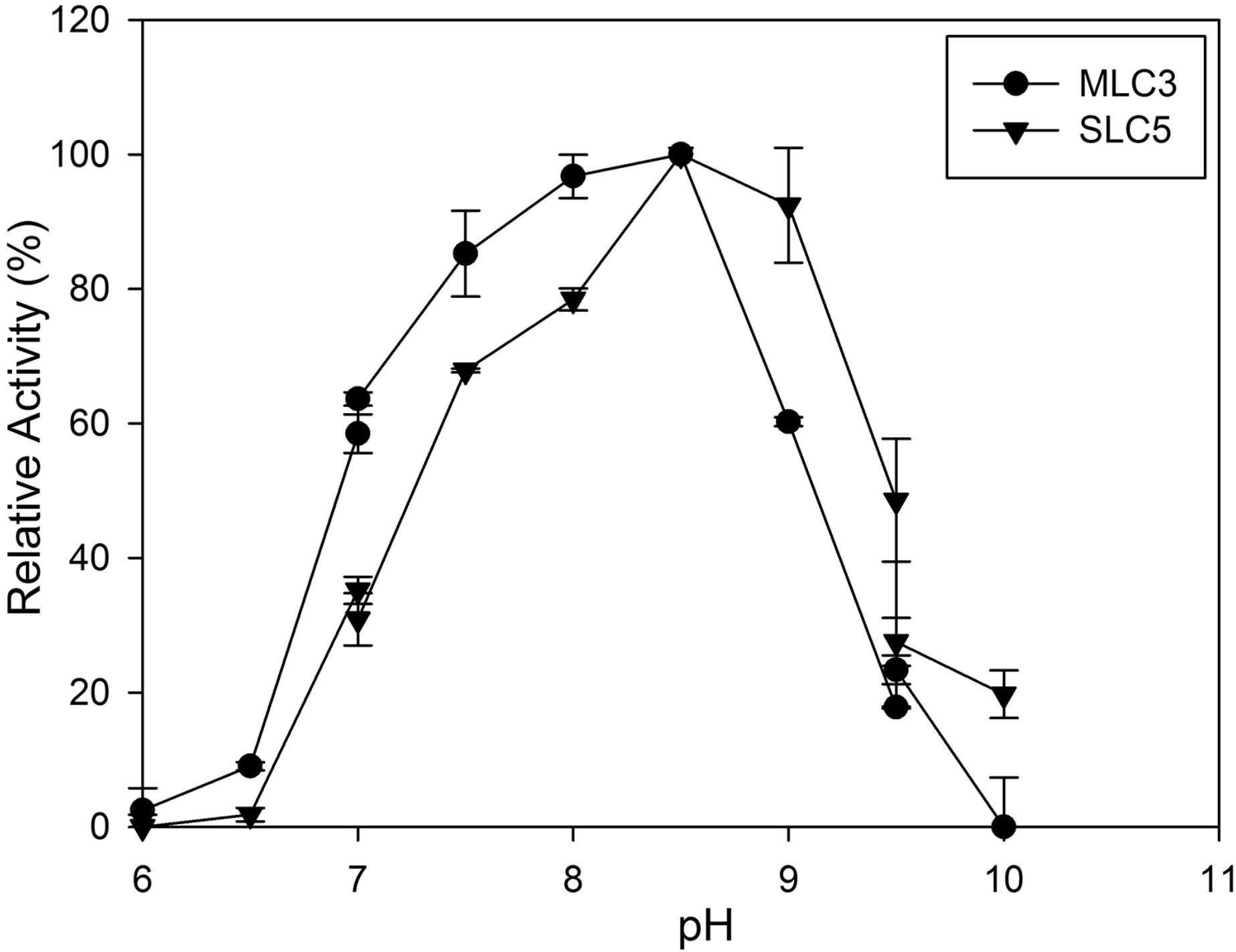

Figure 6

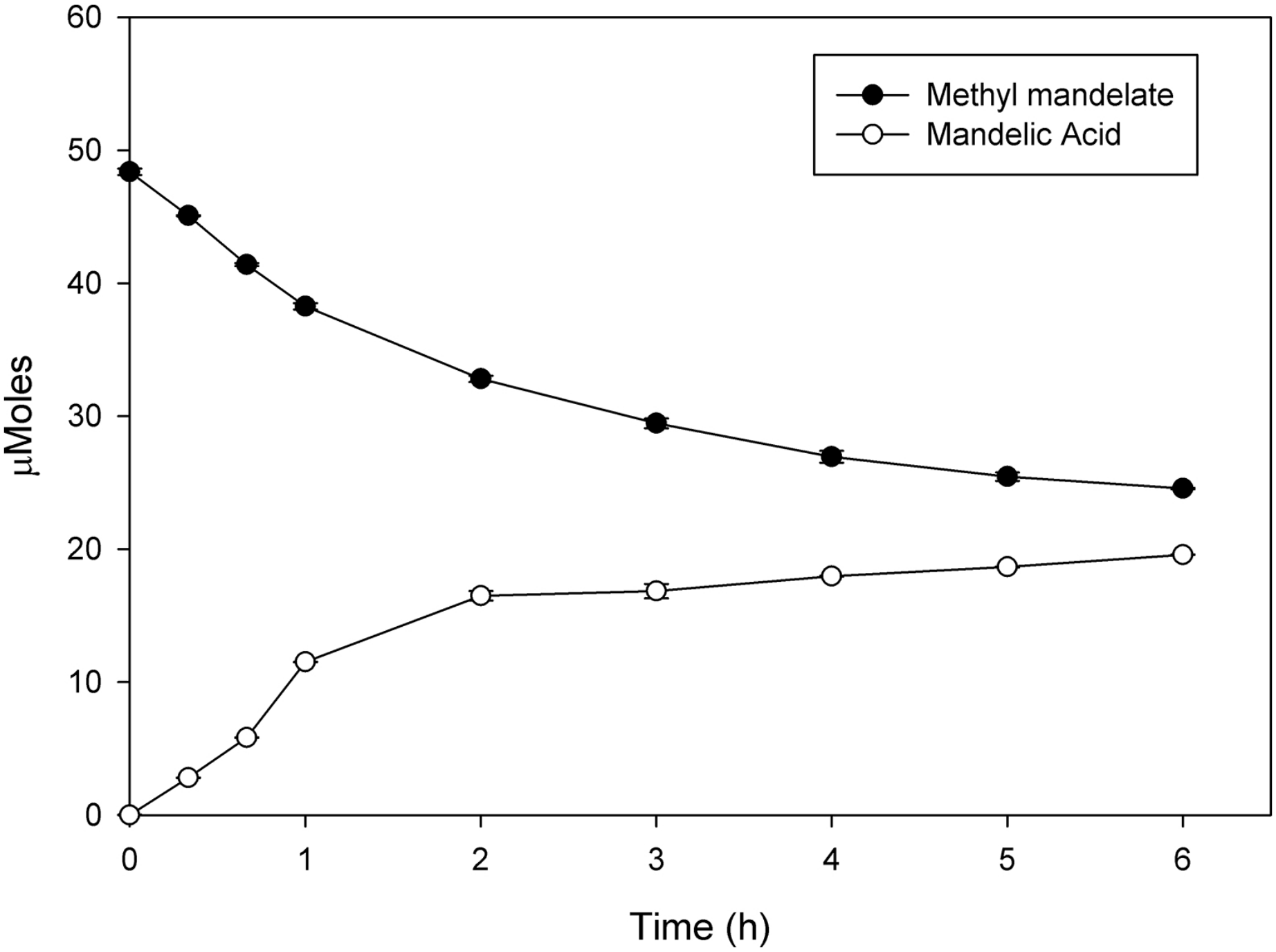
60
50
40
30
20
10
0
µMoles
0
1
2
3
4
5
6
Time (h)
Methyl mandelate
Mandelic Acid

Figure 7