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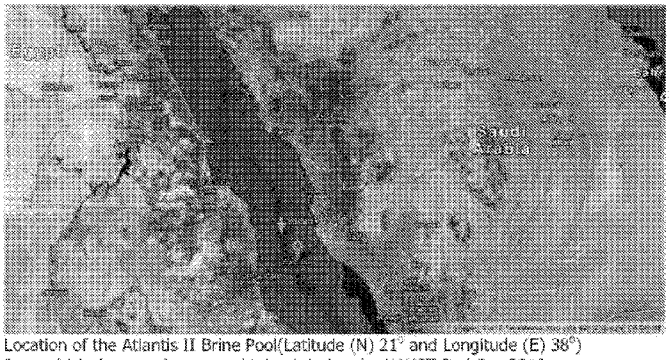
(54) **Title**: ESTERASE RESISTANT TO INACTIVATION BY HEAVY METALS

Figure 1: Atlantis II Brine Pool Sample Site



Location of the Atlantis II Brine Pool(Latitude (N) 21° and Longitude (E) 38°) from which the samples were obtained during the KAUST Red Sea 2010 expedition.

(57) **Abstract**: EstATII is an esterase that a halotolerant, thermophilic and resistant to a spectrum of heavy metals including toxic concentration of metals. It was isolated from the lowest convective layer of the Atlantis II Red Sea brine pool. The Atlantis II brine pool is an extreme environment that possesses multiple harsh conditions such as; high temperature, salinity, pH and high concentration of metals, including toxic heavy metals. A fosmid metagenomic library using DNA isolated from the lowest convective layer this pool was used to identify EstATII. Polynucleotides encoding EstATII and similar esterases are disclosed and can be used to make EstATII. EstATII or compositions or apparatuses that contain it may be used in various processes employing lipases/esterases especially when these processes are performed under harsh conditions that inactivate other kinds of lipases or esterases.

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TITLE

ESTERASE RESISTANT TO INACTIVATION BY HEAVY METALS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. provisional application 61/804,434, filed March 22, 2013, the content of which is incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

A polypeptide esterase that is resistant to inactivation by heavy metals and other extreme conditions. Polynucleotides encoding this esterase.

Description of the Related Art

Until lately, and despite its uniqueness, the Red Sea has received little attention among marine environments. The Red Sea formed 3-5 million years ago when the Arabian and African plates started to split [1]. It is characterized by high temperature and salinity owing to the high rate of evaporation, lack of major river inflows and a low rate of rainfall [1]. The Red Sea is characterized by the presence of deep-sea hypersaline anoxic basins; called brine pools, which are large bodies of water at the bottom of the ocean characterized by high temperature and salinity. To date, twenty-five brine pools have been found in the Red Sea [1, 2]. Atlantis II Deep (Figure 1) is the largest brine pool in the Red Sea, has the highest temperature and is the most dynamic [1, 3]. It has a maximum depth of 2,194 m and is stratified into several layers that increase in temperature and salinity with increasing depth; the brine-seawater interface, upper convective, middle convective and lower convective layers (LCL)[1, 3]. The lowest layer; LCL is characterized by a temperature of 68.2°C, pH value of 5.3 and salinity of 270 psu, which is 7.5 times that of normal seawater [1, 3]. Atlantis II Deep is nearly anoxic and has high concentrations of iron, zinc, copper and other heavy metals [1, 3]. Together, these extreme conditions make the Atlantis II brine pool an attractive site for mining for biocatalysts, such as lipolytic enzymes, which are predicted to possess desirable traits, including and not limited to, thermo-tolerance, halo-tolerance, pH plasticity and resistance to inhibition by heavy metals.

Industrialized societies are moving towards white (industrial) biotechnology, which has proven to be environmentally sound and commercially efficient [4]. This poses a continuous demand for novel biocatalysts, preferably biocatalysts that demonstrate high activity over a wide range of conditions such as temperature, salinity, pH and metal concentration. Biocatalysts of microbial origin represent the majority of biocatalysts used in industrial and biotechnological processes [5]. This owes to the capability of prokaryotes to populate and adapt to different environments, from hydrothermal vents to Antarctic desert soil, from which a wide array of biocatalysts are derived that are robust within a flexible range of conditions; making them desirable for industry [6].

Metagenomics serves as a powerful tool to access the genomes of the unculturable majority of prokaryotes, and to investigate their potential as sources of novel biocatalysts. It has led to the identification and characterization of a vast number of biocatalysts that are active under a wide range of conditions reflecting the environment from which they originate, making them desirable for industrial use [7-10].

Microbial lipolytic enzymes possess a huge potential as industrial biocatalysts. They are characterized by substrate specificity, regio- and enantioselectivity that surpasses that of any other enzyme, making their application potential boundless[11]. Using lipolytic enzymes in industrial and biotechnological applications is estimated to be a billion dollar business[12]. Their applications include and are not limited to leather manufacture, flavor development in the dairy industry, oil biodegradation and the synthesis of pharmaceuticals and chemicals [12-15].

As of 2005, only a dozen thermostable lipases/esterases had been isolated; Rhee J-K et. Al. (2005) [45], *New thermophilic and thermostable esterase with sequence homology to the hormone sensitive lipase family, cloned from a metagenomic library.* Appl Environ Microbiol Vol. 71(2): pp. 817-825. A 2010 paper reported that, surprisingly, only 7 esterases of thermophilic origin had been sequenced. Yu, et al. (2010) [46], *Gene cloning and characterization of a novel thermophilic esterase from Fervidobacterium nodosum Rt17-B1*, Acta Biochim. Biophys. Sin., Vol. 42(4), pp. 288-295 described a new candidate termed FNE acetylesterase, isolated from *Fervidobacterium nodosum* strain Rt17-B12. Another publication, Waters DM et al (2012)[47], *Cloning, Overexpression in Escherichia coli, and Characterization of a Thermostable Fungal Acetylxylan Esterase from Talaromyces emersonii*, Appl. Environ. Microbiol. Vol. 78(10): pp. 3759-3762 recently identified thermostable

esterase from *Talaromyces emersonii* bears sequence homology to acetylxylan esterases.

The global market for lipases is significant. The division of the entire market for lipases is detergent (42%), pulp and paper (about 7%), leather (about 6%), dairy products (about 17%), and sweeteners (about 21%) (otd.unc.edu/documents/11_4_2010_Williams.pptx). The biofuels market, which is expected to grow significantly in its need for novel biocatalysts, is seen as the greatest opportunity for expanding the use of esterases. Over 300 industrial processes have been designed that rely on biocatalysts (Singh RK et al (2013) [48], *From protein engineering to immobilization: promising strategies for the upgrade of industrial enzymes*. Int. J. Mol. Sci. Vol. 14: pp. 1232-1277). Esterases are of particular use in the production of bulk chemicals and pharmaceuticals, where they find very specific niches in chemical production. Examples include precursors for pyrethrin insecticides; in the production of naproxen; solubilization of certain antibiotics; and often as a general mild remover of protective groups on chemical intermediates during various syntheses (Bornscheuer UT (2002) [49] Microbial carboxyl esterases: classification, properties, and application in biocatalysis. FEMS Microbiol. Rev. Vol. 26: pp. 73-81). A recent paper described the use of a thermostable esterase from *Archaeoglobus fulgidus* (Cao H et al (2012) [50] *Biocatalytic synthesis of poly (δ-valerolactone) using a thermophilic esterase from Archaeoglobus fulgidus as catalyst*. Int. J. Mol. Sci. Vol. 13: pp 12232-12241) for producing polymers useful in preparing nanoparticles for targeted therapeutic delivery, as an example. Esterases that have recently received particular attention in industrial use include furoyl esterases, pectin esterases, acetylxylan esterases, and rhamnogalacturonan acetyl esterases. The first two types are commonly used in food processing, while the latter two find use in biomass solubilization. In addition to the biofuels market, enzymatic cleavage of these molecules can contribute to production of components of nutraceuticals, cosmetics, and fine chemicals.

BRIEF SUMMARY OF THE INVENTION

The inventors disclose herein the isolation and biochemical characterization of a novel esterase; EstATII from the lower convective layer of the Atlantis II brine pool. This esterase has been biochemically characterized and is active over a range of temperatures and pH's, retains activity in the presence of agents such heavy metals

including copper, zinc and mercury which inactivate many other esterases. Polynucleotides encoding EstATII and enzymatically active variants of this esterase (EstATII-type esterases) as well as recombinant methods for making it are disclosed. The EstATII-type of esterase may be used to process or transform various substrates on which it is active especially under conditions that inactive other kinds of esterases.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Atlantis II Brine Pool Sample Site. Location of the Atlantis II Brine Pool(Latitude (N) 21° and Longitude (E) 38°) from which the samples were obtained during the KAUST Red Sea 2010 expedition.

