

Enzymes For Oxidizing Beta-Hydroxybutyrate (BHB), Test Strips, And Sensors of Using the Same

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No.
5 63/580,620, filed September 5, 2023, which application is hereby incorporated by
reference in its entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been
submitted electronically in XML format and is hereby incorporated by reference in its
10 entirety. Said XML copy, created on August 26, 2024, is named 081906-1457969-
253310PC_SL.xml and is 61,977 bytes in size.

TECHNICAL FIELD

[0003] The present disclosure relates to enzymes capable of oxidizing beta-
hydroxybutyrate (BHB) and methods thereof. In an aspect, the present disclosure
15 relates to using enzymes described herein for various applications, such as use in
test strips and additional detection platforms, BHB sensors, and systems for
detecting and measuring BHB concentration.

BACKGROUND

[0004] The ketogenic diet is an effective way to propagate weight loss and
20 wellness. The ketogenic diet encompasses minimizing the intake of carbohydrates
while oftentimes increasing the consumption of fat and/or protein in tandem. This
type of dietary therapy forces the body to burn fats, which leads to the production of
ketone bodies (ketones) as the body breaks down fats. For optimized dietary
management, it is important that a subject on a ketogenic diet be able to monitor
25 ketone levels. The present disclosure provides a solution to this industry and
medical need.

[0005] Beta-hydroxybutyrate (BHB) is the conjugate base of beta-hydroxybutyric
acid. BHB is synthesized via the metabolism of fatty acids, and a high level of BHB
indicates that the body is using fats as its main fuel source. Therefore, there is a

need to accurately and reliably measure BHB levels in a subject. This can be through invasive, minimally invasive, or non-invasive mechanisms. The present disclosure solves this need by describing novel enzymes for aiding in the measurement of BHB. Useful systems, devices, and associated methods are further provided herein.

5 BRIEF SUMMARY OF THE DISCLOSURE

[0006] The instant disclosure relates to enzymes with oxidase activity. In an embodiment, enzymes described herein are engineered and/or modified relative to native enzymes. In aspects described herein enzymes are capable of oxidizing beta-hydroxybutyrate (BHB) to produce 3-oxobutanoate. In some embodiments, beta-
10 hydroxybutyrate (BHB) is (R)-beta-hydroxybutyrate. In some embodiments, beta-hydroxybutyrate (BHB) is (S)-beta-hydroxybutyrate. In some embodiments, beta-hydroxybutyrate (BHB) is a mixture of (R)-beta-hydroxybutyrate and (S)-beta-hydroxybutyrate.

[0007] In some embodiments, the enzyme, for example an engineered enzyme,
15 includes (e.g., comprises, consists essentially of or consists of) an amino acid sequence derived from an oxidase of the EC 1.1.3.6 family. In some embodiments, the oxidase from the EC 1.1.3.6 family is a cholesterol oxidase.

[0008] In some embodiments, the enzyme is non-naturally occurring and/or engineered and includes (e.g., comprises, consists essentially of or consists of) one
20 or more amino acid substitutions, deletions, or truncations relative to a native enzyme.

[0009] In an embodiment, the enzyme described herein covers modified Oxidase 8, derived from *Scytonema sp.* In another embodiment, the enzyme is a modified version of SEQ ID NO: 8 which includes one or more amino acid substitutions,
25 deletions, or truncations. In an embodiment, SEQ ID NO: 8 is optimized to yield improved BHB detection activity.

[0010] In some embodiments, the enzyme includes (e.g., comprises, consists essentially of or consists of) an amino acid sequence encompassing one or more mutations corresponding to N137G, Y235Q, and/or A455Y of SEQ ID NO: 8. In other
30 embodiments, enzymes for use herein include SEQ ID NO: 1 – 43. In some aspects, enzymes for use herein include (e.g., comprise, consist essentially of or consist of) any of SEQ ID NO: 24 or SEQ ID NO: 40 – 43, fragments, or modified proteins therein.

[0011] In some embodiments, the enzyme includes (e.g., comprises, consists essentially of or consists of) an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to any of SEQ ID NO: 1 – 43. In some embodiments, the

5 enzyme includes (e.g., comprises, consists essentially of or consists of) an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to any of SEQ ID NO: 24 or SEQ ID NO: 40 – 43, fragments, or modified proteins therein.

[0012] In additional embodiments, the enzyme includes (e.g., comprises, consists essentially of or consists of) an amino acid sequence of any of SEQ ID NO: 1 – 43, wherein the amino acid sequence includes one, two, three, four, five, six, seven, eight, nine, ten, fifteen or more amino acid substitutions. In some embodiments, the enzyme includes an amino acid sequence of any of SEQ ID NO: 24 or 40 – 43, wherein the amino acid sequence includes (e.g., comprises, consists essentially of or

10 consists of) one, two, three, four, five, six, seven, eight, nine, ten, fifteen or more amino acid substitutions. In embodiments, the amino acid substitution(s) are conservative substitutions. In embodiments the substitutions are made only to the portions of the protein which are responsible for binding and/or propagating activity.

[0013] In an embodiment, the disclosure provides for a protein including (e.g., comprising, consisting essentially of or consisting of) an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to any of SEQ ID NO: 1 – 43. In some embodiments, the disclosure provides for an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least

20 98%, at least 99%, or at least 99.5% identical to any of SEQ ID NO: 24 or SEQ ID NO: 40 – 43, fragments, or modified proteins therein.

[0014] In additional embodiments, the disclosure provides for a protein including (e.g., comprising, consisting essentially of or consisting of) an amino acid sequence of any of SEQ ID NO: 1 – 43, wherein the amino acid sequence includes (e.g., comprises, consists essentially of or consists of) one, two, three, four, five, six, seven, eight, nine, ten, or fifteen or more amino acid substitutions. In some

30 embodiments, the disclosure provides for a protein with an amino acid sequence of any of SEQ ID NO: 24 or 40 – 43, wherein the amino acid sequence includes one, two, three, four, five, six, seven, eight, nine, ten, or fifteen or more amino acid

substitutions. In embodiments, the amino acid substitution(s) are conservative substitutions.

[0015] In additional embodiments, engineered or modified enzymes described herein exhibit increased BHB activity relative to the corresponding wild-type enzyme.

- 5 As used herein, wild-type, wildtype, wild type, and WT are all synonymous and refer to an enzyme found naturally (i.e., in its wild or state in nature). As used herein, mutant, mut, MUT are all synonymous and refer to a modified enzyme and/or a mutated enzyme, such as a modified and/or mutant enzyme created by enzyme design and/or enzyme engineering. Directed evolution, rational design, and
- 10 semirational design are the major in silico assisted approaches used widely in enzyme engineering. In an embodiment, the BHB activity is measured by peroxide strips, acetoacetic strips, and/or liquid chromatography–mass spectrometry (LCMS). In an embodiment, the peroxide strips are Bartovation strips and the acetoacetic strips are Bayer Ketostix® strips. In an aspect, the BHB activity for the modified or
- 15 engineered enzyme is about one (1), two (2), three (3), four (4), five (5), six (6), seven (7), eight (8), nine (9), ten (10), twenty (20), thirty (30), forty (40), fifty (50), sixty (60), seventy (70), eighty (80), ninety (90), one hundred (100), two hundred (200), three hundred (300), four hundred (400), five hundred (500), six hundred (600), seven hundred (700), eight hundred (800), nine hundred (900), one thousand
- 20 (1,000), ten thousand (10,000), one hundred thousand (100,000) or more times greater than the corresponding non-modified wild-type when measured by LCMS.
- [0016] In another embodiment, SEQ ID NO: 24 or 40 – 43 and derivatives thereof exhibit an increased BHB activity relative to SEQ ID NO: 8. In an embodiment, the BHB activity is measured by peroxide strips, acetoacetic strips, or liquid
- 25 chromatography–mass spectrometry (LCMS). In an aspect, the peroxide strips are Bartovation strips and the acetoacetic strips are Bayer Ketostix® strips. In an aspect, the BHB activity for SEQ ID NO: 24 or 40 – 43 and their derivatives is about one (1), two (2), three (3), four (4), five (5), six (6), seven (7), eight (8), nine (9), ten (10), twenty (20), thirty (30), forty (40), fifty (50), sixty (60), seventy (70), eighty (80),
- 30 ninety (90), one hundred (100), two hundred (200), three hundred (300), four hundred (400), five hundred (500), six hundred (600), seven hundred (700), eight hundred (800), nine hundred (900), one thousand (1,000), ten thousand (10,000), one hundred thousand (100,000) or more times greater than SEQ ID NO: 8 when measured by LCMS.

[0017] In another embodiment, SEQ ID NO: 24 exhibits increased BHB activity relative to SEQ ID NO: 8. In an embodiment, the BHB activity is measured by peroxide strips, acetoacetic strips, or liquid chromatography–mass spectrometry (LCMS). In an aspect, the peroxide strips are Bartovision strips and the acetoacetic strips are Bayer Ketostix® strips. In an aspect, the BHB activity for SEQ ID NO: 24 is about one (1), two (2), three (3), four (4), five (5), six (6), seven (7), eight (8), nine (9), ten (10), twenty (20), thirty (30), forty (40), fifty (50), sixty (60), seventy (70), eighty (80), ninety (90), one hundred (100), two hundred (200), three hundred (300), four hundred (400), five hundred (500), six hundred (600), seven hundred (700), eight hundred (800), nine hundred (900), one thousand (1,000), ten thousand (10,000), one hundred thousand (100,000) or more times greater than SEQ ID NO: 8 when measured by LCMS.

[0018] In embodiments, enzymes described herein are non-naturally occurring, engineered, isolated, and/or purified.

[0019] The disclosure further provides for sensors capable of detecting and/or measuring BHB concentration, including utilizing an enzyme described herein, for non-wearable sensing systems. An example format of a non-wearable sensing system is a test strip, which detects and or measures BHB concentration. The test strip may be single or multiple use.

[0020] In some embodiments, the test strip includes: a substrate layer; and one or more sensing reagents dispensed upon at least a portion of the substrate layer, wherein at least one of the sensing reagents includes the enzyme.

[0021] In some embodiments, the at least one of the sensing reagents further includes a co-factor, a mediator, and/or another adjuvant or excipient. In some embodiments, the co-factor includes flavin adenine dinucleotide (FAD). In some embodiments, the mediator includes one or more of ferricyanide, phenazine, phenothiazine, thionine, methylene green/blue, tetrathiafulvalene, quinone derivatives, ferrocene, organometallic osmium complexes, and/or organometallic ruthenium complexes.

[0022] In some embodiments, the material of the substrate layer includes one or more of a composite material, a fibrous material, a woven textile, a non-woven textile, a polymer, an adhesive, a film, a gel, PTFE, and /or silicone.

[0023] The sensor, for example, a non-wearable sensor, can be used to detect or measure BHB in any body fluid. The fluid can be obtained through non-invasive,

minimally invasive, or invasive means, such as by finger stick, blood draw, spinal tap, as examples of invasive means, or sweat or saliva collection, as examples of non-invasive or minimally invasive means. Body fluid refers to all body fluid including but not limited to whole blood, serum, plasma, a blood fraction other than serum or
5 plasma, lymph, cerebrospinal fluid (CSF), interstitial fluid (ISF), intracellular fluid, transcellular fluid, saliva, tears, sweat, vaginal discharge, milk, mucus, chyme, pus, bile, semen, urine, amniotic fluid, synovial fluid, peritoneal fluid, pericardial fluid, peritoneum, glandular secretions, exudate, contents of cysts, and/or ascites.

[0024] The disclosure further provides for sensors capable of detecting and/or
10 measuring BHB concentration, including utilizing an enzyme described herein, for continuous, continual, or on-demand detection or measurement. Continuous is meant to be uninterrupted; unbroken; not intermittent or occasional; so persistently repeated at short intervals as to constitute virtually an unbroken series. Continual means repeated regularly and frequently in a steady suggestion. On demand means the
15 detection or measurement can readily be obtained when the sensor or display of sensor data is actuated by the user.

[0025] In some embodiments, the BHB sensor capable of sensing, detecting, and/or measuring BHB concentration includes: a sensor, comprising a sensing electrode; a sensing reagent, wherein the sensing reagent composition includes an
20 enzyme described herein; and a reference electrode. In some embodiments, the sensing reagent is on or adjacent to the sensing electrode. In some embodiments, the sensing reagent is dispensed to be in contact with the sensing electrode at the time of use.

[0026] In some embodiments, wherein the sensing reagent composition further
25 includes a co-factor and/or a mediator.

[0027] In some embodiments, the BHB sensor is configured as a wearable sensor to measure a BHB concentration of a subject and output a data stream in a continuous, continual, or on-demand fashion. In some embodiments, some or all of the sensors are to be located within body tissue (in-the-body sensors) to measure
30 fluid in that tissue. Body tissue can include any tissue, including, but not limited to epidermal, dermal, subcutaneous, muscular, intraperitoneal space. Example embodiments of in-the-body sensors include, but are not limited to, a continuous BHB sensor in a needle-based sensor, a microneedle-based sensor, or an implantable sensor. In other embodiments, the BHB sensor is configured to measure

BHB in body fluid that has been sampled from the body. The sample can be obtained through non-invasive, minimally invasive, or invasive means. Body fluid refers to all body fluid including but not limited to whole blood, serum, plasma, a blood fraction other than serum or plasma, lymph, cerebrospinal fluid (CSF), interstitial fluid (ISF),
5 intracellular fluid, transcellular fluid, saliva, tears, sweat, vaginal discharge, milk, mucus, chyme, pus, bile, semen, urine, amniotic fluid, synovial fluid, peritoneal fluid, pericardial fluid, peritoneum, glandular secretions, exudate, contents of cysts, and/or ascites. The body fluids can either be from direct contact within body tissue internal to the body or from body fluids that are sampled either non-invasively or invasively
10 from the body and sensed "on-body".

[0028] The disclosure further relates to a system for detecting and measuring BHB concentration by utilizing an enzyme described herein.

[0029] In some embodiments, the system for detecting and measuring BHB concentration includes: a BHB sensor, wherein the BHB sensor is configured to
15 continuously, continually, or on-demand measure a BHB concentration of a subject and output a data stream; a sensor can use any method of BHB-measurement, including enzymatic, chemical, physical, electrochemical, spectrophotometric, polarimetric, calorimetric, iontophoretic, radiometric, immunochemical, and the like; and a device connected to the BHB sensor, wherein the device comprises: a
20 processor configured to process the data stream from the BHB sensor; and an interface configured to display and/or communicate measured BHB concentration values.

[0030] The disclosure further relates to a method of detecting and measuring BHB concentration by utilizing an enzyme described herein.

25 [0031] In some embodiments, the method of detecting and measuring BHB concentration includes: obtaining a body fluid from a subject; subjecting the body fluid to a BHB sensor; determining a single point BHB concentration in the body fluid; and displaying and/or communicating the single point BHB concentration on an interface.

30 [0032] In other embodiments, the enzymes, devices, or systems described herein are used in methods of improving health and wellness in a subject in need thereof. In other aspects, the enzymes, devices, or systems described herein are used in methods of weight loss. In other embodiments, enzymes, devices, or systems described herein are used for improving mental and/or metabolic health. In yet other

embodiments, enzymes, devices, or systems described herein are used in methods of controlling intake or monitoring of carbohydrates. In other embodiments, enzymes, devices, or systems described herein are used in methods of monitoring ketone body levels for improvement of cognitive function, to treat various neurological disorders, such as epilepsy, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), traumatic brain injury, Alzheimer's disease, and dementias, to treat mental health, mental illness, psychiatric problems, and psychiatric disorders such as depression, bi-polar disorders, schizophrenia, to improve cardiac metabolism, improve immunotherapeutic, chemotherapeutic and radiotherapeutic response to cancer treatments, modulate inflammatory pathways and immune function, to treat obesity and diabetes, for cholesterol quantification and monitoring, and to monitor and detect alcoholic or diabetic ketoacidosis.