Figure 2: Conserved Motifs found in members of The Hormone Sensitive Lipase (HSL) Family and in EstATII. Multiple sequence alignment of EstATII (SEQ ID NO: 2) with other members of the HSL family: *Pseudomonas* sp. B11-1 (AF034088)(SEQ ID NO: 9), *Archaeoglobus fulgidus* (AE000985)(SEQ ID NO: 10), *Alcaligenes eutrophus* (L36817)(SEQ ID NO: 11), *Moraxella* sp. (X53868)(SEQ ID NO: 12) and *Escherichia coli* (AE000153)(SEQ ID NO: 13) was performed using ClustalWand visualized by BoxShade server. The alignment shows the conserved motif HGG, which is involved in the formation of the oxyanion hole. It also shows the nucleophilic catalytic serine residue in the pentapeptide GDSAG (SEQ ID NO: 8), which is conserved in HSL family.

Figure 3: Phylogenetic Analysis and Classification of EstATII

Multiple sequence alignment of EstAII with 41 lipolytic enzymes (representing the eight families of the bacterial lipolytic enzymes as classified by Arpigny and Jaeger, 1999[17]) was used to construct a phylogenetic tree. EstATII groups with members of family IV also known as the HSL family. The confidence level of the tree was estimated by bootstrapping (10,000 replicates). The tree was constructed using MEGA 5 and the scale represents the number of amino acids substitution.

Figure 4: Overexpression, purification and western blot analysis of recombinant EstATII. Lane 1: Molecular Weight marker, Lane 2: Un-induced sample, Lane 3: Sample induced with 0.5 mM IPTG, Lane 4: Protein Lysate, Lanes 5,6: Flowthrough. Lane 7: Wash step. Lane 8: Purified EstATII. Lane 9: Western Blot analysis of purified EstATII.

DETAILED DESCRIPTION OF THE INVENTION

A polynucleotide comprising or consisting of a sequence that is at least 80%, 82.5%, 85%, 87.5%, 90%, 92.5%, 95%, 97.5%, 98%, 99% or 100% identical to the polynucleotide sequence described by SEQ ID NO: 1 or a fragment thereof that encodes a polypeptide having esterase activity. Polynucleotide encoding immunogenic fragments of the polypeptide described by SEQ ID NO: 2 are also contemplated, especially those encoding immunogenic fragments containing epitopes that specifically identify or distinguish the esterase of SEQ ID NO: 2 from other esterases or lipases. Polynucleotide sequence identity to a reference sequence, such as SEQ ID NO: 1, may be determined using BLASTn using the default setting. Preferred parameters for determining polynucleotide sequence identity when using the BLASTN program (Altschul, S. et al., Journal of Molecular Biology 215 (1990), pages 403-410) are: Expect Threshold: 10; Word size: 28; Match Score: 1; Mismatch Score: -2; Gap costs: Linear.

The polynucleotide sequences of the invention also include those that hybridize under stringent conditions to the polynucleotide sequence of SEQ ID NO: 1 or its full complement where stringent conditions can comprise washing in 4xSSC and 0.1% SDS for 15 mins at 65°C, 2xSSC and 0.1% SDS for 15 mins at 65°C 1xSSC and 0.1% SDS for 15 mins at 65°C, 0.5xSSC and 0.1% SDS for 15 mins at 65°C, or in 0.1x SSC containing 0.1% SDS for 15 mins at 68°C. The polynucleotides that hybridize under stringent conditions may be further selected to encode polypeptides having enzymatic activity, specifically esterase activity.

Polynucleotides according to the invention may be isolated from natural sources, from a library, such as a metagenomic library, or made recombinantly or synthetically using standard techniques such as isolation from a plasmid, amplification, *e.g.*, by the polymerase chain reaction, or by chemical synthesis. Isolated polynucleotides have been removed from other components present in their natural environments or produced during their amplification or synthesis. An isolated polynucleotide may be at least 70%, 80%, 90%, 95% or substantially free of other contaminating polynucleotides or other components. Similarly an isolated polypeptide may be at least 70%, 80%, 90%, 95% or substantially free of other contaminating polypeptides or other components it is associated with prior to its isolation.

The polynucleotides described above may encode a polypeptide comprising at least one of the motifs HGGXFXXXXXXXH (SEQ ID NO: 5), VXXXXYXXXPXXXXPXA (SEQ ID NO: 6), or GDSAGXXL (SEQ ID NO: 7). Advantageously this peptide will exhibit esterase activity even at high temperatures and pressures or in the presence of metals, detergents and chaotropic agents or comprise epitopes that permit it or its fragments to be recognized by a mammalian humoral or cellular immune system.

The polynucleotide described above may be inserted or appear in a vector or DNA construct, such as a bacterial vector (*e.g.*, phage, plasmid or cosmid); a yeast vector; an insect cell vector; a plant cell vector; and a vector for a mammalian cell or other kind of animal cell; wherein any of said vector may optionally comprise one or more regulatory sequences to enhance or control transcription and/or translation of said polynucleotide. Vectors include both cloning and expression vectors as well as vectors that contain chimeric genes containing all or part of the polynucleotide sequences disclosed herein (*e.g.*, those that are at least 80% identical to reference sequence of SEQ ID NO: 1) and optionally polynucleotide encoding other functional sequences, or polynucleotides that encode fusion proteins.

The polynucleotide or vector as described above may be transformed or recombined into a host cell, such as bacterium (*e.g.*, *Escherichia coli* or *Bacillus subtilis*), including a bacterium of Family IV or the Hormone Sensitive Lipase ("HSL") family; a yeast cell, an insect cell, a mammalian cell, an avian cell, a reptilian cell, an amphibian cell, or other kinds of transformable animal, fungal, or plant cells.

The polynucleotide, vectors and host cells described herein may be employed to produce a recombinant or synthetic protein, such as a polypeptide that is at least 80% similar or identical to reference sequence of SEQ ID NO: 2. Such a method will involve expressing the polynucleotide encoding the protein under suitable conditions, such as culturing a host cell containing a vector carrying a polynucleotide encoding the polypeptide and recovering or purifying the protein from the culture medium or from the cells. Advantageously, the recovered protein will have esterase activity as described herein.

Another aspect of the invention is a polypeptide that comprises a sequence that is at least 80%, 82.5%, 85%, 87.5%, 90%, 92.5%, 95%, 97.5%, 98%, 99% or 100% similar to SEQ ID NO: 2 or that is at least 80%, 82.5%, 85%, 87.5%, 90%, 92.5%,

95%, 97.5%, 98%, 99% or 100%identical to the amino acid sequence of SEQ ID NO: 2 or an immunogenic fragment thereof or a fragment thereof having esterase activity. As mentioned above, this polypeptide may contain one of the motifs HGGXFXXXXXXXH (SEQ ID NO: 5), VXXXXYXXXPXXXXPXA (SEQ ID NO: 6), or GDSAGXXL (SEQ ID NO: 7) found in members of the Hormone Sensitive Lipase ("HSL") family. Enzymatically active fragments of the polypeptide of the invention may have the above degrees of similarity or identity to the portion of SEQ ID NO: 2 depicted in Fig. 2 and such fragments may be embedded in longer polypeptide constructs to confer enzymatic activity on the construct.

BLASTP may be used to identify an amino acid sequence having at least 80%, 82.5%, 85%, 87.5%, 90%, 92.5%, 95%, 97.5%, 98%, 99% or 100% sequence similarity or 80%, 82.5%, 85%, 87.5%, 90%, 92.5%, 95%, 97.5%, 98%, 99% or 100% sequence identity to a reference amino acid sequence using a similarity matrix. Similarity matrices include BLOSUM45, BLOSUM62 or BLOSUM80. Unless otherwise indicated a similarity score will be based on use of BLOSUM62. Specific default parameters are Expect threshold = 10; word size = 3; Max matches in a query range = 0; Gap Cost = Existence 11, extension 1; Compositional adjustment = conditional compositional score matrix adjustment;

When BLASTP is used, the percent similarity is based on the BLASTP positives score and the percent sequence identity is based on the BLASTP identities score. BLASTP "Identities" shows the number and fraction of total residues in the high scoring sequence pairs which are identical; and BLASTP "Positives" shows the number and fraction of residues for which the alignment scores have positive values and which are similar to each other. Amino acid sequences having these degrees of identity or similarity or any intermediate degree of identity of similarity to the amino acid sequences disclosed herein are contemplated and encompassed by this disclosure. Variant or engineered polypeptides and polypeptide enzymes of the invention may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more additions, insertions, substitutions, or deletions from the amino acid sequence given by SEQ ID NO: 2 and the invention also encompasses the corresponding polynucleotide sequences encoding these variants.

As used herein the term "enzyme" or "polypeptide enzyme" includes full length proteins such as that described by SEQ ID NO: 2 or by a polypeptide having at least 80% sequence similarity or identity to SEQ ID NO: 2, enzymatically active

fragments comprising a portion of SEQ ID NO: 2 or a sequence having at least 80% identity or similarity to SEQ ID NO: 2, and polypeptide constructs comprising such enzymatically active polypeptides or polypeptide fragments, *e.g.*, dimers, trimers, multimers, aggregates, and other constructs comprising the enzymatically active polypeptide or polypeptide fragment.

Polypeptides according to the invention which exhibit esterase activity that is resistant to inactivation by agents such as high temperature, high pressure, high salinity, extremes in pH, by the presence of metals, such heavy metals, toxic metals (*e.g.*, biologically metals or metals that inactive other kinds of esterases or lipases), radioactive metals or metal isotopes, may be advantageously used in this method in the presence of these agents.

The enzymatically active polypeptides of the invention may be resistant to inactivation by heat, pH, pressure, salinity and the presence of metals, such as toxic heavy metals, and other substances such as surfactants, detergents or chaotropic agents. The enzymes according to the invention include those that are resistant to inactivation in the presence of surfactants, detergents, or chaotropic agents; those that retain activity within the pH range from 3, 4, 5, 6, 7, 8, and ≥ pH 9, preferably between pH 5.5 to 9.0; and those which remain active at salt concentrations ranging from 0, 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 to 6M, which ranges include all intermediate subranges and values. Salts include sodium salts (*e.g.*, sodium chloride), potassium salts (*e.g.*, potassium chloride) and salts of divalent cations such as Mg^{2+} or Ca^{2+} (*e.g.*, $MgCl_2$ or $CaCl_2$) as well as other salts found in saline lakes or seas or portions of these having high salinity. Polypeptide enzymes according to the invention also include those which retain activity at temperatures ranging from the freezing point of a solution containing the enzyme to the boiling point of the solution, *e.g.*, from freezing point to 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95,100°C or boiling point; preferably from 30°C to 80°C; enzymes that retain activity under substantially anoxic conditions, and enzymes that retain activity at standard atmospheric pressure 1.013 bars (14.696 psi) or at higher pressures, *e.g.* suboceanic pressures, *e.g.*, those ranging up to1,086 bars (15,750 psi).

Polypeptide enzymes according to the invention include those resistant to inactivation by metals. Such metals include heavy metals, biologically toxic metals, metals that inactivate enzymes, and radioactive isotope of a metal is selected from the group consisting of aluminum, antimony, arsenic, barium, beryllium, cadmium, lead,

mercury, osmium, thallium, or vanadium; actinium, thorium, uranium, radium, transuranic elements including plutonium and americium, polonium, radioactive isotopes of cobalt, *e.g.*, cobalt-60 and radioactive isotopes of strontium, *e.g.*, strontium-90; chromium, nickel, copper, zinc, and iron. Specifically, heavy metals, a biologically toxic metal, or a radioactive isotope of a metal may be selected from the group consisting of Ca, Mg, Cu, Zn, Co, Mn, Mg, Fe and Ba.

The polypeptide or fragment thereof of disclosed above may be a chimeric protein or a fusion protein that comprises additional fused amino acid residue. Fusion protein constructs may comprise polypeptides from galactosidase, glucuronidase, glutathione-S-transferase, horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), blue fluorescent protein (BFP) or other fluorescent proteins, or luciferase. Fusion proteins may contain tags such as histidine (His) tags, FLAG tags, influenza hemagglutinin (HA) tags, or other tags to facilitate recovery or purification of the fusion protein. Fusion proteins may contain protein cleavage sites to separate enzyme residues from fusion protein segments.

The polypeptides described herein may be in free form, such as those suspended or dissolved in solution or isolated in solid form, such as in a desiccated or freeze-dried form. They may also be covalently or non-covalently attached to a solid substrate, such as to a glass slide, a plastic slide, a tissue culture plate, a microtiter well, a glass tube, a plastic tube, a bead, including latex, polystyrene, or glass beads, a particle, including a microparticle or a nano particle, a chip, such as a silicon chip or array, or other solid substrate. Kits containing the enzymes of the invention may comprise an isolated or purified enzyme in solution or as a desiccated or freeze-dried product, or a solid substrate to which the enzyme is bound, containers for the enzymes, packaging for a solid substrate, kit packaging materials, a positive control containing a ester or ester-containing product, a negative control, and instructions for use.

The polypeptides and polypeptide fragments described herein having esterase activity as well as cells or fragments of cells containing these polypeptides or polypeptide fragments (including polypeptide constructs such as chimeric or fusion proteins, dimers, trimers, multimers, aggregates, *etc.*), may form part of an apparatus or bioreactor such as one that treats, processes, transforms, or degrades a substance or compound that is an ester or that contains ester linkages. Such an apparatus may

contain in addition to the polypeptide esterases one or more containers or contact surfaces for contacting the esterase or a cell or cellular component containing an esterase with an ester or a substance containing an ester. It may also contain an input and output port for inputting a substrate and remove a treated product. The polypeptide esterases herein resistant to inactivation by metals may be usefully employed in apparatuses containing metal surfaces, particles or other components and those resistant to inactivation at high temperatures, high pressures or in the presence of high salinity, detergents or chaotropic agents may be used an apparatuses which require exposure to these agents.

The polypeptides disclosed herein, including fragments and other polypeptide constructs, may be combined with other ingredients such as a buffer solution, excipient or carrier or preservative that preserves or maintains their enzymatic activity. They may also form part of a composition undergoing processing to remove or transform an ester or ester linkage (*e.g.*, a substrate undergoing processing), a pharmaceutical composition (*e.g.*, a drug or product for treating a disease, disorder or condition associated with the presence of an ester), a cleaner or antiseptic (such as a detergent, surface cleaner, or topical antiseptic), a cosmetic composition (such as a shampoo, mouthwash, or skin treating agent) or a food product (*e.g.*, a dairy product or other food or beverages).

Another aspect of the invention is an antibody and antigen binding fragment of an antibody that binds to the polypeptide or polypeptide fragments (including polypeptide constructs such as chimeric or fusion proteins, multimers, aggregates, etc.). The antibody may be of any isotype, such as IgA, IgD, IgE, IgG, IgM, etc. or may be an antibody construct. It may be a monoclonal, monospecific or polyclonal antibody or a fragment of any of these containing at least one antigen binding site.

The antibody may specifically recognize the polypeptide described by SEQ ID NO: 2 or polypeptide having at least 80% sequence similarity or identity to SEQ ID NO: 2 compared to one or more reference proteins, such as those described by Fig. 2. An antibody may bind to a continuous or discontinuous epitope of the polypeptide of SEQ ID NO: 2 or a polypeptide similar to it. For example, it may recognize an continuous epitope having 6, 7, 8, 9, 10, 11 or 12 contiguous amino acid residues described by SEQ ID NO: 2 or a discontinuous or conformation epitope having 15, 20, 25, 30, 40, 50 or more contiguous residues of SEQ ID NO: 2.

The antibody may be unbound, such as an antibody in solution, or bound covalently or non-covalently to a glass slide, a plastic slide, a tissue culture plate, a microtiter well, a glass tube, a plastic tube, a bead, including latex, polystyrene, or glass beads, a particle, including a microparticle or a nano particle, a chip, such as a silicon chip or array, or other solid substrate. Such antibodies or their fragments may be components of an apparatus such as one that purifies or detects a polypeptide having esterase activity. Methods for producing polyclonal and monoclonal antibodies are well-known in the art. For example, polyclonal antibodies may be produced by immunizing a mammal, such as a mouse, rat, guinea pig, or rabbit with the polypeptide of SEQ ID NO: 2 or an immunogenic fragment thereof optionally with an adjuvant, boosting and recovering antibodies after induction of a secondary immune response. Monoclonal antibodies may be made according to the method of Kohler and Milstein; see Köhler, G.; Milstein, C. (1975). "Continuous cultures of fused cells secreting antibody of predefined specificity". *Nature* **256** (5517): 495–497 which is incorporated by reference.

Another aspect of the invention involves the use of the esterases according to the present invention for enzymatic hydrolysis. This method generally involves contacting a compound or substance that is an ester or that contains an ester linkages with the esterase polypeptides described herein, such as those comprising SEQ ID NO: 2 or having at least 80% sequence similarity or identity with SEQ ID NO: 2 (or their active fragments or polypeptide constructs), for a time and under conditions sufficient for enzymatic hydrolysis of the ester. Polypeptides according to the invention which exhibit esterase activity that is resistant to inactivation by agents such as high temperature, high pressure, high salinity, extremes in pH, by the presence of metals, such heavy metals, toxic metals (*e.g.*, biologically metals or metals that inactive other kinds of esterases or lipases), radioactive metals or metal isotopes, may be advantageously used in this method in the presence of these agents.

Such metals include heavy metal, a biologically toxic metal, or a radioactive isotope of a metal is selected from the group consisting of aluminum, antimony, arsenic, barium, beryllium, cadmium, lead, mercury, osmium, thallium, or vanadium; actinium, thorium, uranium, radium, transuranic elements including plutonium and americium, polonium, radioactive isotopes of cobalt, *e.g.*, cobalt-60 and radioactive isotopes of strontium, *e.g.*, strontium-90; chromium, nickel, copper, zinc, and iron. Specifically, heavy metals, a biologically toxic metal, or a radioactive isotope of a

metal may be selected from the group consisting of Ca, Mg, Cu, Zn, Co, Mn, Mg, Fe and Ba.