[0033] In other embodiments, enzymes, devices, or systems described herein are used in conjunction with other weight loss or wellness methods or therapies. In other embodiments, enzymes, devices, or systems described herein are used in conjunction with food or supplements which contain a low carbohydrate amount or sugar alcohols in the formulation.

[0034] In some embodiments, the food item or supplement comprises a ketone supplement or additive. In another embodiment, the food item or supplement comprises an exogenous ketone and/or ketogenic supplement. In some embodiments, the exogenous ketone and/or ketogenic supplement comprises ketone bodies and/or precursors of ketone bodies. In some embodiments, the ketone bodies and/or precursors of ketone bodies comprise one or more of acetone, acetoacetic acid, beta-hydroxybutyrate (BHB), beta-ketopentanoate, beta-hydroxypentanoate, 1,3-butanediol, and medium chain triglycerides (MCT) containing fatty acids with hydrocarbon side chains in the length of 6-12 carbons. In some embodiments, the ketone bodies and/or precursors of ketone bodies are in the form of salts and/or esters. In some embodiments, the MCT comprises one or more of the following: caproic acid (C6), caprylic acid (C8), capric acid (C10), and lauric acid (C12).

[0035] In embodiments, devices, systems, or methods described herein are prescribed to a subject in need thereof by a medical professional. In other embodiments, the patient the patient in need thereof is obese or afflicted with an underlying medical condition such as heart disease.

BRIEF DESCRIPTION OF THE FIGURES

[0036] **Figure 1:** Initial screening of sequence similarity networks (SSNs) composed of 4000 bacterial oxidases.

[0037] **Figure 2:** Comparison between Oxidase 8 (SEQ ID NO 8) and an engineered cholesterol oxidase from *Streptomyces hygrospinosus* (ShCOB) using the Alphafold model.

[0038] **Figure 3:** Visual display of the design of the engineered enzyme (8_MUT) predicted by Alphafold based on wild type Oxidase 8 (8_WT).

[0039] **Figure 4:** Detection results of hydrogen peroxide (H₂O₂; Peroxide) produced by the engineered enzyme containing amino acid sequence SEQ ID NO: 24 (Oxidase8_mut) and the wild type Oxidase 8 (Oxidase8_WT, SEQ ID NO: 8). Commercially available Bartovation hydrogen peroxide strips were used.

[0040] **Figure 5:** Detection results of acetoacetate produced by the engineered enzyme containing amino acid sequence SEQ ID NO: 24 (Oxidase8_mut) and the wild type Oxidase 8 (Oxidase8_WT, SEQ ID NO: 8). Commercially available Bayer Ketostix® urine reagent test strips were used.

[0041] **Figure 6:** The HPLC-MS profile of the reaction mixture of the engineered enzyme containing amino acid sequence SEQ ID NO: 24 (Oxidase8_mut).

[0042] **Figure 7:** Comparing the HPLC-MS profile of the Oxidase8_mut (SEQ ID NO: 24) (8_MUT) reaction mixture with the HPLC-MS profile of the commercially available synthetic acetoacetate (Acetoacetate).

[0043] **Figure 8:** Comparing the HPLC-MS profile of the Oxidase8_mut (SEQ ID NO: 24) (8_MUT) reaction mixture with the HPLC-MS profile of the wild type Oxidase 8 (Oxidase8_WT, SEQ ID NO: 8) (8_WT) reaction mixture.

[0044] **Figure 9:** Comparing the HPLC-MS profile of the Oxidase8_mut (SEQ ID NO: 24) (8_MUT) reaction mixture with the HPLC-MS profile of the Substrate Control.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0045] The disclosure provides for novel proteins and enzymes capable of exhibiting BHB activity. In an aspect, the novel proteins or enzymes are capable of being used with methods, systems, devices, and kits described herein.

[0046] The term “amino acid” refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a

manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refer to compounds that have the same basic chemical structure

5 as a naturally occurring amino acid, i.e., a carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid.

10 [0047] There are various methods in the literature that permit the incorporation of an unnatural amino acid derivative or analog into a polypeptide chain in a site-specific manner, see, for example, WO 02/086075, the content of which is incorporated by reference in its entirety.

[0048] Amino acids may be referred to herein by either the commonly known three

15 letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0049] "Polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. All three terms apply to amino acid

20 polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms encompass amino acid chains of any length, including full-length proteins, wherein the amino acid residues are linked by covalent peptide bonds.

25 [0050] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, (e.g., two proteases of the disclosure and polynucleotides that encode them) refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum

30 correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

[0051] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer,

subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

5 [0052] In the context of the present application, a sequence that is “at least 80% identical to a reference sequence” is a sequence having, over its entire length, 80%, or more, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% , 99.5%, 99.6%, 99.7%, 99.8% sequence identity with the entire length of a reference sequence. Proteins consisting of an amino acid sequence “at least 80%, at least
10 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8% identical” to a reference sequence may comprise mutations such as deletions, insertions and/or substitutions compared to the reference sequence. In case of substitutions, the protein consisting of an amino acid sequence at least 80%, at least
15 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8% identical to a reference sequence may correspond to a homologous sequence derived from another species than the reference sequence.

[0053] In the context of the present application, the “percentage of identity” can be
20 calculated using a global pairwise alignment (*i.e.* the two sequences are compared over their entire length). Methods for comparing the identity of two or more sequences are well known in the art. For example, the “needle” program, which uses the Needleman-Wunsch global alignment algorithm (Needleman and Wunsch, 1970 *J. Mol. Biol.* 48:443-453) to find the optimum alignment (including gaps) of two
25 sequences when considering their entire length, may be used. The needle program is for example available on the ebi.ac.uk World Wide Web site and is further described in the following publication (*EMBOSS: The European Molecular Biology Open Software Suite* (2000) Rice, P. Longden, I. and Bleasby, A. *Trends in Genetics* 16, (6) pp. 276—277). The percentage of identity between two polypeptides, in
30 accordance with the invention, is calculated using the EMBOSS: needle (global) program with a “Gap Open” parameter equal to 10.0, a “Gap Extend” parameter equal to 0.5, and a Blosum62 matrix. Other algorithms that are suitable for determining percent sequence identity and sequence similarity include the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *J. Mol. Biol.*

215:403-410, 1990 and Altschuel *et al.*, *Nucleic Acids Res.* 25:3389-3402, 1977, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

[0054] “At least one” herein refers to one or more of the specified objects such as 1,
5 2, 3, 4, 5 or 6 or more of the specified objects. For example, at least one amino acid substitution herein refers to 1, 2, 3, 4, 5 or 6 or more amino acid substitutions.

[0055] “Amino acid substitutions” may be conservative or non-conservative. In an aspect, substitutions are conservative substitutions, in which one amino acid is substituted for another amino acid with similar structural and/or chemical properties.
10 In an aspect the conservative substitution leads to the same or similar functional properties.

[0056] In an embodiment, conservative substitutions may include those, which are described by Dayhoff in “The Atlas of Protein Sequence and Structure. Vol. 5”, Natl. Biomedical Research, the contents of which are incorporated by reference in their
15 entirety. For example, in an aspect, amino acids, which belong to one of the following groups, can be exchanged for one another, thus, constituting a conservative exchange: Group 1: alanine (A), proline (P), glycine (G), asparagine (N), serine (S), threonine (T); Group 2: cysteine (C), serine (S), tyrosine (Y), threonine (T); Group 3: valine (V), isoleucine (I), leucine (L), methionine (M), alanine (A), phenylalanine (F);
20 Group 4: lysine (K), arginine (R), histidine (H); Group 5: phenylalanine (F), tyrosine (Y), tryptophan (W), histidine (H); and Group 6: aspartic acid (D), glutamic acid (E). In an aspect, a conservative amino acid substitution may be selected from the following of T→A, G→A, A→I, T→V, A→M, T→I, A→V, T→G, and/or T→S.

[0057] In a further embodiment, a conservative amino acid substitution may include
25 the substitution of an amino acid by another amino acid of the same class, for example, (1) nonpolar: Ala, Val, Leu, Ile, Pro, Met, Phe, Trp; (2) uncharged polar: Gly, Ser, Thr, Cys, Tyr, Asn, Gln; (3) acidic: Asp, Glu; and (4) basic: Lys, Arg, His. Other conservative amino acid substitutions may also be made as follows: (1) aromatic: Phe, Tyr, His; (2) proton donor: Asn, Gln, Lys, Arg, His, Trp; and (3) proton
30 acceptor: Glu, Asp, Thr, Ser, Tyr, Asn, Gln (see, for example, U.S. Patent No. 10,106,805, the contents of which are incorporated by reference in their entirety).

[0058] In another embodiment, conservative substitutions may be made in accordance with Table 1. Methods for predicting tolerance to protein modification

may be found in, for example, Guo et al., Proc. Natl. Acad. Sci., USA, 101(25):9205-9210 (2004), the contents of which are incorporated by reference in their entirety.

Table 1: Representative Conservative Amino Acid substitutions

Conservative Amino Acid Substitutions	
Amino Acid	Substitutions (others are known in the art)
Ala	Ser, Gly, Cys
Arg	Lys, Gln, His
Asn	Gln, His, Glu, Asp
Asp	Glu, Asn, Gln
Cys	Ser, Met, Thr
Gln	Asn, Lys, Glu, Asp, Arg
Glu	Asp, Asn, Gln
Gly	Pro, Ala, Ser
His	Asn, Gln, Lys
Ile	Leu, Val, Met, Ala
Leu	Ile, Val, Met, Ala
Lys	Arg, Gln, His
Met	Leu, Ile, Val, Ala, Phe
Phe	Met, Leu, Tyr, Trp, His
Ser	Thr, Cys, Ala
Thr	Ser, Val, Ala
Trp	Tyr, Phe
Tyr	Trp, Phe, His
Val	Ile, Leu, Met, Ala, Thr

5

[0059] In another embodiment, conservative substitutions may be those shown in Table 2 under the heading of “conservative substitutions.” If such substitutions result in a change in biological activity, then more substantial changes, denominated
10 “exemplary substitutions” in Table 2, may be introduced and the products screened if needed.

Table 2: Amino Acid substitutions

Amino Acid Substitutions		
Original Residue (naturally occurring amino acid)	Conservative Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Asp, Lys; Arg
Asp (D)	Glu	Glu; Asn
Cys (C)	Ser	Ser; Ala
Gln (Q)	Asn	Asn; Glu
Glu (E)	Asp	Asp; Gln
Gly (G)	Ala	Ala
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; Norleucine
Leu (L)	Ile	Norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Tyr	Leu; Val; Ile; Ala; Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr; Phe
Tyr (Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; Norleucine

Enzymes

[0060] Enzymes or proteins described herein may be engineered, isolated, or
5 purified. In an aspect, enzymes or proteins described herein may be non-naturally
occurring. In other aspects, enzymes or proteins described herein may be modified
via amino acid substitution, deletion, and/or truncation. In other aspects, enzymes
described herein are optimized to detect or analyze BHB. In other aspects, Enzymes
described herein are designed for use with the systems, devices, kits, and methods
10 described herein.

[0061] In aspects, the disclosure relates to enzymes with oxidase activity, wherein
the enzyme is capable of oxidizing beta-hydroxybutyrate (BHB) to produce 3-
oxobutanoate. In other embodiments, the enzyme comprises, consists of, or consists
essentially of an amino acid sequence comprising one or more mutations

corresponding to N137G, Y235Q, and/or A455Y of SEQ ID NO: 8. In some aspects, an enzyme described herein only comprises a combination of N137G, Y235Q, and/or A455Y mutations of SEQ ID NO: 8. In other aspects, an enzyme described herein comprises, consists essentially of, or consists of a combination of N137G, Y235Q, and/or A455Y mutations of SEQ ID NO: 8 coupled with a N-terminal truncation at amino acid positions 2-32 of SEQ ID NO: 8. In other aspects, an enzyme described herein comprises, consists essentially of, or consists of a combination of N137G, Y235Q, and/or A455Y mutations of SEQ ID NO: 8 coupled with a N-terminal truncation of the amino acid positions selected from the group consisting of amino acid position numbers 2-23, 2-24, 2-25, 2-26, 2-27, 2-28, 2-29, 2-30, 2-31, or 2-32 of SEQ ID NO: 8. In other aspects, an enzyme described herein comprises, consists essentially of, or consists of a combination of N137G, Y235Q, and/or A455Y mutations of SEQ ID NO: 8 coupled with a N-terminal truncation of 10 amino acids, 9 amino acids, 8 amino acids, 7 amino acids, 6 amino acids, 5 amino acids, 4 amino acids, 3 amino acids, 2 amino acids, or 1 amino acid of SEQ ID NO: 8.

[0062] In some aspects, SEQ ID NO: 24 exhibits increased BHB activity over SEQ ID NO: 8 in similar testing conditions. In other aspects, SEQ ID NO: 24 or SEQ ID NO: 40 – 43 exhibits increased BHB activity over SEQ ID NO: 8 in or under the same testing conditions. In other aspects, SEQ ID NO: 24 or SEQ ID NO: 40 – 43 exhibits increased BHB activity over SEQ ID NO: 8 in or under similar testing conditions.

[0063] In some embodiments, the amino acid sequence is derived from an oxidase of the EC 1.1.3.6 family. In some embodiments, the oxidase of the EC 1.1.3.6 family is a cholesterol oxidase. In some embodiments, the amino acid sequence is derived from a cholesterol oxidase originated from *Scytonema sp.*, *Rhodococcus erythropolis* (ReCO), *Brevibacterium sterolicum* (BsCO), or *Streptomyces hygrospinosus* (ShCO).

[0064] In some embodiments, amino acid sequences described herein comprise a truncation at the N-terminal and/or at the C-terminal. In some embodiments, the truncation has a length of at least 3 amino acids, at least 5 amino acids, at least 10 amino acids, at least 15 amino acids, at least 20 amino acids, at least 25 amino acids, at least 26 amino acids, at least 27 amino acids, at least 28 amino acids, at least 29 amino acids, at least 30 amino acids, or at least 31 amino acids. In other embodiments, the truncation has a length of at most 3 amino acids, at most 5 amino acids, at most 10 amino acids, at most 15 amino acids, at most 20 amino acids, at most 25 amino acids, at most 26 amino acids, at most 27 amino acids, at most 28

amino acids, at most 29 amino acids, at most 30 amino acids, or at most 31 amino acids. In other embodiments, the truncation has a length of from 5 to 50 amino acids, from 10 to 50 amino acids, from 15 to 50 amino acids, from 20 to at least 50 amino acids, from 10 to 40 amino acids, from 10 to 35 amino acids, from 15 to 35 amino acids, from 20 to 35 amino acids, from 25 to 32 amino acids, from 28 – 32 amino acids, from 2 to 10 amino acids, or from 2 to 5 amino acids from N-terminal and/or at the C-terminal of an enzyme or protein described herein. In another aspect, the truncations described herein are in one or more of SEQ ID NO: 1 – 43. In one aspect, the truncations described herein are relative to SEQ ID NO: 8, SEQ ID NO: 24, or SEQ ID NO: 40 – 43.