The polypeptide enzymes according to the invention also include those that are resistant to inactivation in the presence of surfactants, detergents, chaotropic agents or other known enzyme or esterase inhibitors; those that retain activity within the pH range from 3, 4, 5, 6, 7, 8, and ≥ pH 9, preferably between pH 5.5 to 9.0; and those which remain active at salt concentrations ranging from 0, 1, 2, 3, 4, 5 to 6M. Salts include sodium salts (*e.g.*, sodium chloride), potassium salts (*e.g.*, potassium chloride) and salts of divalent cations such as Mg^{2+} or Ca^{2+} (*e.g.*, $MgCl_2$ or $CaCl_2$) as well as other salts found in saline lakes or seas or portions of these having high salinity. Polypeptide enzymes according to the invention also include those which retain activity at temperatures ranging from the freezing point of a solution containing the enzyme to the boiling point of the solution, *e.g.*, from freezing point to 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95,100°C or boiling point; preferably from 30°C to 80°C; enzymes that retain activity under substantially anoxic conditions, and enzymes that retain activity at standard atmospheric pressure 1.013 bars (14.696 psi) or at higher pressures, *e.g.* suboceanic pressures, *e.g.*, those ranging up to 1,086 bars (15,750 psi).

In one advantageous embodiment the enzyme according to the invention has lipase/esterase activity at a temperature ranging from 45-75°C and retains this activity even at 80°C. It exhibits high activity under alkaline conditions and maximum activity in a sodium chloride solution at a concentration of 2M NaCl. This embodiment is resistant to inactivation by Ca, Mg, Cu, Zn, Co, Mn, Mg, Fe and Ba. It is active on short chain esters, especially in cleaving acetyl esters, but also exhibits activity against 4-carbon esters and 6-carbon esters and it lacks substantial activity on longer chain esters and thus can be subclassified as an esterase.

Other aspects of the invention include the following.

A method for processing a food comprising contacting the food, nutraceutical, sweetener, or a flavoring with an enzyme or polypeptide enzyme according to the invention or with a cell expressing said enzyme or polypeptide enzyme under conditions suitable for partial or complete cleavage of esters in said food or flavoring, such as cleavage of long chain esters into short chain esters, or under conditions suitable to improve the organoleptic or nutritional properties of the food or flavoring.

A method for processing, transforming, degrading or recycling an organic material, such as leather, cellulose, wood pulp, or paper, comprising contacting the

organic material with an enzyme or polypeptide enzyme according to the invention or with a cell expressing said enzyme or polypeptide enzyme under conditions suitable for partial or complete cleavage of esters in the organic material. Such a method may be applied for bulk solubilization of biomass.

A method for processing, transforming, degrading or recycling a synthetic material, such as a plastic containing ester linkages, comprising contacting the synthetic material with an enzyme or polypeptide enzyme according to the invention or with a cell expressing said enzyme or polypeptide enzyme under conditions suitable for partial or complete cleavage of esters in synthetic material.

A method for processing, transforming, or refining a biofuel or a petrochemical, such as crude oil or other fuel stock that contains esters, comprising contacting the petrochemical with an enzyme or polypeptide enzyme according to the invention or with a cell expressing said enzyme or polypeptide enzyme under conditions suitable for partial or complete cleavage of esters in synthetic material.

A method for processing a chemical substrate that contains esters as well as metals or salts, comprising contacting the chemical substrate with an enzyme or polypeptide enzyme according to the invention or with a cell expressing said enzyme or polypeptide enzyme under conditions suitable for partial or complete cleavage of esters in the chemical substrate. A chemical substrate may be one used to produce a pharmaceutical product, a nutraceutical, a bulk chemical, or a fine chemical.

A method for processing waste material that contains esters and optionally metals and/or salts, comprising contacting the waste with an enzyme or polypeptide enzyme according to the invention or with a cell expressing said enzyme or polypeptide enzyme under conditions suitable for partial or complete cleavage of esters in the waste material. Other products and methods of use as described in the background section above are also specifically contemplated.

EXAMPLES

Example 1. Sample collection, DNA isolation and Fosmid Library Construction.

Water samples were collected from Atlantis II brine lower convective layer (LCL) during the KAUST Red Sea 2010 expedition (Latitude (N) 21° and Longitude (E) 38°). Collected water samples were immediately processed by serial filtration on mixed Cellulose Esters filters (Nitrocellulose/Cellulose Acetate) with pore sizes of 3,

0.8 and 0.1μm. Filters were stored in sucrose buffer followed by DNA extraction. DNA extraction was carried out using the Epicentre Metagenomic DNA Isolation Kit for Water, from the 0.1 μm filters. Fosmid library construction was carried out using Copy Control Fosmid Library Production Kit (Epicentre), in which the metagenomic DNA was sheared, size selected of ~40 kb size DNA fragments and subsequently cloned into fosmids and transformed into *E. coli* host cells. The constructed library (fosmid vector pCC2FOS) was spread over 11 large petri dish plates. Then colonies were picked individually and each colony was transferred to one well of the 96 well ELISA plates, such that each well contains only one fosmid. The result was a total of 11196 well ELISA plates with total of 10,656 clones.

Results. Screening Metagenomic Library for Lipolytic Activity

The constructed fosmid library comprised 10,656 clones that were manually placed into 111 96-well plates for ease of handling. Functional screening of the fosmid library on tributyrin agar detected a total of five recombinant clones forming a clear halo zone indicative of putative lipolytic activity.

Example 2. Functional Screening for Lipolytic Activity, Sequencing and Identification of Lipolytic Gene

Transformants were grown on LB agar plates supplemented with 12.5μg chloramphenicol/ml and 1% Tributyrin (Sigma-Aldrich). Plates were incubated at 37°C for 3 days and the appearance of a clear halo zone around a transformant was indicative of a candidate lipolytic activity. Candidate transformants were selected for fosmid isolation using the Wizard® Plus SV Minipreps DNA Purification System (Promega).

Fosmids were digested using *Bam*HI to assess their diversity (data not shown) and subjected to pyrosequencing using the GS FLX Titanium pyrosequencer (454 Life Sciences). Table 1a summarizes the pyrosequencing data. Sequences obtained were assembled using GS FLX de novo assembler. Open reading frames (ORFs) were identified using the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) provided by the National Center for Biotechnology Information (NCBI). The putative function of each ORF was annotated by comparing the amino acid sequences to the non-redundant protein database using BLASTP.

Table 1a: Characteristics of 454 pyrosequencing data

Total Number of Reads	31767
Total Number Of Bases	7755522
Number of Aligned Reads	28166 (88.66%)
Number of Aligned Bases	6936553 (89.44%)
All Contig Metrics	
Number Of Contigs	11
Number Of Bases	107205
Large Contig Metrics	
Number Of Contigs	4
Number Of Bases	90337
Average Contig Size (bp)	22584
Largest Contig Size (bp)	32374

Example 3. Sequence Analysis and Phylogenetic Tree Construction

Domain search was conducted by the Conserved Domain (CD)-search tool provided by NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Prediction of signal peptide sequence was performed using SignalP 3.0 servers. For phylogenetic analysis, sequences of 43 bacterial lipolytic enzymes (representing the eight families of bacterial lipolytic enzymes as classified by Arpigny & Jaeger, 1999[17]) were retrieved from the GenBank sequence database. The selected 43 enzymes are present in Figure 3. Multiple sequence alignment of retrieved sequences and EstATII was performed using ClustalW version 1.83 [18]. Phylogenetic tree was constructed using the neighbor-joining method using the software MEGA version 5.05 [19]. Bootstrapping (10,000 replicates) was used to estimate the confidence of the tree.

Results: Sequencing, Identification and Sequence Analysis

Fosmids of the five positive recombinant clones were pyrosequenced to identify the genes responsible for putative lipolytic activity. Following assembly, four large contigs (> 10 kb) and seven smaller contigs (ranging from 1.4 – 4.9 kb) were obtained; the largest contig obtained being approximately 32 kb (Table 1b). A 945 bp ORF encoding a putative esterase/lipase (designated EstATII) was identified.

Table 1b: Contigs generated from 454 pyrosequencing data and utilized in this study.

Contig #	Length (bp)	Number of Aligned Reads
1	32374	5647
2	23734	2626

3	21070	5060
4	13159	10136
5	4915	977
6	4767	2319
7	1606	1074
8	1431	22
9	1407	23
10	1403	21
11	1339	49

The maximum identity to sequences in the database was 65% with an alpha/beta hydrolase domain-containing protein from *Pseudomonas mendocina*. The highest identity to a lipolytic enzyme in the database was 56% to an esterase from *Pseudomonas aeruginosa*. EstATII was the only lipolytic enzyme detected, however other ORFs encoding for sulfatases were detected which could be responsible for false positive activity as previously reported [22]. EstATII consists of 945 bp corresponding to 314 amino acids. A domain search conducted using CD-search tool detected an alpha/beta hydrolase fold domain [Pfam ID: pfam07859] between residues 85 and 286, which is the catalytic domain found in members of the alpha/beta hydrolases family. An esterase/lipase domain (cd00312) was also detected. In addition, two prokaryotic Clusters of Orthologous Groups (COGs) were identified; COG0657 and COG2272 which are involved in lipid metabolism.

The catalytic triad residues were identified in EstATII; Ser160, Asp204 and His282. The catalytic nucleophilic residue Ser160 was found in the consensus pentapeptide GDSAG (SEQ ID NO:8), which is characteristic of the hormone sensitive lipase (HSL) family. Another motif characteristic of the HSL family (HGG), which contributes to the formation of the oxyanion hole, was also identified in the sequence (Figure 2). EstATII was predicted to be soluble since a signal peptide was not detected. A transmembrane domain was identified using TMAP program[23]. This domain is a stretch of 24 amino acids at N-terminal site from amino acid 29-52 (data not shown). The membrane imbedded domain was repeatedly reported in other identified esterase[24].