[0065] In some embodiments, the enzyme comprises an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.6%, at least 99.7%, or at least 99.8% identical to any of SEQ ID NO: 1 – 43. In some embodiments, the enzyme comprises an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.6%, at least 99.7%, or at least 99.8% identical to any of SEQ ID NO: 24 or SEQ ID NO: 40 – 43. In embodiments, enzymes described herein comprise N137G, Y235Q, and/or A455Y mutations relative to SEQ ID NO: 8, wherein the enzyme further comprises an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.6%, at least 99.7%, or at least 99.8% to SEQ ID NOs: 8, 24, or 40 – 43.

[0066] In additional embodiments, the enzyme comprises an amino acid sequence of any of SEQ ID NO: 1 – 43, wherein the amino acid sequence comprises one, two, three, four, five, six, seven, eight, nine, or ten or more amino acid substitutions. In other embodiments, the enzyme comprises an amino acid sequence of any of SEQ ID NO: 1 – 43, wherein the amino acid sequence comprises at most one, at most two, at most three, at most four, at most five, at most six, at most seven, at most eight, at most nine, or at most ten or more amino acid substitutions.

[0067] In additional embodiments, the enzyme comprises an amino acid sequence of any of SEQ ID NO: 24 or 40 – 43, wherein the amino acid sequence includes one, two, three, four, five, six, seven, eight, nine, or ten or more amino acid substitutions. In embodiments, the amino acid substitution(s) are conservative substitutions. In additional embodiments, the enzyme comprises an amino acid sequence of any of

SEQ ID NO: 24 or 40 – 43, wherein the amino acid sequence comprises at most one, at most two, at most three, at most four, at most five, at most six, at most seven, at most eight, at most nine, or at most ten or more amino acid substitutions.

BHB sensor, and method of detecting and measuring BHB concentration.

5 [0068] In general, a biosensor is a device that measures biological or chemical reactions by generating signals proportional to the concentration of an analyte in the reaction. According to the IUPAC definition, a biosensor is a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds. For an overview of
10 biosensors see, e.g., Bhalla et al., Introduction to biosensors, June 30, 2016, Essays Biochem. 60(1):1-8, which is hereby incorporated by reference in its entirety.

[0069] In other aspects, the disclosure provides for a sensor, such as a nonwearable sensor, capable of detecting and/or measuring BHB concentration including utilizing enzymes described herein. Such sensors, for example, non-
15 wearable sensors, may be in the format of a test strip or a one-touch fingertip sweat sensor. In other aspects, the enzyme comprises one or more of SEQ ID NO: 1 – 43, fragments, or modified enzymes of SEQ ID NO: 1 – 43 as described herein. A detailed discussion of suitable applications of the enzyme in various nonwearable sensors may be found in Yin *et al.* (WO 2022/170361), the content of which is hereby
20 incorporated by reference in its entirety.

[0070] The nonwearable sensor can be used to detect or measure BHB in body fluid, in which the body fluid comprises one or more of whole blood, serum, plasma, a blood fraction other than serum or plasma, lymph, cerebrospinal fluid (CSF), interstitial fluid (ISF), intracellular fluid, transcellular fluid, saliva, tears, sweat, vaginal
25 discharge, milk, mucus, chyme, pus, bile, semen, urine, amniotic fluid, synovial fluid, peritoneal fluid, pericardial fluid, peritoneum, glandular secretions, exudate, contents of cysts, and/or ascites.

[0071] In some embodiments, the test strip includes: a substrate layer; and one or more sensing reagents dispensed upon at least a portion of the substrate layer,
30 wherein at least one of the sensing reagents includes the enzyme.

[0072] In some embodiments, the at least one of the sensing reagents further includes a co-factor, a mediator, and/or another adjuvant or excipient. In some embodiments, the co-factor includes flavin adenine dinucleotide (FAD). In some

embodiments, the mediator includes one or more of ferricyanide, phenazine, phenothiazine, thionine, methylene green/blue, tetrathiafulvalene, quinone derivatives, ferrocene, organometallic osmium complexes, and/or organometallic ruthenium complexes.

5 [0073] In other embodiments, the test strip further comprises one or more tetrazolium salts. In some embodiments, wherein one or more of the tetrazolium salts are selected from the group consisting of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) and 3-(4,5-dimethylthiazolyl-1-2)-2,5-diphenyltetrazolium bromide (MTT).

10 [0074] The material of the substrate layer may comprise one or more of a composite material, a fibrous material, a woven textile, a non-woven textile, a polymer, an adhesive, a film, a gel, PTFE, and /or silicone. The disclosure further provides for a BHB sensor capable of sensing, detecting, and/or measuring BHB concentration by utilizing enzyme described herein in a continuous, continual, or on-
15 demand manner. In other aspects, the enzyme comprises one or more of SEQ ID NO: 1 – 43, fragments, or modified enzymes of SEQ ID NO: 1 – 43 as described herein.

[0075] In some embodiments, the BHB sensor capable of sensing, detecting, and/or measuring BHB concentration comprises: a sensor comprising a sensing
20 reagent, wherein the sensing reagent composition comprises said engineered enzyme; and a reference electrode.

[0076] In some embodiments, the sensing reagent composition further comprises one or more of a co-factor, mediator, adjuvant, or excipient.

[0077] In some embodiments, the wearable BHB sensor is configured to
25 continuously, continually, or on-demand measure a BHB concentration of a subject and output a data stream. In some embodiments, wherein the continuous BHB sensor is an implantable or non-implantable device. In some embodiments, the wearable BHB sensor is a needle-based sensor or a micro-needle-based sensor. In some embodiments, a sensor can use any method of BHB-measurement, including
30 enzymatic, chemical, physical, electrochemical, spectrophotometric, polarimetric, calorimetric, iontophoretic, radiometric, immunochemical, and the like. In some embodiments, the wearable BHB sensor is configured to measure a BHB concentration in a body fluid. In other embodiments, the body fluid comprises one or more of whole blood, serum, plasma, a blood fraction other than serum or plasma,

lymph, cerebrospinal fluid (CSF), interstitial fluid (ISF), intracellular fluid, transcellular fluid, saliva, tears, sweat, vaginal discharge, milk, mucus, chyme, pus, bile, semen, urine, amniotic fluid, synovial fluid, peritoneal fluid, pericardial fluid, peritoneum, glandular secretions, exudate, contents of cysts, and/or ascites.

- 5 [0078] The disclosure further provides for a system for detecting and measuring BHB concentration by utilizing enzymes described herein. In other aspects, the enzyme comprises one or more of SEQ ID NO: 1 – 43, fragments, or modified enzymes of SEQ ID NO: 1 – 43 as described herein.

- [0079] In some embodiments, the system for detecting and measuring BHB
10 concentration comprises: a BHB sensor, wherein the BHB sensor is configured to measure a BHB concentration of a subject and output a data stream; and a device connected to the BHB sensor, wherein the device comprises: a processor configured to process the data stream from the BHB sensor; and an interface configured to display and/or communicate measured BHB concentration values.

- 15 [0080] In some embodiments, a BHB sensor or BHB sensing system is created by integrating said engineered enzyme into a variety of sensing platforms and/or devices. Platforms and/or devices that can adopt said engineered enzyme include, but are not limited to, pH-change sensors such as the ones described in Chodavarapu *et al.*, US 7,794,584, electrochemical sensors such as the ones
20 described in Simpson *et al.*, US 7,081,195, Lebel *et al.*, US 6,915,147, and Jina *et al.*, US 2010/0049021, optical sensors such as the ones described in Petrich *et al.*, US 10,724,943, implantable sensor platforms such as the ones described in Jain, EP 2079358, chip-shaped blood analysis devices such as the ones described in Ogawa *et al.*, US 7,582,259, and monitoring systems where the control terminal is remotely
25 coupled such as the ones described in Karan *et al.*, US 2012/0245447. The content of each of these patents and applications is hereby incorporated by reference in their entireties. In some embodiments according to the present disclosure, the BHB sensors, BHB sensing systems, BHB devices, and applicable techniques are non-invasive or minimally invasive, wearable biosensing and/or chemical monitoring
30 sensors, systems, devices and techniques.

[0081] In some embodiments of the present disclosure, the electrochemical biosensors or chemical sensors used to measure BHB qualitatively and/or quantitatively are fingertip sensors.

[0082] In some embodiments, the BHB sensor or BHB sensing system is a one-touch fingertip sweat sensor and personalized data processing method, system, or device such as those described in WIPO International Patent Application No. PCT/2022/070554, filed on February 07, 2022, and published as Publication No.

5 WO/2022/170361 on August 11, 2022; or in U.S. Patent Application No. 18/264,755, filed on August 08, 2023, and published as Publication No. US 2024/0049994 A1 on February 15, 2024. The content of each of these applications is hereby incorporated by reference in their entireties.

[0083] In some embodiments, the BHB sensor or BHB sensing system utilizes
10 reverse iontophoresis, which is a non-invasive or minimally invasive process of extraction of biomarkers. Reverse iontophoresis is a technique by which a small current flow is applied to the skin with the effect of extracting polar and non-polar molecules to the anode or cathode, where they can be sensed by the electrochemistry. For detailed descriptions of applicable iontophoretic techniques,
15 systems, and devices see, e.g., U.S. Patent No. 10,722,160 issued on July 28, 2020, U.S. Patent Application Publication No. US 2021/0076988 published on March 18, 2021, and WIPO International Patent Application No. PCT/US2015/063836, filed on May 31, 2017, and published as Publication No. WO/2016/090189 on June 9, 2016. The content of each of these patents and applications is hereby incorporated by
20 reference in their entireties.

[0084] In some embodiments, the electrochemical biosensors or chemical sensors are wearable, epidermal electrochemical sensor devices for detecting BHB in sweat. For detailed descriptions of applicable epidermal electrochemical sensors devices see, e.g., U.S. Patent No. 9,820,692 and U.S. Patent No. 11,185,286. The content of
25 each of these patents is hereby incorporated by reference in their entireties.

[0085] The disclosure further provides for methods of detecting and measuring BHB concentration by utilizing an enzyme described herein. In other aspects, the enzyme comprises one or more of SEQ ID NO: 1 – 43, fragments, or modified enzymes of SEQ ID NO: 1 – 43 as described herein.

30 [0086] In some embodiments, the method of detecting and measuring BHB concentration comprises: obtaining a body fluid from a subject; subjecting the body fluid to a BHB sensor; determining a single point BHB concentration in the body fluid; and displaying and/or communicating the single point BHB concentration on an interface.

Test kits.

[0087] The disclosure further relates to test kits suitable for use in, for example, automatic analyzers, systems, and devices.

[0088] In some embodiments, test kits described herein comprises a reagent A and a reagent B. In some embodiments, the test kit is activated by mixing the reagent A with the reagent B.

[0089] In some embodiments, wherein the reagent A comprises an enzyme described herein. In some embodiments, an enzyme described herein is dissolved in a buffer solution. In some embodiments, reagent A comprises NaCl and/or KCl. In some embodiments, wherein the concentration of NaCl and/or KCl is 0.1-5 M. In some embodiments, wherein the reagent A comprises citric acid, acetic acid, KH_2PO_4 , N-cyclohexyl-2-aminoethanesulfonic acid (CHES), 2-(N-morpholino)ethanesulfonic acid (MES), phosphate, and/or tris(hydroxymethyl)aminomethane. In some embodiments, wherein the reagent A has a pH of 3-11, optionally 4-10, optionally 5-9, optionally 6-8.

[0090] In some embodiments, the reagent B comprises a co-factor, a mediator, an excipient, an adjuvant, or a carrier. In an embodiment the composition containing the enzyme is dissolved in water. In some embodiments, the co-factor comprises one or more of flavin adenine dinucleotide (FAD), semiquinone form flavin adenine dinucleotide (FADH), and/or quinone form flavin adenine dinucleotide (FADH_2). In some embodiments, wherein the mediator comprises one or more of ferricyanide, phenazine, phenothiazine, thionine, methylene green/blue, tetrathiafulvalene, quinone derivatives, ferrocene, organometallic osmium complexes, and/or organometallic ruthenium complexes. In some embodiments, the reagent B comprises NaCl and/or KCl. In some embodiments, the concentration of NaCl and/or KCl is 0.1-5 M. In some embodiments, the reagent B comprises citric acid, acetic acid, KH_2PO_4 , N-cyclohexyl-2-aminoethanesulfonic acid (CHES), 2-(N-morpholino)ethanesulfonic acid (MES), phosphate, and/or tris(hydroxymethyl)aminomethane. In other embodiments, the reagent B has a pH of 3-11, optionally 4-10, optionally 5-9, optionally 6-8. In some embodiments, the reagent B comprises one or more tetrazolium salts. In some embodiments, wherein one or more of the tetrazolium salts are selected from the group consisting of 2-(p-iodophenyl)-3-(p-

nitrophenyl)-5-phenyltetrazolium chloride (INT) and 3-(4,5-dimethylthiazolyl-1-2)-2,5-diphenyltetrazolium bromide (MTT).

Pharmaceutical compositions

- [0091] In another embodiment, the invention relates to applications of the enzyme
5 as an ingredient of a composition.
- [0092] In some embodiments, the composition comprises one or more of active ingredients. In some embodiments, the active ingredients further comprise one or more of other enzymes such as glucose oxidase, glucosyl transferase, fructosyl transferase, catalase, amylase, lactase, lipase, and/or protease.
- 10 [0093] In some embodiments, the composition may further comprise one or more excipients, one or more adjuvants, and/or one or more carriers. Excipients, adjuvants, and/or carriers commonly known in the field may be found from, for example, Margolin *et al.* (US 7,718,169), the content of which is incorporated herein by reference.
- 15 [0094] In some embodiments, the one or more excipients comprises one or more of microcrystalline cellulose, Maltrin, Croscopovidone, colloidal silicon dioxide, magnesium stearate, talc, sucrose, trehalose, lactose, sorbitol, lactitol, mannitol, inositol, salts of sodium and potassium, such as acetate, phosphates, citrates and borate, glycine, arginine, polyethylene oxide, polyvinyl alcohol, polyethylene glycol, hexylene glycol,
20 methoxy polyethylene glycol, gelatin, hydroxypropyl- β -cyclodextrin, polylysine, polyarginine, amino acids such as glycine, arginine, aspartic acid, glutamic acid, lysine, asparagine, glutamine, proline, carbohydrates such as glucose, fructose, galactose, mannose, arabinose, xylose, ribose, lactose, trehalose, maltose, sucrose, maltodextrins, dextrans, starch, glycogen, alditols such as mannitol, xylitol, lactitol, sorbitol, cyclodextrins such as methyl cyclodextrin, hydroxypropyl- β -cyclodextrin and
25 alike, inorganic molecules such as sodium chloride, potassium chloride, magnesium chloride, phosphates of sodium and potassium, boric acid, ammonium carbonate and ammonium phosphate, organic molecules such as acetates, citrate, ascorbate, lactate, glucuronic acid, galacturonic acid, emulsifying or solubilizing/stabilizing
30 agents such as acacia, diethanolamine, glyceryl monostearate, lecithin, monoethanolamine, oleic acid, oleyl alcohol, poloxamer, polysorbates, sodium lauryl sulfate, stearic acid, sorbitan monolaurate, sorbitan monostearate, and other sorbitan derivatives, polyoxyl derivatives, wax, polyoxyethylene derivatives, sorbitan

derivatives, viscosity increasing reagents such as agar, alginic acid and its salts, guar gum, pectin, polyvinyl alcohol, polyethylene oxide, cellulose and its derivatives propylene carbonate, polyethylene glycol, hexylene glycol, tyloxapol or salts of such compounds. Additional examples of excipients are described in the Handbook of

- 5 Pharmaceutical Excipients, published jointly by the American Pharmaceutical Association and the Pharmaceutical Society of Great Britain.