Bacterial lipolytic enzymes were classified into eight families by Arpigny & Jaeger in 1999 [17]. In order to determine whether EstATII classifies as a member of one of these families, a multiple sequence alignment of EstATII together with 43 sequences of bacterial lipolytic enzymes, representing the eight families, was

performed. A phylogenetic tree was constructed and EstATII grouped with members of family IV which is also known as the HSL family (Figure 3).

Example 4. Cloning of EstATII gene

The gene was amplified using the forward primer (EstF) 5'-ATG TCC AGG TAC GTT GAT GAG C-3' and the reverse primer (EstR) 5'-TCA GCT TAC CGA GTC GGT CT-3'using *Taq* polymerase (Fermentas). The primers were designed based on a 945 bp ORF in Contig 1 (Table 1-b), which was annotated as a putative lipolytic sequence by BlastP. The amplified fragment was cloned into the pET-SUMO vector (Champion™ pET SUMO Protein Expression System kit, Invitrogen) according to manufacturer's instructions. Recombinant plasmids were transformed into *E. coli* BL21 (DE3) chemical competent cells. Colony PCR using the gene primers was performed to verify the presence of the insert, while colony PCR using the gene forward primer and the vector reverse primer was performed to verify the orientation of the gene.

Example 5. Overexpression and Purification of Recombinant EstATII enzyme

200 ml of *E. coli* BL21(DE3) harboring the pET-SUMO/EstATII plasmid were grown in LB at 37°C until the culture reached an OD_{600}= 0.4-0.6. The culture was induced by adding isopropyl-b-D thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and further incubated for 3 hours at 37°C. The cells were harvested by centrifugation at 10,000 × g for 15 min at 4°C. The purification procedure was performed using the His SpinTrap™ (GE Healthcare) according to manufacturer's instructions. The purified protein was dialysed against 50 mM NaH_2PO_4 buffer, pH 8.0, analyzed by SDS-PAGE and stored at 4°C until further use. Protein isolation was confirmed by Western Blot analysis using an anti-His-G antibody (Invitrogen).

Results: Overexpression and Purification of EstATII

In order to investigate the biochemical characteristics of EstATII, the gene was expressed as an N-terminal His-tag recombinant protein in the expression vector pET-SUMO in *E. coli* BL21 (DE3) cells. The overexpressed protein had the expected molecular weight of approximately 46 kDa and western blot analysis of the purified protein showed a single band at the expected size (Figure 4).

Example 6. Characterization of EstATII

Enzyme activity was determined by measuring the formation of *p*-nitrophenol (pNP) from the enzymatic hydrolysis of the *p*-nitrophenyl ester; *p*-nitrophenyl butyrate (Sigma-Aldrich). The reaction mixture contained *p*-nitrophenyl butyrate (pNPB) to a final concentration 0.1 mM and 50 mM Tris-HCl pH=8. In this study, the standard reaction was conducted at 65°C, initiated by the addition of EstATII and terminated by the addition of 10% SDS. Measurements were done at 410 nm using UV-spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences) unless stated otherwise[20]. All experiments were performed in triplicates. Substrate specificity of EstATII was determined using *p*-nitrophenol esters with varying side chain length. Short-chain fatty acid esters used were PNP-acetate (C2), PNP-butyrate (C4) and PNP-valerate (C5) and long-chain fatty acid esters used were PNP-decanoate (C10), PNP-dodecanoate C12, PNP-myristate (C14) and PNP-palmitate (C16).The optimum temperature for the activity of EstATII was determined at a temperature range 30-80°C. The optimum pH for the activity of EstATII was measured at a pH range (3-9.5) using the following buffers: 50 mM sodium acetate (pH 3-5.5), 50 mM sodium phosphate (pH 6, 7.5) and 50 mM Tris-HCL (pH 7.5-9.5). Formation of pNP was measured at 348 nm (the pH-independent isosbestic wavelength of pNP)[21].The effect of NaCl concentration on enzyme activity was measured at different NaCl concentrations (0-4.5 M) under standard assay conditions. To assess the effect of metal ions on enzyme activity, cations were added to a final concentration of 1mM, and relative activity measured at the above-described standard conditions. The effect of detergents (at final concentrations 0.1 and 1%) and inhibitors (final concentration 1 mM) on enzyme activity was tested at the above-described standard assay conditions.

Results: Biochemical Characterization of EstATII

Effect of temperature and pH on the Activity of EstATII

The effect of temperature on the activity of EstATII was assayed at temperatures ranging from 30°C to 80°C. The activity of the enzyme increased reproducibly with the increase in temperature until 65°C, after which the activity started to drop. High activity of the enzyme (>70%) was observed at temperatures ranging from 45°C to 75°C. The apparent optimum temperature of EstATII is 65°C. The enzyme remained active even after reaching 80°C (Table 2).

The effect of pH on the activity of EstATII was assayed at pH range 3-9. The enzyme exhibited significant activity (>50%) at pH= 7-9, with the highest activity at pH=8.5. No activity was observed at pH lower than 5.5 (Table 2).

Effect of NaCl concentration on the activity of EstATII

The activity of EstATII was assayed at different molar concentrations of NaCl ranging from 0M – 4.5M. The enzyme showed highest activity in the presence of 2M NaCl. Enzyme activity was maintained up to 4.5M NaCl (Table 2).

Substrate specificity of EstATII

To assess whether EstATII is a lipase or an esterase, its substrate specificity was investigated using an array of p-nitrophenol esters with varying chain length. EstATII was active towards short-chain fatty acid esters (C2, C4 and C5), however, under the conditions it showed no activity towards long-chain fatty acid esters (C10, C12, C14 and C16) indicating that EstATII is an esterase not a lipase[25] (Table 2). However, these results do not preclude activity on other substrates, such as longer chain fatty acid esters under different conditions, such as conditions found in extreme environments such as deep sea brine pools. Biocatalysts such as EstATII-type esterases often reflect the conditions of the environment from which they were isolated and therefore can be potentially used for industrial and biotechnological applications that employ extreme conditions.

Effect of metal ions on the activity of EstATII

The effect of metal ions was assessed with and without EDTA chelation. Enzyme activity was not affected by EDTA chelation, suggesting that EstATII is not a metalloenzyme [9]. Enzyme activity was promoted by Barium (158%), Manganese (111%) and Cobalt (104%). The enzyme was resistant to inhibition by the rest of the metal ions tested; activity remained above 60%(Table 3).Upon investigating the effect of heavy metal ions on the activity of EstATII in comparison to other esterases (thermophilic and mesophilic), it was noticed that Copper, Zinc and Mercury exhibit a strong inhibitory effect on the activity of most esterases included in the comparison (<50%) (Table 3). Although EstATII shows significant resistance to all heavy metal ions tested, resistance to these three heavy metals is of particular interest due to their strong inhibitory effect on most identified esterases.

Table 3: Activity of Mesophilic and Thermophilic Esterase in comparison to the Red Sea EstAII activity

		Temp° C	pH	Ca^{2+}	Mg^{2+}	Cu^{2+}	Zn^{2+}	Co^{2+}	Mn^{2+}	Hg^{2+}	Fe^{3+}	Ba^{2+}	Ref.
Thermophilic	EstATII	65	8.5	94.8 ± 3	90.6 ± 3.6	94.8 ± 0.1	85.2 ± 4.4	104 ± 2	111.8 ± 0.1	60.9 ± 0.4	96.2 ± 0.7	158.3 ± 2.2	
	G.obscurus	80	8	91.7	100	76.9	43.9	114.9	97.4	49.9	N/A	N/A	26
	LKE-028	70	11	167.7	173.3	94.1	43.6	124.7	N/A	89.3	84.4	33.9	27
	EstA3	70	9.5	80.3	82.8	24.9	37.1	61.8	66.6	31.3	54.1	N/A	38
	EstA	60-65	9.5	152	170	N/A	17	N/A	N/A	N/A	N/A	N/A	39
	EstCS2	55	9	119	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	29
	DR8806	50	8	64.5	60.7	34.9	26	N/A	47.1	21.9	N/A	N/A	28
	A. gonensis A4	60-80	5.5	86 ± 4	N/A	69 ± 3	59 ± 4	65 ± 5	96 ± 4	47 ± 2	N/A	N/A	40
	EstR	60	9	87	87	93	87	N/A	95	N/A	73	N/A	35
	EstY	50	9	~90	N/A	N/A	~ 15	N/A	N/A	N/A	~40	N/A	30
	Fusarium	50	8	96.6 ± 2.6	94.9 ± 2.7	11.7 ± 0.4	75.2 ± 1.0	98.1 ± 1.4	91.9 ± 2.6	38.4 ± 1.5	N/A	N/A	41