[0095] In some embodiments, wherein the one or more adjuvants comprises one or more of water-in-oil and oil-in-water emulsions, aluminum salt adjuvants, liposomes, and/or CpG oligodeoxynucleotide adsorbed to aluminum salt.

- 10 [0096] In some embodiments, wherein the one or more carriers comprises one or more of polymers used for encapsulation of protein crystals for delivery of proteins, including controlled release biological delivery. In some embodiments, wherein the polymers include biocompatible and biodegradable polymers, or mixtures thereof. In some embodiments, wherein a rate of dissolution and, therefore, delivery of enzymes
- 15 will be determined by the particular encapsulation technique, polymer composition, polymer crosslinking, polymer thickness, polymer stability, enzyme crystal geometry and degree, if any, of enzyme crosslinking.

[0097] In some embodiments, wherein the one or more active ingredient(s) may be present in the composition in association with a polymeric carrier.

- 20 [0098] Useful polymeric carriers include, for example, polymers used for encapsulation of protein crystals for delivery of proteins, including controlled release biological delivery. Such polymers include biocompatible and biodegradable polymers, or mixtures thereof. Preferably, the polymeric carrier is a biodegradable polymer. The rate of dissolution and, therefore, delivery of enzymes will be
- 25 determined by the particular encapsulation technique, polymer composition, polymer crosslinking, polymer thickness, polymer stability, enzyme crystal geometry and degree, if any, of enzyme crosslinking. Other useful carriers include water.

Ketogenic diets and methods of treating diseases.

- [0099] The disclosure further provides for ketogenic diets and methods of treating
- 30 diseases via the use of devices, systems, methods, or kits integrated with enzymes described herein. In an embodiment, ketogenic diets are used in tandem with the devices and systems described herein. A representative description of nutritional ketosis and diseases treatable with the same may be found in, for example, at

D'Agostino *et al.* (US 2020/0268701), the content of which is incorporated by reference in its entirety.

Ketosis

[0100] The term “ketone” and/or “ketone body” refers to water-soluble molecules or compounds that contain the ketone groups produced from fatty acids. As generally known in the field, ketone groups contain one or more carbonyl group(s) -C(=O)-. In the context of this application, “ketone” and/or “ketone body” include the following non-exhaustive compounds: acetone (2-propanone, dimethyl ketone, or beta-ketopropane), acetoacetic acid (3-oxobutanoic acid, acetonecarboxylic acid, or diacetic acid), acetoacetate, beta-hydroxybutyric acid (3-hydroxybutyric acid), beta-hydroxybutyrate (BHB, 3HB, or 3-hydroxybutyrate), beta-ketopentanoate (3-oxopentanoate, 3-oxovaleric acid, or 3-ketovaleric acid), and beta-hydroxypentanoate (3-hydroxyvalerate, 3-hydroxy valeric acid, or beta-hydroxyvaleric acid). For a review of ketones see, e.g., L. Laffel, Nov.-Dec. 1999, Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes, *Diabetes Metab Rev.* 15(6):412-426, which is hereby incorporated in its entirety.

[0101] BHB is an anionic small molecule acid metabolite with a hydroxyl group and is the major ketone body that is distributed in the human brain and its primary energy source when glucose is absent. For a review of BHB see, e.g., J.C. Newman and E. Verdin, August 21, 2017, β -Hydroxybutyrate, *Ann Rev Nutr.* 37:51-76.

[0102] The term “acetoacetate” refers to the conjugate base of acetoacetic acid and is used interchangeably with the term “3-oxobutanoate.”

[0103] In an embodiment, enzymes, methods, devices, and systems described herein are related to ketosis and may be used in, for example, nutritional or therapeutic ketosis. Nutritional, or therapeutic, ketosis is the physiological state of elevated blood ketone body levels (typically above 0.5 mmol/L) resulting from ketogenic diets, calorie restriction, therapeutic fasting and/or supplementation with ketogenic precursors. Ketone bodies represent alternative energy substrates for both peripheral tissues and the central nervous system. The two most abundant and physiologically significant ketone bodies are acetoacetate and beta-hydroxybutyrate (BHB), while the third ketone body, acetone, is produced as a byproduct that the lungs breathe off. The body produces ketone bodies during nutritional or therapeutic

ketosis in the range of about 0.3 – 16 mmol/L. The metabolism of ketone bodies is associated with anticonvulsant effects, enhanced brain metabolism, neuroprotective, muscle sparing properties and improvement in cognitive and physical performance. Science-based improvements in efficiency of cellular metabolism, managed through ketone supplementation, could have beneficial impacts on physical, cognitive health, psychological health, warfighter resilience and a long-term impact on health with respect to the common avoidable diseases such as obesity, neurodegenerative diseases, autoimmune diseases, diabetes and cancer.

[0104] Under normal conditions of a standard diet, the brain is exclusively dependent upon the metabolism of glucose to supply its metabolic energy. Though the brain is only 2% of bodyweight, it represents 25% of total glucose consumption. Ketones can replace glucose to supply most of the brain's metabolic energy needs (>50%) during periods of limited glucose availability resulting from starvation/fasting, caloric restriction or carbohydrate restriction as in ketogenic diets. During carbohydrate deprivation, glucose availability decreases causing a metabolic shift towards fatty acid beta-oxidation and the production of ketone bodies for energy homeostasis.

[0105] Dietary carbohydrates (carbs) include simple sugars, such as table sugar (sucrose) and complex carbohydrates (starch) found in foods like potatoes and pasta. Carbohydrate and sugar consumption have dramatically increased in the last two centuries in Western societies. When sugars and carbohydrates are consumed by humans, the pancreas secretes insulin, a hormone used to convert the sugars and carbohydrates into glucose. The glucose is then used by the body as a fuel source. In most Western diets, glucose is the body's primary fuel source.

[0106] In periods of fasting, extreme exercise, and/or low carbohydrate consumption, glucose stores in the body are rapidly used and can become quickly depleted. Failure to replenish glucose stores as they become depleted causes the body to turn to an alternative method to generate energy by creating ketone bodies. Ketone bodies can be used by every cell of the body as a replacement fuel to satisfy the body's energy needs, including the needs of the brain. During a prolonged fast, for example, blood ketone levels will increase to as high as 2 or 3 mmol/L, or even higher. It is conventionally understood and agreed that when blood ketones rise above 0.5 mmol/L, the heart, brain and peripheral tissues are using ketone bodies (beta hydroxybutyrate and acetoacetate) as the primary fuel source. This condition is

referred to as ketosis, or “nutritional ketosis.” This is distinguished from diabetic or alcoholic ketoacidosis, which is the runaway accumulation of ketone bodies and associated drop in blood pH. Diabetic ketoacidosis is associated with the absence of insulin as occurs in those suffering from type 1 diabetes. Ketoacidosis typically results in blood ketone levels more than 15 mmol/L in combination with metabolic derangement and electrolyte imbalance.

[0107] When in ketosis, the body essentially burns fat for fuel. This is accomplished because fat stores in the body are utilized to create the water-soluble ketone bodies beta-hydroxybutyrate (BHB) and acetoacetate (also known as acetylacetonate).

These ketone bodies are then used by the body as its primary energy source.

[0108] The body enters a state of ketosis when it has no dietary source of glucose or sugar and its stores of glycogen have been depleted. This typically occurs during fasting, exercise, and/or pursuing a carbohydrate restricted ketogenic diet. Upon transitioning into ketosis, the body begins cleaving fats into fatty acids and glycerol and transforms the fatty acids into acetyl CoA molecules which are then eventually transformed into ketone bodies in the liver. In other words, during ketogenic metabolism in the liver, the body uses dietary and bodily fats as its primary energy source. Consequently, once in ketosis, one can easily induce loss of body fat by reducing dietary fat intake and adjusting carbohydrate intake low enough to sustain ketosis.

[0109] In an embodiment, enzymes, methods, devices, and systems described herein are related to and may aid in methods of weight loss, regulation and/or monitoring of weight loss and regulation and/or monitoring of carbohydrate intake. In other embodiments, enzymes, methods, devices, and systems described herein are related to regulation and/or monitoring of ketosis over hours, days, weeks, months, or years. In some embodiments, the ketone concentration is monitored for the purpose of detecting and preventing ketoacidosis.

Ketogenic diets and weight loss

[0110] The disclosure provides for methods of treating weight loss in subjects in need thereof by utilizing the enzymes, methods, devices, or systems described herein.

[0111] A ketogenic diet is one that is high in dietary fat and low in carbohydrates with moderate levels of protein (approximately 1-2 g/kg). The classical ketogenic diet

consists of a strict regimen of 4 parts fat to 1 part protein with less than 25 - 50 grams of carbohydrates per day. It has been suggested that the ideal macronutrient ratio to maintain a ketogenic diet is 65-85 percent of calories from fats, 10-20 percent of calories from proteins, and 5 percent of calories from carbohydrates. In

5 embodiments, the disclosure provides for methods of assisting subjects to monitor and/or calibrate carbohydrate intake of about 10 to about 30 grams, about 15 to about 40 grams, about 20 to about 50 grams, about 30 to about 60 grams, or about 40 to about 80 grams of carbohydrates per day by utilizing enzymes, systems, and devices described herein. This diet protocol may be used in conjunction with the
10 devices and systems described herein.

[0112] An advantage of pursuing weight loss through a ketogenic diet is that a ketogenic diet may result in loss of fat stores while maintaining and protecting muscle mass. Some studies have suggested that the muscle sparing properties of a ketogenic diet result in improvement in physical performance. Athletes who maintain
15 nutritional ketosis maintain lower insulin levels and can better utilize fatty acids and ketones for fuel, effectively sparing blood glucose, which optimizes and prolongs physical and mental performance. This state is referred to as being "keto adapted." Keto adaptation occurs when the body adjusts to ketosis by building up the necessary fat-burning enzymes, hormone levels are changed to accommodate
20 ketosis, glycogen stored in muscles and liver is reduced, and the body is carrying less water.

[0113] Individuals on the standard American diet can expect to get peak fat oxidation while exercising from between 60 to 65 percent of their maximum oxygen consumption (VO_2 max); higher exertion levels will then deplete glycogen stores.
25 Keto-adapted individuals draw proportionally more substrate from fats and ketones (sparing glycogen) and can shift the peak to much higher VO_2 levels and thus sustain effort for an extended duration. Transitioning to a keto-adapted state (blood ketones >0.5 mmol/L) typically requires 1 to 2 weeks with severe restriction of carbohydrates (<25 g/day) and moderate protein restriction (1 g/kg/day) with the
30 balance of macronutrient from fat. A sustained physiological decrease in glucose and insulin are required for sustained hepatic ketogenesis, which is very difficult for most humans.

[0114] Vlahakos (U.S. 6,613,356), the content of which is incorporated by reference in its entirety, provides a weight-loss composition using n-butyrate ions from

potassium butyrate or related compounds. Butyric acid stimulates receptors in the stomach that the stomach is full, and food is stagnant in the stomach. Thus, consuming butyric acid precursors prior to eating reduces food consumption. Testing showed the compositions improved a patient's ability to withstand rigorous exercise, improved hypercholesterolemia and hypertriglyceridemia, and reduced fatigue.

[0115] Another advantage to pursuing weight loss through a ketogenic diet is that being in ketosis reduces hunger. Indeed, hunger is the major barrier that is often cited for the inability to maintain a traditional calorie restricted diet.

[0116] Because the presence of blood ketones can be easily measured by using one of many ketone test strips or devices available on the commercial market, those desirous of pursuing a state of ketosis can easily measure their progress. Just as those on a traditional diet can weigh themselves and gain positive feedback by measuring weight loss, those pursuing a state of ketosis can also be encouraged by measuring their blood ketone levels. However, when transitioning into ketosis, it may take from several days to two weeks or longer for any measurable increase in blood ketone levels to manifest through a urine test.

Methods of implementing ketogenic diets and monitoring ketosis by a BHB sensor

[0117] In some embodiments, a ketogenic diet comprises about 5 – 10% carbohydrates; about 55 – 85% fats; and about 10 – 40% proteins. In some embodiments, the ketogenic diet comprises: 15-50 g carbohydrates; 60-400 g fats; and 40-100 g proteins. In some embodiments, the ketogenic diet provides 1500 – 3000 kcal per day. In some embodiments, the ketogenic diet includes intermittent or prolonged fasting. In aspects, these parameters may be modulated and/or monitored via methods, systems, and devices described herein.

[0118] In some embodiments, the ketogenic diet is consumed each day for a period of at least 2 days, at least 3 days, at least 4 days, at least 5 days, at least 6 days, at least 7 days, at least 8 days, at least 9 days, at least 10 days, at least 11 days, at least 12 days, at least 13 days, at least 14 days, or at least 3 weeks, at least 4 weeks, at least 5 weeks, at least 6 weeks, at least 7 weeks, at least 8 weeks, at least 9 weeks, at least 10 weeks, at least 11 weeks, or at least 12 weeks.

[0119] In some embodiments, ketosis monitoring is performed after 1 day, after 2 days, after 3 days, after 4 days, after 5 days, after 6 days, after 7 days, after 8 days,

after 9 days, after 10 days, after 11 days, after 12 days, after 13 days, or after 14 days of following the ketogenic diet.

[0120] In some embodiments, ketosis monitoring is performed by using a beta-hydroxybutyrate (BHB) sensor to detect a BHB concentration. In some
5 embodiments, the BHB concentration is a blood BHB concentration or an interstitial fluid (ISF) BHB concentration. In some embodiments, the ketosis monitoring is performed at least once every day, at least twice every day, at least 3 times a day, at least 4 times a day, at least 5 times a day, at least once every 2 days, at least once every 3 days, at least once every 4 days, at least once every 5 days, at least once
10 every 6 days, or at least once every 7 days, or at least once every 2 weeks, at least once every 3 weeks, or at least once every 4 weeks. In some embodiments, the ketosis monitoring is performed at 7:00 am, at 8:00 am, at 9:00 am, at 10:00 am, at 11:00 am, at 12:00 pm, at 1:00 pm, at 2:00 pm, at 3:00 pm, at 4:00 pm, at 5:00 pm, at 6:00 pm, at 7:00 pm, at 8:00 pm, at 9:00 pm, and/or at 10:00 pm. In some
15 embodiments, the ketosis monitoring is performed 3 hours before breakfast, 2 hours before breakfast, 1 hour before breakfast, right before breakfast, right after breakfast, 1 hour after breakfast, 2 hours after breakfast, 3 hours after breakfast, 3 hours before lunch, 2 hours before lunch, 1 hour before lunch, right before lunch, right after lunch, 1 hour after lunch, 2 hours after lunch, 3 hours after lunch, 3 hours before dinner, 2
20 hours before dinner, 1 hour before dinner, right before dinner, right after dinner, 1 hour after dinner, 2 hours after dinner, or 3 hours after dinner.

Effects of ketosis on cognitive and physical performance

[0121] Performance studies have shown improved motor function, endurance, and cognitive function with ketone supplementation. Resilience of cardiopulmonary and
25 neurological function under extreme environments of oxidative stress (hyperoxia) has been achieved in rats given ketone supplementation. Many people on a ketogenic diet report greater mental clarity, an enhanced ability to multi-task, and a more favourable and balanced mood.

[0122] Other advantages to the ketogenic diet including anti-aging and mood
30 stabilizing effects. Other studies have demonstrated superior performance with respect to endurance time, volume of oxygen consumed, heart rate, blood lactate levels and power output when blood ketone levels are elevated.

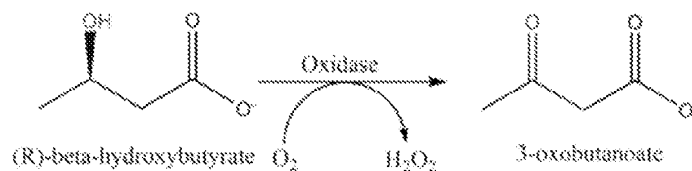
[0123] To this end, and in an embodiment, enzymes, methods, devices, and systems described herein are related to and may aid in methods of increasing endurance, cognitive function, and mood improvement.

EXAMPLES

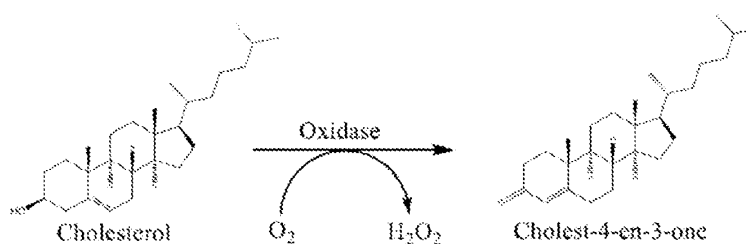
- 5 [0124] The following examples illustrate particular aspects of the disclosure and are not intended in any way to limit the disclosure.

Example 1: Enzyme Design

- [0125] (R)-beta-hydroxybutyrate (R-BHB) can be converted into 3-oxobutanoate via
10 the oxidation of a secondary alcohol, as illustrated below.



- [0126] There are naturally existing alcohol oxidases that can facilitate the oxidation of secondary alcohols to ketones. For example, cholesterol oxidase, which belongs to the oxidoreductase family EC 1.1.3.6, has the ability to convert cholesterol into
15 cholest-4-en-3-one. The reaction of converting cholesterol into cholest-4-en-3-one is illustrated below. This ability makes cholesterol oxidase a potential candidate for developing optimized engineered enzymes able to convert (R)-beta-hydroxybutyrate into 3-oxobutanoate.



- 20 [0127] Any suitable assay for determining the presence and/or amount of hydrogen peroxide (H_2O_2) produced by the enzymatic reactions can be utilized according to the present disclosure. For example, the resultant hydrogen peroxide can be detected using sensitive and stable fluorescent probes. See, e.g., Allain et al., Clin Chem

1974, 20:470-475; Amundson et al., J Biochem Biophys Meth 1999, 38:43-52; and dos Santos Ferreira et al., Clin Chim Acta 2015, 446:263-266. The content of each of these applications is hereby incorporated by reference in their entireties.

[0128] To identify a suitable candidate oxidase for further engineering, a screening of sequence similarity networks (SSNs) composed of 4000 bacterial oxidases was performed. The SSNs method is described in Atkinson *et al.*, *PLoS ONE* 2009 Feb., 4(2), e4345, and is incorporated herein by reference. The initial screening result is shown in Fig. 1. 39 amino acid sequences were identified as potential candidates. These amino acid sequences are shown in Table 3.

[0129] The cholesterol oxidase of Uniprot ID: A0A1Z4IKQ1 (Oxidase 8), derived from the species *Scytonema sp.*, was identified by using the AlphaFold protein structure database for structural prediction. The AlphaFold prediction method is described in detail in Varadi *et al.*, *Nucleic Acids Res.*, Vol. 50, Issue D1, 7 January 2022, pages D439–D444, and in Jumper *et al.*, *Nature*, Vol. 596, pages 583–589 (2021), each of which is incorporated herein by reference. The AlphaFold models were then visualized using PyMol (<http://citebay.com/how-to-cite/pymol/>). A comparison between Oxidase 8 and an engineered cholesterol oxidase from *Streptomyces hygrospinosus* (ShCOB, SEQ ID NO: 21, see Heath *et al.*, *Chembiochem*. 2022 Apr 5;23(7):e202200075) using the AlphaFold model is shown in Fig. 2.

[0130] *In silico* design of an engineered enzyme was then analyzed. Mutant N137G was introduced to create space in the enzyme's active site for BHB, and mutants Y235Q and A455Y were introduced to produce hydrogen bonding to the carboxylic acid of wildtype BHB (reference sequence = SEQ ID NO: 8). A 31 amino acid N-terminus truncation (amino acid positions 2-32 of SEQ ID NO: 8 [oxidase 8 wt]) was introduced. A visual display of the design of the engineered enzyme as predicted by AlphaFold is shown in Fig. 3. The amino acid sequences of the engineered enzymes are also provided in Table 3. As provided in Table 3, SEQ ID NO: 24 (aka 8_MUT or Oxidase8_mut) comprises all three of these amino acid substitutions as well as the truncation of amino acid positions 2-32 when compared to SEQ ID NO: 8 (aka herein as 8_WT). Accounting for the truncated 31 amino acids, the three mutations correspond to N106G, Y204Q, and/or A424Y in the resultant SEQ ID 24. Alternatively, if the 'M' at amino acid position number 1 (i.e., methionine or Met) is

ignored and accounting for the truncated 31 amino acids, the three mutations correspond to N105G, Y203Q, and/or A423Y in the resultant SEQ ID 24.

Table 3: Amino acid sequences of wild type and mutant oxidases

SEQ ID	Amino Acid Sequence	Representative Modifications
SEQ ID NO: 1	MSHQLNVHANRRRAVLRGVALGLGAIGLGTLGVP GTAAAAPLRGKTAVVVGSGFGGAVAALRLGQAG ISTTVLERGRRWPIDPAGTTTFSTINQPDGRSAWF SDRPNINQLTRMIPIQRYPGIVDRVHGNIDALYG AGVGGGSLAFGAFTQPRRRDFEQVFPSAIDYS ELDRVYYPRAKSMLGTSPLPDDLLAHPFYKGAR AWLDDIRDFGGEAVRHDFCVDWDVVRDELSGLK PASYSIGEGPYGSNSGAKNSVDHNYLPAAEATG NVTVSAMHEVTEIREIPGQEKFEIAVKVIDESGAA VSHKTLVADYVFMAAGSFYTSALLATAKAKGTLT RLNEHVGKGFGNNGDFLMARTALRRDYGSAQG GPGVAVMYDDENPDGPISISWEASPFPAIAGGIT SANLIQVMSDERGSIDYNPATGRAELNYPHTEAS SDVDARAKRFAGRFHERADVRHGPSVSGIPVHT RLADFGSGCTWHGLGGMVMGRACDHGKVDG YSNLFVVDGALLPGSSALVNPALTITAIAERCLDR FVANQT	
SEQ ID NO: 2	MEPVVQGDDEPAESVDAVVVGSGFSGSVASYR LAAGGQSVVLMERGRAYPPGSFARTPSEMGRN FWDPKGLYGLFDAWTFRGLEGIVSSGLGGGSL IYANVLLRKDKNWFVQESPVPGGGYESWPFSRQ DLEPYDAAEAMLRPVYPYRDTPKTLAMEAAA RALGLSITRPPIAVTFAASPGAAPARNQVLPPAPY GSVHGEGETVRTTCTLSGECDIGCNTGAKNTLDH NYLSAAKAAGADIRTHHDVRGIRPLPGGGYEVRY VHDPADTAPGLLREQLIHCRRILAAAGTFGTTL LLNRNRLPALSPALGTRFSGNGDLLGFVLDKD PDGVTRTLSGSVGPVITTAIRTPDSTDGGRGY YIEDAGYPSFMNWLETGQFRQVAKRAGKVMVQ LLKDRLFQAQRSNISADLAAALGDGRLSASSVPL LGMGRDIPDGVMTLQGGRLLAINWTLATSAAYFG RMQATMQAIAATELGGSFKQNPLWWSKRVITVHP LGGAPAGRNSSEGVCDSEYGEVFGYPGLHVVDG AVMPGPVGANPALTIAFAERACDRILNGTGADR QRGGDLTPGFPPGNTGGVTDDGITGYQPGPLR ALVPDATKGAKAPAEEGTFVPRAASRSLGLPSAV SPEVGISPEMMLPESEALQEAIEEETELAETGP SSVSFTEQMHGWFSAGVTDPEEGRSLGRDRSR RMMFELTITAADIDEFVRDPLHPATAEGYVLADQ FGGRLPVERGWFNLFVKDGPPEPAGRKMLYRLW LRDPGGTPLTFVGHKDVRNDAGFDVWGDTTTTLY VTVLRGHVPSPDPKLPVSTAPQAAAPHGPAVVA GAGMLFIRPWDFARQLTTFRVAGPNPASALAAF GKVFLGELWQVYGRRIHGRSAGRQ	
SEQ ID NO: 3	MEKQPKACLSSPITDMQPEYEVVIGSGYGGGSA ASRLARAGKKVCLLERGKEFQPGDFPEDQAQAA	

SEQ ID	Amino Acid Sequence	Representative Modifications
	KEMQFNLPQQHLGSKTALYEFHNMKDINVFVGC GLGGTSLVNANVCIEPDKRVFNDEVWPREIRNDL DSVARGVQRAREMLRPECYPEGKNGYPVLPKTE AMKVAASALHEKFSFVPINVSFHTHINHVGVSQH KCDLCGDCVTGCNYGAKNTTLMNYLPDAINHRA QIFTEVAVQYLEKVNNKWL VYYCLQNAEREKFDA PFLFVKADMVVLGAGSLGSTEILLKSKERGLALSP MLGKRFTGNGDVLGFGFNNDCVINGIGYGKNKP GGKIKAVGPCIGGVIDLRGKEKLEDGFVIEDGSIP GALAGVLPGAFISFARLLGKDTDKNLKDFLVEKF RKIKAVIMGAYSGALNNTLTYLVMSHDDANGTMR VEGDRIRVDWPEVGKQPIFKTVNNKLQEATKALG GTYLTNPSWSKALNFDLVTVHPLGGCVMGESAE KGVVNHAGQVYAGEQGTELHPGLYVLDGAIIPRS VGVNPLLTISALAERCCELIAQDYKLTIPYHFAVS PQEQMIKPVGIQFTEKMTGYFSTREKQDYTHAFE LGQQTDSPCEFTLTIIISDNLQRMLLSDRHEAKMV GTVTVPALSKNPLVVSEGIFNLFEEDQLAGGVLK MNYNMKLHAVDGQKFYFNGYKEVYDNP GFDLW TDTSTLFITIYEGSDKKGPVAGKGILKIQPKDFAAQ ITTMKALNTANPLETAQALKDFGLFFTKSLYQQYA	
SEQ ID NO: 4	MSTRFDTD AIVVSGSGFGGAVAAARLAQAGFSVT VLERGRRWRPGGFPRRPHMEDGWLWEVDQGL YDIRWLDRMASVQAAGWGGGSLAYANVFARPF EGALDERWPDRLRRAELDPYALAAHMMMEVAPV GDDPRTGRPAPRTELVERLTRDMDMPDATVRP NLAVTFGDPDTWRPNAHGVRRRGCAFVGECVIG CNHGAKNTLDHTYLAVAEAAGARAVTDAEVRRIE PRDGGYAVTAATPSDPDAPLREWTAPRVVLAAG AVASNELLRSRDVHGTLPLSLRLLGQGFGSGNG DFLT LAELRGARQDMTTGPTITTSTVLDVPEGRR PWVYQVQDGAFFPPLHTLVDTLTPVPRVRGWW RRKVRRTTPRSVFTVLAMGTDSGNGTLRLDRSG KATLSWRNRWQAHLYRSQLRVGPLIARFLDARL YSPFTWSLLRRTVTVHALGGVRAGADAATGVVD EVGEVHGYPGLFVMDGSVLPATGVNPSATILAV AERSIETVIRRSGRHGWRAPWDVAVPGDVPED AAFAYMSERHAATKGDGVVFRERMATRRRDRP GVVLTAAEIRSMDRFLAGAAHTVSMRGVIDAEG VASRAAIAGTSLFPGGGPEAMSYALRFTGDDG RAWRLTGVKTVRSRTPMALLGLTTLRGELSPD DGGAGTRFVLSIGARDLARLGTSIRGTGFTRARR LRAAARFTAFFARSALRR	
SEQ ID NO: 5	MINAAIAAIPTHAQVSQAFPDIDSNLFLGKYTERA KRMLRISYRDMDWFEQTPWFQYARVGRQYAAN AGYRV DYNGSAYSFDYMKKEEAGEVPRSAFD FE QQFGNNYGRSGSVDQTYIAAALATGKATLKPLTE VIRIRRERSGRYVISIREIGRYGEHVQQSEISCNEL HLNAGVLGTTELLLRARDTGTLPNLN EEEIGRGYG NNGDIMVAHALAPTD PAGTQQSLMGLINLDGRD DPDKPVYASMFSIPLPVETFALGYVVMVRTGDRA EAVYNPDSDSIAINWPRGHTDHLHDKAQVWFDK VTQANGVEYRDDLFQGN AANTVHPLGGVVR	

SEQ ID	Amino Acid Sequence	Representative Modifications
	GKATDAYGRVKGYDRLYVNDASLLPGYLGCNPFMSITALAERNIEGILLGRT	
SEQ ID NO: 6	MRRRALFKAAGVAALFAGAGSLLGGRPAAADPVWNGLFREWWPEIFAPLPDPPEHSPALVVGSGFGAAVTALRLAEAGVATTVLERSRWPNDPWREIFTGDDLPDGRGFWHRTSFTGVTKVPMHFASFGGVLDCTEYPGIDVWRAAAVGGGSVIFTGAMIAPQRKLFDEVFRGSVDYDELDRVYYPRVRQMLRLSTMADIYHSAPFTHSRAWDEQVRKAGYEPQPNDSIFTWDILRGELAGTTRPSATVARSNLGNSNGAKFDLNQNYLKYAQDTGKAAVFPGHRVDAIAQESGGRYAVTVTKLAPTGDVLATRTLTCDRFLGAGSIGTSELLVRAQATGALPNLNEHVGDGWTNGDWWLARGASSLTGLGQGVPASARIYDETDAPVTLESWYIPGIPVETGALASLGMVLDPTRTRFGYNAADGRVGLSWDRVNRDQVWAAARTVDQRIAERAGSLVGYGPIGYDANALFTAHLPLGGAVLGKATDGYGRVHGHPGLYVMDGAAIPGSTATVNPSTITALAERNIERIRDGR	
SEQ ID NO: 7	MSNRRATTPAGPDHVDREERHGVIIIGSGVGGSVTAFRLAEAGVDNVLERGRWPITPAENTFPRWSPDPRLWVGRRPPVPLLSRVPWLGSVTDAQAQKLLPRSTGLLDVLPNRNLTVCGAGVGGGTLVYGGMLPQPRAEVFRRAPFAELDYEEFDRTYYPRARSRLGATEFPDDLARSEYRSSEIWRDALVDAGLSPERVLSTFDVDRVRAELNGTARPSAVIGQYFLTGCDSGSKLSVDRTYLERAEATGRTEVRPLHEVTGISQLADGRYHLWDHLDTGAVLEKVAITCDRLVLAAGGVHTARMLVTAKATGGLPRLNEFVGAQWGTNGDQMYTVKVKAMPPEGPHGGPPAFLTRSRDGTAMVTHGGGGGLGKSWILVPGMGLPDRFGRWTWCSVTGRPQLEWARGSDESAHRATKDLVHQMARHVDGGATVHDPLAAHPVVLHPLGGAVLTKATDAYGRLHGYDGLYCLDSALMPGSTAAVNPVLTIAAVVERCLDHVVDDFR	
SEQ ID NO: 8	MSPVLKRRQFLQGSLAAAASVGVSASVRASAVRSKENYVEAIVIGSGFGGAVASLRLGQAGIETIVLERGRRWQITDAGDTFSTYQQPDGRSTWLSPTTVFNKVPIDIYTGVLVDVKIGDGIIAYRGAGVGGGSLVYNAVTYQPTRELFYQVFPRTINYEELDSVYYPRVRSILNPSPIPDDILQTNYYLSSRIFLEQAAKAGLKARKLDIAVDWDIVRQEIAGQKVPSAITGQVYYGINSGAKNSLDRNYLSMAEATGKVEIRPLHVVTSSIECDKERFRITCHQINEQGEVLAQKSFVCRYLFLAAGSIGTTELLLRKANGTLRRLNNQVGKFWGTNGDVVSAVITNGPTNPTQGGPAATVIEHFDNPIAPVVSEQLPFPIVPEGIISSIGQAIKPEGYLTYNASTQSADLFWPSDSANNQKNAQALQYTYQLLNQANGTTLAQPLDFSATAHPLGGATIGQVCNTYGQVYGYRNLFVVDGSFIPGSTACTNPSFTIAALAERSMERFLNRSA	Oxidase 8, derived from <i>Scytonema sp.</i>
SEQ ID NO: 9	MANNYDVIVIGSGFGGAVTACRLAEKGMKVLVLE RGRRWANAYPRKPPEDFIFDPEQPHKRNGWID	