Mesophilic	EstIM1	40°C	8	95	88	76	18	87	90	8	N/A	84	33
	Est_p1	40°C	8.57	86.5 ± 1	101 ± 0.03	11.3 ± 2	24.6 ± 5	79.6± 14	85.5 ± 3	N/A	N/A	N/A	34
	EstAS	35°C	9	100.5 ± 3.4	81.7 ± 2.9	7.8± 2.3	114. 7± 1.3	117.8 ± 2.1	192.9 ± 3.8	N/A	N/A	N/A	9
	EstA	45°C	6.5	102.9 ±6.7	139.5 ± 7.8	99.3 ± 8.1	89.7 ±8.7	116.5 ± 6.6	142.8 ± 9.5	N/A	N/A	N/A	36
	EstB	45°C	7.5	107.6 ±8.3	86.1 ± 6.4	27.2± 2.3	11.4 ±1.1	116± 7.2	88.7 ± 4.9	N/A	N/A	N/A	36
	EstEH112	35°C	8	102	102	97	96	102	101	N/A	N/A	N/A	37
	EstPc	35°C	8.5	N/A	94	49	0.80	93	121	N/A	N/A	N/A	31
	rEst97	35°C	7.5	96.4 ± 6.3	96.7 ±2.8	68.3± 3.3	7.4± 0.5	80.4± 4.3	97.1 ±3.3	N/A	71.4 ± 9.9	N/A	42
	HBB-4	N/A	N/A	102.9 ± 0.29	93.21 ±1.56	10.78 ± 1.46	N/A	84.11 ± 1.33	159.7 ± 3.31	3.41 ± 0.28	72.07 ± 1.13	N/A	43
	EstKT4	40°C	8.5	83	81	68	38	57	27	N/A	N/A	N/A	44
	EstKT7	35°C	8	68	75	25	22	115	65	N/A	N/A	N/A	44
	EstKT9	45°C	8.5	90	96	77	26	78	92	N/A	N/A	N/A	44
	EstC	35	8.5-9	88 + 1	93 + 0.6	75 + 0.1	18 + 1	56 +1	88 + 1	N/A	N/A	N/A	32

Effect of detergents, reducing and modifying agents on the Activity of EstATII

The effect of detergents (ionic and non-ionic) was tested at two concentrations: 0.1% and 1%. At 0.1%, Tween 80 showed no effect on enzyme activity. Triton X-100 and Tween 20 were tolerated by the enzyme (42.3% and 87.5% respectively), while SDS dramatically inhibited enzyme activity to 6%. At 1%, EstATII was tolerant to the effects of Tween 20 and 80 (40.1% and 54.5% respectively), while SDS and Triton X-100 abolished enzyme activity. Reducing agent β-mercaptoethanol (at final concentration 1 mM) enhanced enzyme activity to 117.9%, while 1 mM DTT slightly inhibited activity to 78.9%. DEPC, the histidine residue modifier, reduced activity by half. This result is in agreement with the involvement of a histidine residue in the catalytic triad (Table 2).

Table 2: Effect of Temperature, pH, substrate chain length and some additives on EstATII activity

Variable	Relative Activity
Temperature (°C)	
30	56.3 ± 0.8
35	59.9 ± 1
40	65.3 ± 1.8
45	86.9 ± 0.9
50	90.9 ± 2
55	93.9 ± 1
60	95.9 ± 0.3
65[a]	100 ± 0.2
70	91.6 ± 0.15
75	76.7 ± 0.3
80	46.7 ± 1.3
pH	
3	0
4	0
5	0
5.5	13.1 ± 0.2
6	38.5 ± 1.2
6.5	43.2 ± 2
7	81.5 ± 1
7.5 *(Na Phosphate)*	88 ± 1
7.5 *(Tris.HCl)*	66.1 ± 0.2
8	84.7 ± 1.1
8.5 [a]	100 ± 3
9	60 ± 2
Substrate Specificity	

pNP-acetate (C2)	100 ± 3
pNP-butyrate (C4)	61.9 ± 0.8
pNP-valerate (C5)	40.5 ± 1.2
pNP-decanoate (C10)	0
pNP-dodecanoate (C12)	0
pNP-myristate (C14)	0
pNP-palmitate (C16)	0
Sodium Chloride [b]	
0.5M	102.2 ± 2
1M	99.9 ± 0.5
1.5M	120 ± 1
2M	122.9 ± 1
2.5M	102.4 ± 1.8
3M	73.8 ± 3.8
3.5M	65.3 ± 4.6
4M	53.8 ± 1
4.5M	39.7 ± 1.7
Detergents (0.1%)	
SDS	6.1 ± 0.4
Triton X-100	42.2 ± 1.1
Tween 20	87.5 ± 2.3
Tween 80	100.7 ± 2.3
Detergents (1%)	
SDS	0
Triton X-100	4.3 ± 1
Tween 20	40.1 ± 0.9
Tween 80	54.5 ± 0.7
Reducing and Modifying agents (1 mM)	
β-mercaptoethanol	117.9 ± 0.3
DTT	78.9 ± 4.2
DEPC	48
CTAB	13.1 ± 1.9

[a] Optimum condition (defined as 100%)
[b] Activity in the absence of NaCl is defined as 100%

As shown above, the inventors have identified a novel esterase (EstATII) from the Atlantis II brine pool in the Red Sea, using a function-based approach. Sequence and phylogenetic analysis of EstATII revealed that it is a new member of the Hormone-Sensitive Lipase family (Family IV). EstATII groups with members of the HSL family and was found to harbor conserved motifs characteristic of this family. Characterization of EstATII reflected the environment from which it was isolated; shows that it is thermophilic with an optimum temperature 65°C and halotolerant maintaining significant activity (>50%) up to 4M NaCl.

In addition and of particular interest, EstATII shows significant resistance to inhibition by all heavy metal ions tested in this work (Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, $Fe^{3+}$and Ba^{2+}). In this aspect, EstATII was compared to 23 recently characterized esterases of both thermophilic and mesophilic nature. They represent different families and are isolated from either a single isolate or a metagenomic library. These include selected thermophilic esterases from *G. obscurus* [26], *Rhodococcus sp.* LKE-028 [27], *Bacillus subtilis* DR8806 [28], as well as, EstCS2 from compost soil metagenomic library [29] and EstY from metagenomic library of Yangtze River [30]. Examples of mesophilic esterases selected for this comparison include EstPc, a cold-adapted esterase from *Psychrobacter cryohalolentis* K5T [31], EstC; a cold-active esterase from *Streptomyces coelicolor* A3(2) [32], as well as, EstIM1 from a metagenomic library of mountain soil [33] and Est_p1 from a metagenomic library of neritic sediments of the South China sea [34]. In our comparison, an enzyme was considered significantly inhibited by a given heavy metal ion, if its relative activity dropped below 50%. It was found that most esterases included for comparison were significantly inhibited by three of the tested metal ions; Copper, Zinc and Mercury. Copper significantly inhibited 37.5% of thermophilic esterases and 46% of mesophilic esterases (~43% of all esterases). Zinc inhibited 60% of thermophilic esterases and 75% of mesophilic esterases (~68% of all esterases). Mercury significantly inhibited ~71.5% of thermophilic esterases and 100% of mesophilic esterases (~78% of all esterases). 19 out of the 23 esterases discussed in our analysis exhibited significant inhibition by at least one of the three heavy metal ions. Only 4 esterases showed resistance to inhibition by all metal ions tested; EstATII (this study), EstR (isolated from Ralstonia sp. M1) [35], EstA (isolated from a metagenomic library of the South China sea) [36] and EstH112 (isolated from the metagenome of a Korean intertidal flat sediment) [37]. Some comparisons using certain metals, detergents and/or inhibitors were not made since they were not performed in prior studies. The demonstrated resistance of EstTAII to inhibition by metal ions, in addition to retention of significant activity when exposed to some of the detergents and inhibitors tested, show that EstATII-like esterases are useful biocatalyst under conditions adverse to enzymatic activity of other esterases.

Incorporation by Reference

Each document, patent, patent application or patent publication cited by or

referred to in this disclosure is incorporated by reference in its entirety especially for describing the subject matter surrounding the citation of the reference in the text. However, no admission is made that any such reference constitutes background art and the right to challenge the accuracy and pertinence of the cited documents is reserved.

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CLAIMS

1. A polypeptide of SEQ ID NO: 2, a polypeptide that comprises a sequence that is least 85% similar or identical to the amino acid sequence of SEQ ID NO: 2, or an immunogenic fragment thereof or a fragment thereof having esterase activity.

2. The polypeptide of claim 1 that is at least 90% similar or identical to the amino acid sequence of SEQ ID NO: 2 or an immunogenic fragment thereof or a fragment thereof having esterase activity.

3. The polypeptide of claim 1 that is at least 95% similar or identical to the amino acid sequence of SEQ ID NO: 2 or an immunogenic fragment thereof or a fragment thereof having esterase activity.

4. The polypeptide of claim 1 that is at least 97.5% similar or identical to the amino acid sequence of SEQ ID NO: 2 or an immunogenic fragment thereof or a fragment thereof having esterase activity.

5. The polypeptide of claim 1 that is at least 99% similar or identical to the amino acid sequence of SEQ ID NO: 2 or an immunogenic fragment thereof or a fragment thereof having esterase activity.

6. The polypeptide or fragment thereof of claim 1 is a fusion protein or a chimeric protein that further comprises additional fused amino acid residues, such as a GST protein sequence, FLAG peptide sequence, or hexa-his peptide sequence to facilitate affinity purification with a nickel or cobalt resin, or other fused amino acid sequences.