SEQ ID	Amino Acid Sequence	Representative Modifications
	FRIFDDMAVAQAGVGGGSLIYANISVEAKEELF AQGWPAEITYQELKPYDDKVGLMLSVNPIPNDQL TGRFKVMKEGAEALGYEARFRKLPMAVRFDEN WSYDLDDPFNDKHSRTFINEHGVEQGTCTVHCGN CDIGCQVKAKNTLDLNYIPRAEKHGAEVRLHLV RNISPENGGYRVQFDRIENGRLVPGSETARKVIV AAGSLGSTELLRSRDQYKTLPAISKFLGYNWSS NGDFLTPAFYDDRTISPTQGPTITCAIDFLDGDVD NKQFFIEDGGFPNILRNVLAAGLQSGTKNQVYKV LLQDLGNLLKKEDPLGNVMPWFSQGIDAADGRL YLKRSWLPPWRRRLRLDWDIERSEAVINAIVDMH KRLSKATGGKPWWPSSWTILKNLVTPLPLGGCN MGTTSNGVNVNHRGEVFGYPNLYVADGAIPEAI GLNPSRTIAALAERIAELMND	
SEQ ID NO: 10	MGDNTLPKRGSNGISRRGFLGRTGSIIGAVALAG HLAPPAQAAAAAAAAAPGPIGDGARVPVLVIGTG YGGSVAAALRLAQAGVDVQMIEMGMNWDTPGPD GKVFPKVTSPDYRSFWLRTRTKAPLSNFLGFPID KDVPKYTGILDAEDFAGITVYQGRGVGGGSLVN GMAVTPKRANFAAVLPSVNADEMYDTYYPRAN AGLGVGMIDPAWFDTADCYQFSRVGRKHAQRS GFAWTFVPDYYDWDHMKREAAGTATKSALAGEI LYGNNAAGKSLVQTYLAQARATGRVTISPLHRVS TVSPAAGGGYTVQIDQLNTTGDTVATKTVTADRV FFAAGSVGTSKLLVKKATGALPNLNGEVGKGW GENGNVMCGRANHMWDPTGKLQSTIPCSGIDN WDAGGAFAEVAPLPTGIETYASFYLSITKNPNRA EFTWNAATGRADLSWQTAWKQPSITMAKTIFDKI NSKEGTIYRTDLFGGNKIWNNTLYHPLGGAVLD RATDNYGRLHGYTGLYVIDGALIPGNASVNPFTI TALAERNIEKIIATDLS	
SEQ ID NO: 11	MSKIDSTGGDVSRRGFLTASGALLGTAALGLHSP AARAAGPISSGAHVAALVIGTG YGGSVAAALRLAQ AGVDVHLIEMGMAWDTPGTDGKIFCNTREPDYR SYWLRTRTKAPLNYFLGFPIDRDIPRHTGILDAEE FGGITVYQGRGVGGGSLVNGGMAVTPKRENFA AVLPSVNPDEMYATYYPRANAALGVGTVDPAWF DTTACYQYARVGRKHAQRSGFPFVFPDYYDW DYMKEAAAGTVPQSALAGEILYGNNHGKRSLQK TYLARATATGRVTISALHRVTSVTPAGGAYTVTV EQLDTTGGTVATKTVTADRVFFAAGSVGTSKLLV KLKATGALPALNGEIGKGWGDNGNVMCGRANH LWDPTGSLQSAIPCCGIDNWTAGGAFAEVAPLPT GIETFASFYLSITDNPHRAQFTWNAGAGRVELNW QTAWKQPSIAMAKSIFDKINAKEGTIYRTDLFGVY KIWDHLYHPLGGAVLNKATDNYGRLTAYPGL YVIDGALIPGNTTVNPFVTITALAERNIERIIAADL	
SEQ ID NO: 12	MKYDFDYAVIGSGFGGSVSALRLAEKGYSCVF EKGKRFQKNDFPDNTNWLWNWLMPRINFTGI QQLTLLHNVFILSGTGVGGGSLVYCAVLFEPDPT FYYDPQWAGLNGDWKKKLAPFFKEAKRMLGVT ENPKLWKSDELLKDYAKEIGREKYFRPTQVGIYF GEPGVTVSDPFFNGKGPARTGCDFGGQCMVGC	

SEQ ID	Amino Acid Sequence	Representative Modifications
	KNGGKNTLDRNYLYLAENLGVKIVPETEVVRVKE DGNGGYELTTRNSIGLIRGNIQTIHVRGVVFAAGA LGTNKLLECKRSGALSKISDQLGKVGRTNSEVM VGARARSGDSEYCEGIAIASSLFVDDVTHIEPVRF ARGADAMFWLSTLATDGGTRLRPLKHIWNCLT HPIDYLRSRNPVGWAKRAILLVMQTLDNQLHFH WKRRWWFPFTRSLTSKGTVKSTPTYIAAANDAA KGIAKRINGIPQSAVTEVLFNIPLSAHILGGAVIGK SPQHGVVDKYGKVFGYENMYITDGSIVPANLGV NPSLTITALAEYVMSNVPEKENNV	
SEQ ID NO: 13	MSEISRRSLLAGAAATAGLAAVGAARTPARAAK ARVPVTRSQERVVIIGSGFGGGVTALRFAQAGVP CLVLERGIWWRTGPNAEFFPHAASPDKRMFWL GSTPQIFGISADPFEHYTGLLEQVAGNGMDMIVA AGVGGGSLVYQGMTLQPTRDAFNANLPEQLDYT RMDQVYYPRVAQMLQIETAPDELINSPTYQPARV FAKDVRAGHPLSKIPMPIDWDYALRELKGEMK PSYTNAGDCALGVNNGGKHSVDVTYIAAAQATGL VTVATQHNVTDVAMTSDGRWEIHVDRIGHTDGT LEKKIITAKALVMAAGTAGTTRLLMRAAAKGQIPD MPDGLGTGWGSNGDRIYVWTDLEDDFGDPQGG PVVYGSLEWDDPNTANTVIQASLPPTGGDIRSTM IVGYGVSKGRGRFVYDSGKDDAVLQWPKGADD TIYKRINERITKIAGSLSVLTDTTGAYPSTWHPLG GAPMGTVCDLSGRVQGHRLYVLDGALIPGTTA ACNPSMTIAAIAERATDDIVAHDAAGTVF	
SEQ ID NO: 14	MNRTKTSLAAILSEGRLYFSKSKLPNSEAPPIKKKI ELSRSVIIGSGYGAAVAALRLCEAGKKVLILEMGI NWKESGIPYSGLLRPGRSAAWLKRKTIAPFMNVF PLKPFTGALDKFNFKNIDVWVGRGVGGGSIVNG GMAVIPKESYFKEVFPDLDAEFYDFYFPLASKE LKVNGIDEQFVKDCPYKFRVGESEAHKAGFKT VRVPNVYDFKYMEREYKNEVPRSALNTEVIYGN NHGKNSLDKTYLKKALATGNLEILDLCVDVIKSN DDNSYSLEVRQINTSGKTVADKIVHCNKLILGAGT MGTCLKLLGSQSNTFPLDHTVGKNWGNNGNFM TGRNWWVRPLSGGTGALQSTIPVGAVDNWEDSE HSFFTEIAPLPMGVDVATALYLLINRVDKKGSVTY DKKQSRILDWNESHTLKMKANANYFVKMKNHA NGGTRSHLLFKNGFGANICYHPLGGCVLGESTN LFGKLKEHDNLYVMDGSLIPGSIGVNPFMITIAIA EYCIENLIEQGFTQVS	
SEQ ID NO: 15	MQHVTRRSFLTGAAAAAGLALTGTAGAHATPGIP ARVNNVPLTHEEHRVVIGTGFGGGVTALRLAQ AGVRVHMLERGIRWPTGPNASTFPRVDNPDKRF LWHRSNPEVFGRHAFEPYTGLVEACVGDNMTA LCAAGVGGGSLVYQGMTLQPDEAVFNAHFPNEL DWARMDRVHYPRVAQMLRLAVAPDELVDSPNY ATSRIFADRARKAGFPIDKIPMPIDWDYALAEALRG EMAPAYTDGSGAMGVNNGGKHSVDVTYIAEAEA TGLVTVATQHEVTDIERAEDGRWTVHVNHIDTSG AVVENKILTTDALVLSAGTMNTTRLLMKAAAKDLI SDLPDGVGQGWGSNADRIYLWTNLEEEFGAVQ	

SEQ ID	Amino Acid Sequence	Representative Modifications
	GGPVVYGSKNWDDPNSAYTVIQASIPGFYGIDM RSTVLVGYGVSEGRGHFVYDAARDDAVLRWPH EGDSVLQTGHDPTVRRRIGERSILDTNSIVPST WHPLGGASMGTVCDLEGRVFGQRGLYVLDGAL MPGNTAACNPSTIAAVVERALDDIVVRDVGTVF	
SEQ ID NO: 16	MNASPDPAASGVGRRQVLGATALGGLGLALGP GRAQATTVPTRVPVTEQRRERAVVSGSGGGGV TALRLGQAGVRTLVLERGVRWPTGPNADTFCRF ENIDNRSAWLTRRSNLPGVIKFWEPYTGIIAIDG NGMTINCAAAVGGGSLNYHGMSLQPNRANFAAS IPLLADHYDELDRTHYRTVARMLGLSTIPDDILAT DPYKSSRVFLDVAPKAGLETFMVPLPIDWSFVRG ELEGRYKPTYLTSDIAFGVNNGGKNSIDVTYLA AESTGNVQVAPLHIVRDVSLGAGGRWWLHVDRI DTGGKVLEKKRITADAVFLAAGSPGTTRLMVKAR AKRLIPDLPAVGTWGNNGDRIYEWGLDADL GTRQGGPACVGGFDPNSPIPYTHAGQPIQIGGI TMMNVVVGFGINKPAGTWSYDVLRDDAKLTWPSS GDADLQKLIKARVQEFTKVRGGVMIDTNALGAST WHSLGGVPMGDAVDLHGRVLGHRGLYVMDGA RIPGSTGACNPSTIAALAEHSMATIVREDIGRVF	
SEQ ID NO: 17	MADSTSGGLSRRRFLAGTGSAGLGMGLPGRA TAAPGTAAPGAAGPIGDGARVPALIIGTYGGSV AALRLAQAGVDVHVMEMGMAWDTGPDGKLF NTRTPDHRSYWLRTKQIPISNLFGLDKDVPR YTGILDAEDFGGILVYQGRGVGGGSLVNGGMAV TPKRSRFGAVLPSVDADEMYGVYPRANAGLV NEIDRAWFDTDCYQYARVGRKHAQRSGFEFVY VPNVYDFDYMKREAAGTVPQSALAEILYGN GKKSLSQKTYLAQAKATGRVAISPLHKVTSVSPAS NGGYTVVIDQLDTTGAVTATKTVADRFFAAGS VGTSKLLVQMKATGRLPNLNGEIGKGWGDNGNV MCGRANHLWDPTGKLQSAMPTGGIDNWDAGGA FAEVAPIPTGIETYASFYLSITNTPHRAEFTWNA AGRVDLSWQTAWKQASIDRAKTIFDKINQKEGTI YRTDLFGASKIWGDHLYHPLGGAVLNKATDNY GRLRGHPGLYIDGSLIPGNTSVNPFVTITALAER NIEEIVATDL	
SEQ ID NO: 18	MSQHPSWFTEAHQASGSSIGYRVEKLLHAEKTP LQTIEIYQTTDWGNLMVIDGCVMLTSRDNFLYHE MMTHPALFTHARAKRVVIGGGDCGTLREVLKHE EVEHAVQVEIDERVTRLAEQYFPELCDANNDPRA ELLFIDGIKYMAEAEPESLDLIIVDSTDPVGP AEGLFNAAFYASCYKALRHGGILVQQSESPIAHLE LIKSMRSAMRTAGFSAVKTLFPQPCYPTGWW SCTMVRKGGDLAGFRERGALSKNFPTRYNADIH KAALAQPEFMREALGE	
SEQ ID NO: 19	MIVSAGTLGTNELLRLCRDFHKTLPASRTLGRNF SGNGDFLSFVVDGDKPADPNYGPVITQGIDYNLF KNFNREHAFFLEDASFPSYLAWYIEGLRPGFSHF NAFFRTAVMLFRRFLKGITGQIGYAFNELKGD L SYTSGVLLCMGVDKSSGQLVLDKNHYLDIKW PYLDNMPLYQAILNMGERFRKWAGGTAFMPMPNWI	

SEQ ID	Amino Acid Sequence	Representative Modifications
	WPLRKNVTVHALGGCRLADDDPCMGVTSAGDS FGQVFGYQGLYVADGALLPTSVGANPVATISALS ERVAAGITGIEPDAGL	
SEQ ID NO: 20	MPVNRRRILGLAGRGTLAALGA AVLGGVGSRTY AAAVSRTHFRALVIGSGFGGSVAALRLGQAGVD TLVVERGREWPVAPRRHDFGSMNGVTDTMFWF RRTTRWPALPPTPVRPAPGVMEVSEERGLDIAC GAGVGGGSIVYTGTVAPPRRYFERLYPAGLPY EEFTRRWFPVRRMLGASPMPRDVYRSMPFTH SRLWDEHLATAGFRTSPLDSTFDWDVVRRELAR AAPLSATVGESDFGCGNGAKKSLTRSYPALAT GHVQVRALHEVRSIGRRRDGRFRVEVRRLLTTGG AVLGTDEFTCDMLFVAAGTLNTNRLVVAARERG DLAGLPASLGTGFGDNGDQATLRSQTLRFHGGG QGSPCASGAFLPDQFELPLAESWLPAYHALP AIVTFSVTVDTDNRGTFRYDPRSGRVSLSDWSP EKSAAAARSALSSRVIDANPGTLPVTLTTHPFT GHPLGGCVIGRTTDLEGRVHGVRLYVLDGSLIP GNAGGANPSLTIAALAERAMAGIIAQAG	
SEQ ID NO: 21	MFENQQNQHLRRRLGLAALSGAAVTGLTTISA APRAAADKRSRADSGSFVPAVVIGTGYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRRSSWFKSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVIYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNP NIMTGRANHWNPNGAHQSSIPALGIDWDNPD APVFAAIAPMIAGLETWVSLYLAIKPNQGRGSFVY DKATDRAMLRWTRQNA PAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCYHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	ShCob
SEQ ID NO: 22	MAPSRTLADGDRVPALVIGSGYGGAVAALRLTQ AGIPTQIVEMGRSWDTPGSDGKIFCGMLNPKR SMWLADKTDQPVSNFMGFGINKSIDRYVGVLD ERFSGIKVYQGRGVGGGSLVNGGMAVTPKRNY FEEILPSVDSNEMYNKYFPRANTGLGVNNIDQAW FESTEWYKFARTGRKTAQRSGFTTAFVPNVYDF EYMKKEAAGQVTKSGLGGEVIYGNAGKKSLDK TYLAQAAATGKLTITTLHRVTKVAPATGSGYSVT MEQIDEQGNVATKVVTADRVFFAAGSVGTSKLL VSMKAQGHLPNLSSQVGEWGNGNIMVGRAN HMWDATGSKQATIPTMGIDNWADPTAPIFAEIAP LPAGLETYVSLYLAIKPNPERARFQFNSGTGKVD LTWAQSQNQKGIDMAKKVFDKINQKEGTIYRTDL FGVYYKTWGDDFTYHPLGGVLLNKATDNFGRLP EYPGLYVVDGSLVPGNVGVNPFVTITALAERNMD KIISSDIQ	Cholesterol oxidase 1COY derived from <i>Brevibacterium sterolicum</i> , https://www.rcsb.org/structure/1COY