7. The polypeptide of claim 1 that is bound to a glass slide, a plastic slide, a tissue culture plate, a microtiter well, a glass tube, a plastic tube, a bead, including

latex, polystyrene, or glass beads, a particle, including a microparticle or a nano particle, a chip, such as a silicon chip or array, or other solid substrate.

8. An apparatus comprising the polypeptide of claim 1 or fragment thereof as an active component and one or more containers or contact surfaces for contacting said polypeptide with a substance containing an ester.

9. An apparatus comprising a cell containing or expressing the polypeptide of claim 1 or fragment thereof as an active component and one or more containers or contact surfaces for contacting said cell with a substance containing an ester.

10. A composition comprising the polypeptide or fragment thereof of claim 1 and a secondary component, wherein said secondary component may be a pharmaceutical product or composition, a nutritional product or composition, or a carrier, excipient or buffer.

11. The composition of claim 10 that comprises a dairy product or other food or drink component containing esters; a pharmaceutical product containing a pharmaceutically acceptable carrier or excipient; or a detergent, cleaning product, or other product for breaking down materials or contaminants containing esters.

12. An antibody that binds to the polypeptide of claim 1 or a fragment thereof comprising an antigen binding site.

13. The antibody of claim 12 that is a monoclonal, monospecific or polyclonal antibody or a fragment thereof comprising an antigen binding site.

14. The antibody or fragment thereof of claim 12 that does not bind to one or more of the polypeptides described in Fig. 2.

15. The antibody of claim 12 that is bound to a glass slide, a plastic slide, a tissue culture plate, a microtiter well, a glass tube, a plastic tube, a bead, including

latex, polystyrene, or glass beads, a particle, including a microparticle or a nano particle, a chip, such as a silicon chip or array, or other solid substrate.

16. An apparatus comprising the antibody of claim 12 or a fragment thereof comprising an antigen binding site.

17. A method of enzymatic hydrolysis comprising contacting a compound or substance containing an ester link with the polypeptide or polypeptide fragment of claim 1 or with a cell expressing said polypeptide for a time and under conditions sufficient for enzymatic hydrolysis of the ester.

18. The method of claim 17, wherein said contacting occurs in the presence of a heavy metal, a biologically toxic metal, or a radioactive isotope of a metal.

19. The method of claim 18, wherein said heavy metal, a biologically toxic metal, or a radioactive isotope of a metal is selected from the group consisting of aluminum, antimony, arsenic, barium, beryllium, cadmium, lead, mercury, osmium, thallium, or vanadium; actinium, thorium, uranium, radium, transuranic elements including plutonium and americium, polonium, cobalt-60 and other radioactive isotopes of cobalt, strontium-90 and other radioactive isotopes of strontium; chromium, nickel, copper, zinc, and iron.

20. The method of claim 18, wherein said heavy metal, a biologically toxic metal, or a radioactive isotope of a metal is selected from the group consisting of Ca, Mg, Cu, Zn, Co, Mn, Mg, Fe and Ba.

21. The method of claim 17, wherein said contacting occurs in the presence of at least one surfactant, detergent, or chaotropic agent.

22. The method of claim 17, wherein said contacting occurs at a pH ranging from pH 3 to $\geq$ pH 9, preferably between pH 5.5 to 9.0.

23. The method of claim 17, wherein said contacting occurs at a salt concentration ranging from 0, 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 to 6M.

24. The method of claim 17, wherein said contacting occurs at a temperature ranging from the freezing point of a solution containing said polypeptide to the boiling point of said solution, *e.g.*, from freezing point to 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95,100°C or boiling point; preferably from 30°C to 80°C.

25. The method of claim 17, wherein said contacting occurs under substantially anoxic conditions.

26. The method of claim 17, wherein said contacting occurs at a pressure of at least standard atmospheric pressure.

27. A method for processing a food comprising contacting the food or a flavoring with the polypeptide of claim 1 or with a cell expressing said polypeptide under conditions suitable for partial or complete cleavage of esters in said food or flavoring, such as cleavage of long chain esters into short chain esters, or under conditions suitable to improve the organoleptic or nutritional properties of the food or flavoring.

28. A method for processing, transforming, degrading or recycling an organic material, such as leather, cellulose, wood pulp, or paper, comprising contacting the organic material with the polypeptide of claim 1 or with a cell expressing said polypeptide under conditions suitable for partial or complete cleavage of esters in the organic material.

29. A method for processing, transforming, degrading or recycling a synthetic material, such as a plastic containing ester linkages, comprising contacting the synthetic material with the polypeptide of claim 1 or with a cell expressing said polypeptide under conditions suitable for partial or complete cleavage of esters in synthetic material.

30. A method for processing, transforming, or refining a petrochemical, such as crude oil or other fuel stock that contains one or more esters, comprising contacting the petrochemical with the polypeptide of claim 1 or with a cell expressing said

polypeptide under conditions suitable for partial or complete cleavage of esters in synthetic material.

31. A method for processing a chemical substrate that contains one or more esters as well as metals or salts, comprising contacting the chemical substrate with the polypeptide of claim 1 or with a cell expressing said polypeptide under conditions suitable for partial or complete cleavage of esters in the chemical substrate.

32. A method for processing waste material that contains one or more esters and optionally metals and/or salts, comprising contacting the waste with the polypeptide of claim 1 or with a cell expressing said polypeptide under conditions suitable for partial or complete cleavage of esters in the waste material.

33. A polynucleotide having a sequence that is at least 85% similar or 85% identical to the polynucleotide sequence described by SEQ ID NO: 1 or a fragment thereof that encodes a polypeptide having esterase activity.

34. The polynucleotide of claim 33 that has a sequence that is at least 90% similar or 90% identical to the polynucleotide sequence described by SEQ ID NO: 1 or a fragment thereof that encodes a polypeptide having esterase activity.

35. The polynucleotide of claim 33 that has a sequence that is at least 95% similar of 95% identical to the polynucleotide sequence described by SEQ ID NO: 1 or a fragment thereof that encodes a polypeptide having esterase activity.

36. The polynucleotide of claim 33 that has a sequence that is at least 99% similar or 99% identical to the polynucleotide sequence described by SEQ ID NO: 1 or a fragment thereof that encodes a polypeptide having esterase activity.

37. The polynucleotide of claim 33 that encodes a polypeptide comprising at least one of the motifs HGGXFXXXXXXXH (SEQ ID NO: 5), VXXXXYXXXPXXXXPXA (SEQ ID NO: 6), or GDSAGXXL (SEQ ID NO: 7).

38. A vector or DNA construct comprising the polynucleotide sequence of claim 33.

39. The vector of claim 38 selected from the group consisting of a bacterial vector; a yeast vector; an insect cell vector; a plant cell vector; and a vector for a mammalian cell or other kind of animal cell; wherein any of said vector may optionally comprise one or more regulatory sequences to enhance or control transcription and/or translation of said polynucleotide.

40. A host cell comprising the polynucleotide of claim 33 or a vector containing it.

41. The host cell of claim 40 selected from the group consisting of a bacterium, including a bacterium of Family IV or the HSL Family; a yeast cell, an insect cell, and a mammalian or other kind of animal cell.

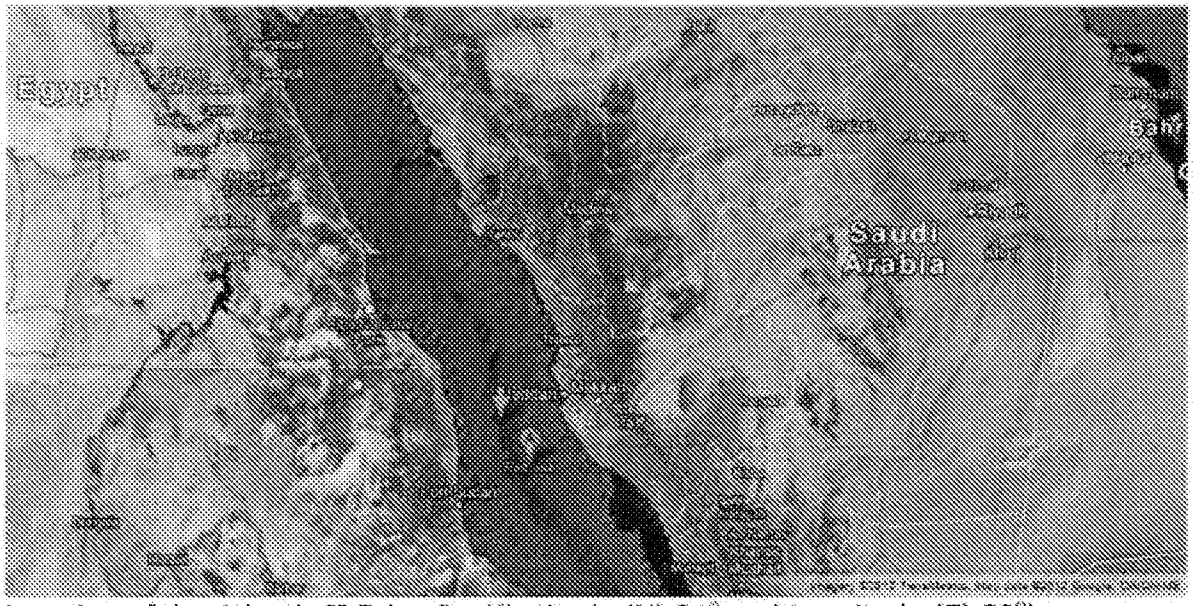
42. A method for making a protein encoded by the polynucleotide of claim 33 comprising:

expressing said polynucleotide, and

recovering the expressed protein,

wherein said expressed protein optionally has esterase activity.