SEQ ID	Amino Acid Sequence	Representative Modifications
SEQ ID NO: 23	MAPSRTLADGDRVPALVIGSGYGGAVAALRLTQ AGIPTQIVEMGRSWDTPGSDGKIFCGMLNPDKR SMWLADKTDQPVSNFMGFGINKSIDRYVGVLD ERFSGIKVYQGRGVGGGSLVNGGMAVTPKRNY FEEILPSVDSNEMYNKYFPRANTGLGVNNIDQAW FESTEWYKFARTGRKTAQRSGFTTAFVNPVYDF EYMKKEAAGQVTKSGLGGEVNYGNNAGKKS LDKTYLAQAAATGKLTITTLHRVTKVAPATGSGYSVT MEQIDEQGNVATKVVTADRVFFAAGSVGTSKLL VSMKAQGHLPLNLSSQVGEWGNGNIMVGRAN HMWDATGSKQATIPTMGIDNWADPTAPIFAEIA LPAGLETYVSLYLAIKTNPERARFQFNSGTGKVD LTWAQSQNQKGIDMAKKVFDKINQKEGTIYRTDL FGVYYKTWGDDFTYHPLGGVLLNKATDNFGRLP EYPGLYVWDGSLVPGNVGVNPFVTITALAERNMD KIISSDIQ	I219N substituted 1COY
SEQ ID NO: 24	MSKENYVEAIVIGSGFGGAVASLRLGQAGIETIVL ERGRRWQITDAGDTFSTYQQPDGRSTWLSPTTV VFNKVPIDIYTGVLVDVKIGDGIIAYRGAGVGGGSL VYGAVTYQPTRELFYQVFPRNTINYEELDSVYYPR VRSILNPSPIDDLQTNYYLSSRIFLEQAAKAGLK ARKLDIAVDWDIVRQEIAGQKVPSAITGQVQYGIN SGAKNSLDRNYLSMAEATGKVEIRPLHVVTISIEE CDKERFRITCHQINEQGEVLAQKSFVCRYLFLAA GSIGTTELLLRKANGTLRRLNNQVGKFWGTNG DVVSAVITNGPTNPTQGGPAATVIEHFDNPIAPVV SEQLPFPVPEGISSIGQAIKPEGYLTYNASTQS ADLFWPSDSANNQKNAQALQYTYQLLNQANGTT LAQPLDFSATYHPLGGATIGQVCNTYGGVYGYR NLFVVDGSFIPGSTACTNPSFTIAALAERSMERFL NRSA	Mutant oxidase 8 (8_MUT) with substitutions at N137G, Y235Q, A455Y, and an N- terminal truncation at amino acid positions 2-32, all amino acid positions of which are based on SEQ NO: 8 (8_WT) as the wildtype, template sequence
SEQ ID NO: 25	MSPVLKRRQFLQGS�AAASVGVS AVRASAVRS KENYVEAIVIGSGFGGAVASLRLGQAGIETIVLER GRRWQITDAGDTFSTYQQPDGRSTWLSPTTVVF NKVPIDIYTGVLVDVKIGDGIIAYRGAGVGGGSLVY GAVTYQPTRELFYQVFPRNTINYEELDSVYYPRVR SILNPSPIDDLQTNYYLSSRIFLEQAAKAGLKAR KLDIAVDWDIVRQEIAGQKVPSAITGQVQYGINSG AKNSLDRNYLSMAEATGKVEIRPLHVVTISIEECD KERFRITCHQINEQGEVLAQKSFVCRYLFLAAGSI GTTELLLRKANGTLRRLNNQVGKFWGTNGDVV SAVITNGPTNPTQGGPAATVIEHFDNPIAPVVSEQ LPFPVPEGISSIGQAIKPEGYLTYNASTQSADL FWPSDSANNQKNAQALQYTYQLLNQANGTTLAQ PLDFSATYHPLGGATIGQVCNTYGGVYGYRNLF VVDGSFIPGSTACTNPSFTIAALAERSMERFLNR SA	Oxidase 8 with substitutions at N137G, Y235Q, A455Y
SEQ ID NO: 26	MFENQQNQHLRRRLGLAALSGAAVTGLTTISA APRAAAADKRSRADSGSFVPAVVIGTGYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRRSSWFKSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL	I261N substituted ShCOB

SEQ ID	Amino Acid Sequence	Representative Modifications
	KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVNYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAAIAPMIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWTRQNAFAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCYHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	
SEQ ID NO: 27	MFENQQNQHLRRLGLAALSGAAVTGLTTISA APRAAAADKRSRADSGSFVPAVVIGTGYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRSSWFKSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVQYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAAIAPMIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWTRQNAFAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCYHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	I261Q substituted ShCOB
SEQ ID NO: 28	MFENQQNQHLRRLGLAALSGAAVTGLTTISA APRAAAADKRSRADSGSFVPAVVIGTGYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRSSWFKSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVRYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAAIAPMIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWTRQNAFAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCYHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	I261R substituted ShCOB
SEQ ID NO: 29	MFENQQNQHLRRLGLAALSGAAVTGLTTISA APRAAAADKRSRADSGSFVPAVVIGTGYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRSSWFKSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVKYGN	I261K substituted ShCOB

SEQ ID	Amino Acid Sequence	Representative Modifications
	NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAAIAPMIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWWTREQNAPAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCYHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	
SEQ ID NO: 30	MFENQQNQHLRRRLGLAALSGAAVTGLTTISA APRAAADKRSPRADSGSFVPAVVIGTGYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRRSSWFKSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVAYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAAIAPMIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWWTREQNAPAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCYHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	I261A substituted ShCOB
SEQ ID NO: 31	MFENQQNQHLRRRLGLAALSGAAVTGLTTISA APRAAADKRSPRADSGSFVPAVVIGTGYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRRSSWFKSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVIYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAEIAPIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWWTREQNAPAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCYHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	I404E substituted ShCOB
SEQ ID NO: 32	MFENQQNQHLRRRLGLAALSGAAVTGLTTISA APRAAADKRSPRADSGSFVPAVVIGTGYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRRSSWFKSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVIYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA	1489F substituted ShCOB

SEQ ID	Amino Acid Sequence	Representative Modifications
	GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAAIAPMIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWWTREQNAPAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCFHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	
SEQ ID NO: 33	MFENQQNQHLRRRLGLAALSGAAVTGLTTISA APRAAAADKRSPRADSGSFVPAVVIGTYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRRSSFWSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVIYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAAIAPMIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWWTREQNAPAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCAHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	I489A substituted ShCOB
SEQ ID NO: 34	MFENQQNQHLRRRLGLAALSGAAVTGLTTISA APRAAAADKRSPRADSGSFVPAVVIGTYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRRSSFWSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVIYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAAIAPMIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWWTREQNAPAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCQHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	I489Q substituted ShCOB
SEQ ID NO: 35	MFENQQNQHLRRRLGLAALSGAAVTGLTTISA APRAAAADKRSPRADSGSFVPAVVIGTYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRRSSFWSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVIYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD	I489N substituted ShCOB

SEQ ID	Amino Acid Sequence	Representative Modifications
	APVFAAIAPMIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWWTREQNAPAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCNHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	
SEQ ID NO: 36	MFENQQNQHLSRRRLGLAALSGAAVTGLTTISA APRAAAADKRSPRADSGSFVPAVVIGTGYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRRSSWFKSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVQYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAEIAPMIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWWTREQNAPAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCYHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	I261Q, A404E substituted ShCOB
SEQ ID NO: 37	MFENQQNQHLSRRRLGLAALSGAAVTGLTTISA APRAAAADKRSPRADSGSFVPAVVIGTGYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRRSSWFKSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVNYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAEIAPMIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWWTREQNAPAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCYHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	I261N, A404E substituted ShCOB
SEQ ID NO: 38	MFENQQNQHLSRRRLGLAALSGAAVTGLTTISA APRAAAADKRSPRADSGSFVPAVVIGTGYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRRSSWFKSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVRYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAEIAPMIAGLETWWSLYLAITKNPQRGSFVY DKATDRAMLRWWTREQNAPAVAAAKSLFDRINKA	I261R, A404E, Y489F substituted ShCOB

SEQ ID	Amino Acid Sequence	Representative Modifications
	NTTMYRYDLFGPQLKNFADDFCFHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	
SEQ ID NO: 39	MFENQQNQHLRRRLGLAALSGAAVTGLTTISA APRAAADKRSRADSGSFVPAVVIGTGYGAAV SALRLGEAGVETLMLEMGQLWNKPAEDGNVFC GMLTPDRRSSWFKSRTEAPLGSFLWLDVINRDIE PYAGVLDRVHFDQMSVYVGRGVGGGSLVNGGM AVVPKRAYFEEVLPQVDAAQMYERYFPRANAAL KVNHIDPAWFEKTEWYNFARVSREQAGKAGLST TFVPNVYDFDHMQREAAGTAPKSALAGEVKYGN NHGKQSLDKTYLAAALGTGKVTIETLHRVTAIRQ QADGSYVLSVDQSDANGTVIAHKEIACRHLFLGA GSLGSTELLVRARDTGALPHLNAEVGEGWGPNG NIMTGRANHWWNPTGAHQSSIPALGIDDWDNPD APVFAEIAPMIAGLETWVSLYLAIKPNQRGSFVY DKATDRAMLRWTREQNAPAVAAAKSLFDRINKA NTTMYRYDLFGPQLKNFADDFCYHPLGGCVLGK ATDDYGRVAGYHNLYVTDGALIPGSIGVNPFTIT ALAERNIERVIAEDVRTAA	I261K, A404E substituted ShCOB

Example 2: Enzyme Expression and Purification

[0131] The method of expressing and purifying the engineered enzyme is described in Yoshikuni *et al.*, U.S. Patent Publication No. 2022/0348970 A1, the contents of which are hereby incorporated herein by reference in their entireties.

[0132] Overnight cultures of BLR cells suspended in a 2 mL volume were transformed with a pet29b+ plasmid (encoding polypeptides of interest with a C-terminal His-tag) and grown in Terrific Broth with 50 µg/ml kanamycin. Cultures were diluted 1:1,000 in 500 ml of Terrific Broth with 1 mM MgSO₄, 1% glucose and 50 µg/ml antibiotic and then grown at 37°C. for 24 hours. Cultures were pelleted down at 5,000 G for 10 minutes and resuspended in auto-induction media (TB broth, 1 mM MgSO₄, 1×NPS and 1×5052) for induction at 18°C for 24 hours. At the end of induction, cells were centrifuged, the supernatant was removed and cells were resuspended in 40 mL lysis buffer (1x PBS, pH 7.5, 5 mM Imidazole) and 1 mM phenylmethylsulphonyl fluoride. The cell lysate suspension was sonicated for 2 min and followed by centrifugation at 5,000 G. The supernatant was loaded onto a gravity flow column with 500 µL Cobalt beads and was washed with 15 mL of lysis buffer five times. Proteins were eluted with 1 mL of elution buffer (1x PBS, pH 7.5, 200 mM Imidazole). Protein concentrations were determined using a Synergy H1 spectrophotometer (Biotek) by measuring absorbance at 280 nm using calculated

extinction coefficients. Enzymes containing a concentration of at least 0.1 mg/mL were then assayed.

Example 3: Enzyme Activity

5 [0133] The activity of the enzymes was evaluated by measuring the production of hydrogen peroxide and acetoacetic acid. Commercial test strips were used for detecting the presence of hydrogen peroxide and acetoacetic acid.

[0134] To set up the substrate, 100 mM of BHB was dissolved in 400 mM sodium phosphate at pH 7 and 100 mM NaCl. In a 96 well-plate, 100 μ L of 0.5 mg/mL
10 enzyme, and 100 μ L of dissolved substrate were combined. The reaction was allowed to react for 24hrs at 21°C. After the incubation period, 10 μ L of each reaction was placed in an individual test strip for colorimetric analysis using the hydrogen peroxide and acetoacetic acid detection strips.

15 Detection of hydrogen peroxide:

[0135] Bartovation hydrogen peroxide strips (<https://bartovation.com>) were used to detect the presence of hydrogen peroxide. These strips were calibrated at the 0, 1, 3, 10, 50, and 100 ppm. The test strip turns blue if hydrogen peroxide is presented, and bluer color indicates higher concentration of hydrogen peroxide.

20 [0136] As shown in Fig. 4, the engineered enzyme containing amino acid sequence SEQ ID NO: 24 (Oxidase8_mut) generated hydrogen peroxide with the presence of 2-pentanol, (R)-2-pentanol, BHB, or (R)-beta-hydroxybutyrate (R-BHB), as the test strip turned blue when contacting with the reaction mixture of these reactants. In contrast, the wild type Oxidase 8 (Oxidase8_WT, SEQ ID NO: 8) exhibited a lesser
25 activity in oxidizing BHB, as the brighter blue color indicated; and did not oxidize R-BHB, as the test strip did not turn blue. While the mutated enzyme of SEQ ID NO: 24 yielded a positive test result, none of SEQ ID NO: 1 – 23 and 25 – 39 yielded a positive result.

30 Detection of acetoacetic acid:

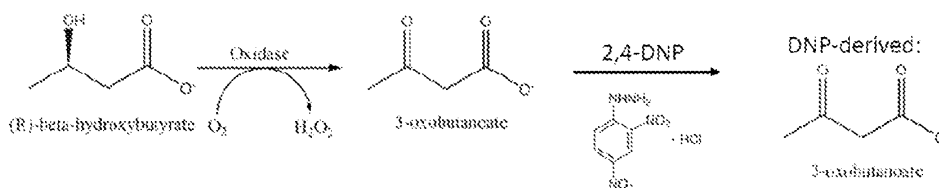
[0137] Bayer Ketostix® urine reagent test strips were used to detect the presence of acetoacetic acid in the urine. These strips are calibrated at the 0, 5, 15, 40, 80, 100 mg/dL. The test strip turns red if acetoacetic acid is presented.

[0138] As shown in Fig. 5, the engineered enzyme containing amino acid sequence SEQ ID NO: 24 (Oxidase8_mut) generated acetoacetic acid when R-BHB was used as the reactant, as the test strip turned red. In contrast, the wild type Oxidase 8 (Oxidase8_WT, SEQ ID NO: 8) did not produce acetoacetic acid when R-BHB was used as the reactant, as the test strip did not turn red.