Figure 1: Atlantis II Brine Pool Sample Site



Location of the Atlantis II Brine Pool(Latitude (N) 21° and Longitude (E) 38°) from which the samples were obtained during the KAUST Red Sea 2010 expedition.

Figure 2: Conserved Motifs found in members of The Hormone Sensitive Lipase (HSL) Family and in EstATII

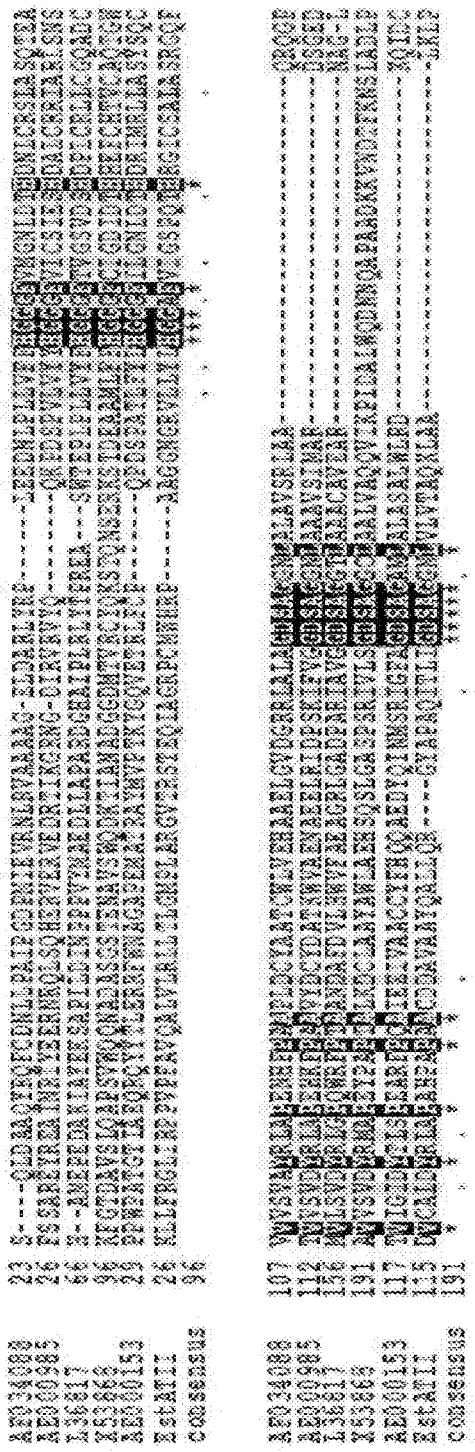


FIGURE 3: Phylogenetic Analysis and Classification of EstATH

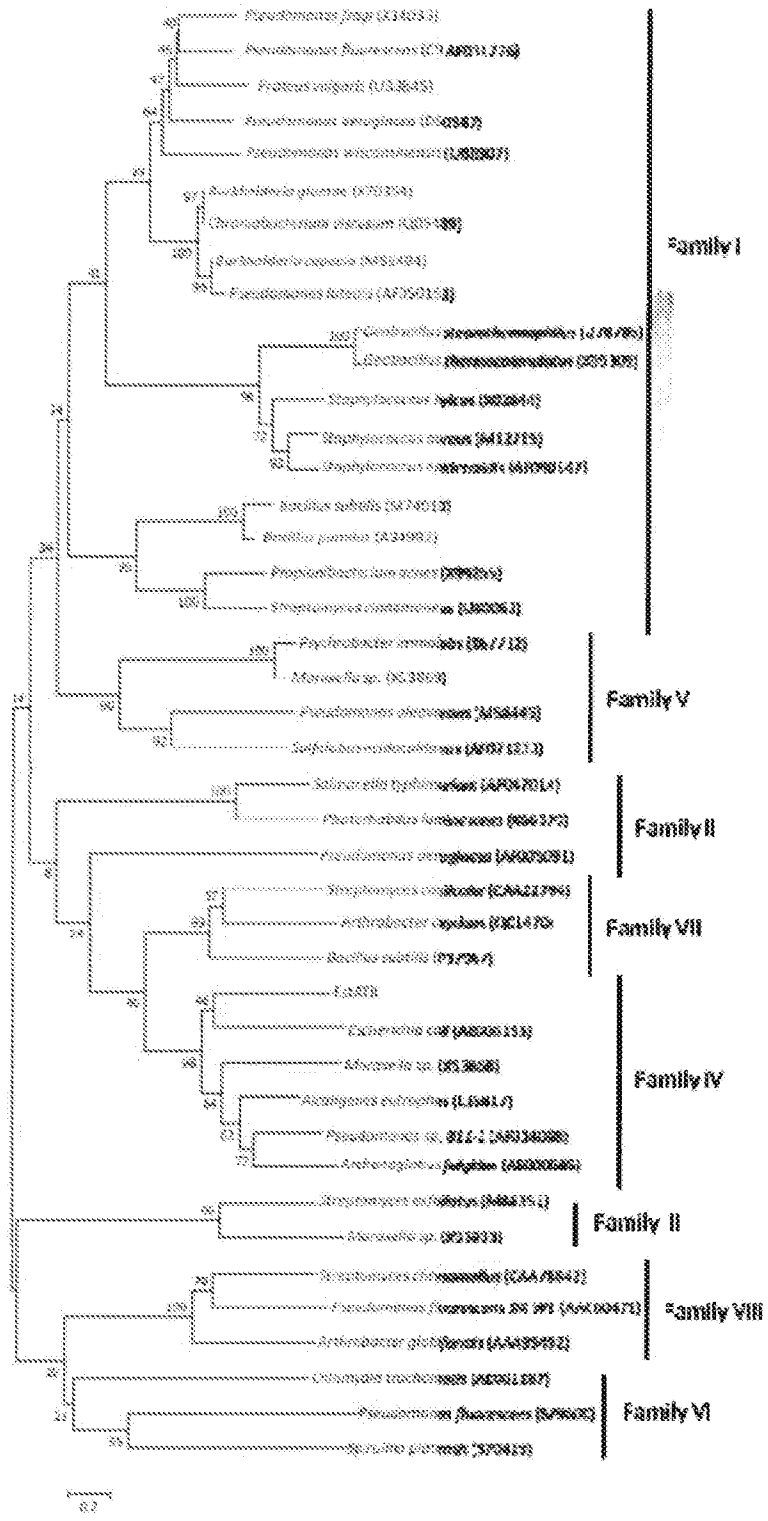
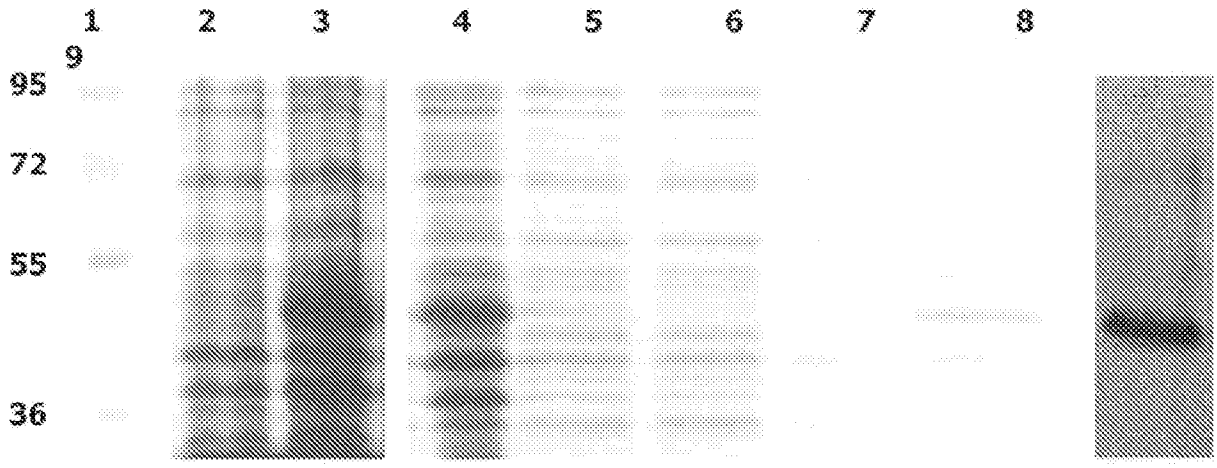


Figure 4: Overexpression, purification and western blot analysis of recombinant EstATII



Lane 1: Molecular Weight marker, Lane 2: Un-induced sample, Lane 3: Sample induced with 0.5 mM IPTG, Lane 4: Protein Lysate, Lanes 5,6: Flowthrough. Lane 7: Wash step. Lane 8: Purified EstATII. Lane 9: Western Blot analysis of purified EstATII.