HPLC-MS analysis of acetoacetate:

[0139] The method of detecting acetoacetate by using high-performance liquid chromatography-mass spectrometry (HPLC-MS) is described in detail by U *et al.*, *Nat. Prod. Chem. Res.* 2019 7(2):364, the content of which is incorporated herein by reference.

[0140] 2,4-Dinitrophenylhydrazine (2,4-DNP) is commonly used to detect ketones and aldehydes as it readily reacts with the C=O carbonyl group, forming a hydrazone compound. The reaction of reacting R-BHB with 2,4-DNP is shown below.



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[0141] The reaction shown above was utilized in the HPLC-MS analysis. A derivation mix (100 mM 2,4-DNP in methanol) was prepared. After the 24 hr incubation at 21°C, the samples were prepared for HPLC-MS analysis using 200 μ L reaction mix, 100 μ L 4M Acetate, 700 μ L derivation mix. This was allowed to react for 1 hour at 60 °C. The reaction was then centrifuged for 10 minutes at 5000 G. Supernatant was collected to be used in HPLC-MS analysis.

[0142] The column, Agilent Eclipse Plus C18 RRHD 1.8 μ m, was used for separation. The analysis was run at 0.3 mL/min flow rate with Mobile Phase A (H₂O, 0.1% formate) and Mobile Phase B (100% ACN, 0.1% formate). Using a gradient process, the analysis was started at 80% MPA/ 20% MPB to 0%MPA/100% MPB over the course of 10 minutes. To detect the Acetoacetic acid, Single Ion Mode was used in the MS to look for the derivatized product. Fig. 6 shows the HPLC-MS profile of the reaction mixture of the engineered enzyme containing amino acid sequence SEQ ID NO: 24 (Oxidase8_mut); Fig. 7 compares the HPLC-MS profile of the Oxidase8_mut reaction mixture with the HPLC-MS profile of the commercially

30

available synthetic acetoacetate; Fig. 8 compares the HPLC-MS profile of the Oxidase8_mut reaction mixture with the HPLC-MS profile of the wild type Oxidase 8 (Oxidase8_WT, SEQ ID NO: 8) reaction mixture; Fig. 9 compares the HPLC-MS profile of the Oxidase8_mut reaction mixture with the HPLC-MS profile of the substrate control. Figs. 6-9 together show that the engineered enzyme Oxidase8_mut SEQ ID NO: 24 did produce acetoacetic acid.

Results of hydrogen peroxide testing, detection of acetoacetate, and HPLC-MS analysis:

[0143] BHB activity was analyzed based on the hydrogen peroxide testing protocol (Bartovation hydrogen peroxide strips), the acetoacetate detection protocol (Bayer Ketostix®), and HPLC-MS protocols as described herein. The results are provided in Table 4. Oxidase 8 can sometimes produce peroxide when incubated with BHB, but at trace, very minor amounts as indicated in Fig. 4. This most likely explains the positive peroxide strip activity as noted in Table 4 for the wildtype SEQ ID NO: 8.

Table 4: Review of representative activities

SEQ ID:	Peroxide strip activity	Acetoacetate activity	HPLC-MS activity
SEQ ID NO: 8	Yes	No	No
SEQ ID NO: 24	Yes	Yes	Yes

Example 4: Further Enzyme Design

[0144] Additional enzymes suitable for use in the current invention and associated methods include those set forth in Tables 5 and 6.

Table 5: Amino acid sequences of representative enzymes according to the disclosure

SEQ ID	Amino Acid Sequence	Representative Modifications
SEQ ID NO: 40	MSKENYVEAIVIGSGFGGAVASLRLGQAGIETIVLERGRRWQITDAGDTFSTYQQPDGRSTWLSPTTVVFENKVPIDIYTGVLVDVKIGDGIAYRGAGVGGGSLVYGAVTYQPTRELFYQVFPRTINYEELDSVYYPRVRSILNPSPIPDDILQTNYLSSRIFLEQAAKAGLKARKLDIAVDWDIVRQEIAGQKVPSAITGQVYYGINSGAKNSLDRNYLSMAEATGKVEIRPLHVVTSEECDKERFRITCHQINEQGEVLAQKSFVCRYLFLAAGSIGTTELLLRKANGTLRRLNNQVGKFWGTNGDVVSAVITNGPTNPTQGGPAATVIEHFDNPIAPVVSEQLPFPVPEGISSIGQAIKPEGYLTYNASTQSADLFWPSDSANNQKNAQALQYTYQLLNQANGTT	Oxidase 8 with N137G substitution and N-terminal truncation at amino acid positions 2-32, all amino acid positions of which are based on SEQ NO: 8 (8_WT) as the wildtype,

	LAQPLDFSATAHPLGGATIGQVCNTYGQVYGYR NLFVVDGSFIPGSTACTNPSFTIAALAERSMERFL NRSA	template sequence
SEQ ID NO: 41	MSKENYVEAIVIGSGFGGAVASLRLGQAGIETIVL ERGRRWQITDAGDTFSTYQQPDGRSTWLSPTTV VFNKVPIDIYTGVLVDVKIGDGIAYRGAGVGGGSL VYNAVTYQPTRELFYQVFPRTINYEELDSVYYPR VRSILNPSPIPDDILQTNYYLSSRIFLEQAAKAGLK ARKLDIAVDWDIVRQEIAGQKVPSAITGQVQYGIN SGAKNSLDRNYLSMAEATGKVEIRPLHVVT SIEE CDKERFRITCHQINEQGEVLAQKSFVCRYLFLAA GSIGTTELLLRKANGTLRRLNNQVGKFWGTNG DVVSAVITNGPTNPTQGGPAATVIEHFDNPIAPVV SEQLPFPIVPEGISSIGQAIKPEGYLTYNASTQS ADLFWPSDSANNQKNAQALQYTYQLLNQANGTT LAQPLDFSATAHPLGGATIGQVCNTYGQVYGYR NLFVVDGSFIPGSTACTNPSFTIAALAERSMERFL NRSA	Oxidase 8 with Y235Q substitution and N-terminal truncation at amino acid positions 2-32, all amino acid positions of which are based on SEQ NO: 8 (8_WT) as the wildtype, template sequence
SEQ ID NO: 42	MSKENYVEAIVIGSGFGGAVASLRLGQAGIETIVL ERGRRWQITDAGDTFSTYQQPDGRSTWLSPTTV VFNKVPIDIYTGVLVDVKIGDGIAYRGAGVGGGSL VYNAVTYQPTRELFYQVFPRTINYEELDSVYYPR VRSILNPSPIPDDILQTNYYLSSRIFLEQAAKAGLK ARKLDIAVDWDIVRQEIAGQKVPSAITGQVYGIN SGAKNSLDRNYLSMAEATGKVEIRPLHVVT SIEE CDKERFRITCHQINEQGEVLAQKSFVCRYLFLAA GSIGTTELLLRKANGTLRRLNNQVGKFWGTNG DVVSAVITNGPTNPTQGGPAATVIEHFDNPIAPVV SEQLPFPIVPEGISSIGQAIKPEGYLTYNASTQS ADLFWPSDSANNQKNAQALQYTYQLLNQANGTT LAQPLDFSATYHPLGGATIGQVCNTYGQVYGYR NLFVVDGSFIPGSTACTNPSFTIAALAERSMERFL NRSA	Oxidase 8 with A455Y substitution and N-terminal truncation at amino acid positions 2-32, all amino acid positions of which are based on SEQ NO: 8 (8_WT) as the wildtype, template sequence

Table 6: Amino acid sequences of representative enzymes according to the disclosure

SEQ ID	Amino Acid Sequence	Representative Modifications
SEQ ID NO: 43	MSKENYVEAIVIGSGFGGAVASLRLGQAGIETIVL ERGRRWQITDAGDTFSTYQQPDGRSTWLSPTTV VFNKVPIDIYTGVLVDVKIGDGIAYRGAGVGGGSL VYX ¹ AVTYQPTRELFYQVFPRTINYEELDSVYYPR VRSILNPSPIPDDILQTNYYLSSRIFLEQAAKAGLK ARKLDIAVDWDIVRQEIAGQKVPSAITGQVX ² YGINSGAKNSLDRNYLSMAEATGKVEIRPLHVVT SIEECDKERFRITCHQINEQGEVLAQKSFVCRYLF LAAGSIGTTELLLRKANGTLRRLNNQVGKFWGT NGDVVSAVITNGPTNPTQGGPAATVIEHFDNPIA PVVSEQLPFPIVPEGISSIGQAIKPEGYLTYNAS TQSADLFWPSDSANNQKNAQALQYTYQLLNQAN GTTLAQPLDFSATX ³ HPLGGATIGQVCNTYGQVY GYRNLFVVDGSFIPGSTACTNPSFTIAALAERSM ERFLNRSA	Oxidase 8 wherein X ¹ , X ² , X ³ may be substituted, for example, by conservative substitutions and N-terminal truncation at amino acid positions 2-32

[0145] In an aspect, the disclosure provides combinations wherein X¹ may be N, G, P, A, or S; X² may be Y, Q, N, K, E, or D; and X³ may be A, Y, W, F, or H.

Further Numbered Embodiments of the Disclosure

5 [0146] Other subject matter contemplated by the present disclosure is set out in the following numbered embodiments:

1. An enzyme with oxidase activity, wherein said enzyme is engineered to exhibit improved beta-hydroxybutyrate (BHB) activity.
2. The enzyme of embodiment 1, wherein said enzyme is capable of
10 oxidizing beta-hydroxybutyrate (BHB) to produce 3-oxobutanoate.
3. The enzyme of any one of embodiments 1 or 2, wherein the amino acid sequence is derived from an oxidase of the EC 1.1.3.6 family.
4. The enzyme of any one of embodiments 1 – 3, wherein the oxidase of
the EC 1.1.3.6 family is a cholesterol oxidase.
- 15 5. The enzyme of any one of embodiments 1 – 4, wherein the enzyme is non-naturally occurring and includes one or more amino acid substitutions, deletions, or truncations relative to a native or wild-type enzyme.
6. The enzyme of any one of embodiments 1 – 5, wherein the amino acid sequence of the enzyme comprises one or more amino acid substitutions
20 corresponding to N137G, Y235Q, and/or A455Y of SEQ ID NO: 8.
7. The enzyme of any one of embodiments 1 – 6, wherein the amino acid sequence of the enzyme comprises an N-terminal truncation of at most 3 amino acids, at most 5 amino acids, at most 10 amino acids, at most 15 amino acids, at most 20 amino acids, at most 25 amino acids, at most 26 amino acids, at most 27
25 amino acids, at most 28 amino acids, at most 29 amino acids, at most 30 amino acids, or at most 35 amino acids in one or more of SEQ ID NO: 8, 24, or 40 – 43.
8. The enzyme of any one of embodiments 1 – 6, wherein the amino acid sequence of the enzyme comprises an N-terminal truncation corresponding to amino acid positions 2-32 of SEQ ID NO: 8.
- 30 9. The enzyme of any one of embodiments 1 – 8 comprising an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.6%, at least 99.7%, or at least 99.8% identical to one of SEQ ID NOs: 8, 24, 40, 41, 42, or 43.

10. The enzyme of any one of embodiments 1 – 8 comprising the amino acid sequence of one of SEQ ID NOs: 8, 24, 40, 41, 42, or 43, wherein said amino acid sequence comprises one, at most two, at most three, at most four, at most five, at most six, at most seven, at most eight, at most nine, or at most ten amino acid substitutions.
11. The enzyme of embodiment 10, wherein the amino acid substitutions are made only to the portions of the protein which are responsible for binding and/or propagating BHB activity.
12. The enzyme of any one of embodiments 10 or 11 wherein the amino acid substitution(s) are conservative substitutions.
13. The enzyme of any one of embodiments 1 – 11, wherein the enzyme exhibits increased BHB activity as measured by peroxide strips, acetoacetic strips, or liquid chromatography–mass spectrometry (LCMS).
14. The enzyme of embodiment 13, wherein the enzyme exhibits increased BHB activity as measured by liquid chromatography–mass spectrometry (LCMS).
15. The enzyme of embodiment 14, wherein the engineered enzyme exhibits about one, about two, about three, about four, about five, about ten, or about twenty times greater BHB activity than the corresponding non-modified wild-type when measured by LCMS.
16. The enzyme of embodiment 14, wherein the enzyme comprises a sequence at least 90%, at least 95%, or at least 98% identical to SEQ ID NO: 24 and wherein SEQ ID NO: 24 exhibits increased BHB activity as measured by liquid chromatography–mass spectrometry (LCMS) as compared to SEQ ID NO: 8.
17. The enzyme of any one of embodiments 1 – 2, wherein the beta-hydroxybutyrate is (R)-beta-hydroxybutyrate.
18. The enzyme of any one of embodiments 1 – 2, wherein the beta-hydroxybutyrate is (S)-beta-hydroxybutyrate.
19. The enzyme of any one of embodiments 1 – 2, wherein the beta-hydroxybutyrate is a mixture of (R)-beta-hydroxybutyrate and (S)-beta-hydroxybutyrate.
20. A non-wearable BHB sensor capable of detecting and/or measuring BHB concentration comprising utilizing the enzyme of any one of embodiments 1 – 19.

21. The non-wearable BHB sensor of embodiment 20, wherein the non-wearable BHB sensor is a test strip.

22. The test strip of embodiment 21, wherein said test strip comprises: a substrate layer; and one or more sensing reagents dispensed upon at least a portion of the substrate layer, wherein at least one of the sensing reagents comprise an enzyme of any one of embodiments 1 – 19.

23. The test strip of embodiment 21 or 22, wherein the at least one sensing reagents comprising a co-factor, a mediator, an adjuvant, a carrier, and/or an excipient.

24. The test strip of embodiment 23, wherein the co-factor comprises flavin adenine dinucleotide (FAD).

25. The test strip of any one of embodiments 23 – 24, wherein the mediator comprises one or more of ferricyanide, phenazine, phenothiazine, thionine, methylene green/blue, tetrathiafulvalene, quinone derivatives, ferrocene, organometallic osmium complexes, and/or organometallic ruthenium complexes.

26. The test strip of any one of embodiments 22 – 25, wherein a material of the substrate layer comprises one or more of a composite material, a fibrous material, a woven textile, a non-woven textile, a polymer, an adhesive, a film, a gel, PTFE, and /or silicone.

27. The non-wearable BHB sensor of embodiment 20, wherein the non-wearable sensor is a fingertip sweat sensor.

28. The non-wearable BHB sensor of embodiment 20, wherein the non-wearable sensor can be used to detect or measure BHB in any body fluid.

29. The non-wearable BHB sensor of embodiment 28, wherein the body fluid can be obtained through non-invasive, minimally invasive, or invasive means, optionally via finger stick, blood draw, spinal tap, sweat and/or saliva collection.

30. The non-wearable BHB sensor of embodiment 29, wherein the body fluid comprises one or more of whole blood, serum, plasma, a blood fraction other than serum or plasma, lymph, cerebrospinal fluid (CSF), interstitial fluid (ISF), intracellular fluid, transcellular fluid, saliva, tears, sweat, vaginal discharge, milk, mucus, chyme, pus, bile, semen, urine, amniotic fluid, synovial fluid, peritoneal fluid, pericardial fluid, peritoneum, glandular secretions, exudate, contents of cysts, and/or ascites.

31. A wearable BHB sensor capable of detecting, measuring, and/or monitoring BHB concentration, comprising: a sensor, comprising a sensing electrode; a sensing reagent, wherein the sensing reagent composition comprising the enzyme of any one of embodiments 1 – 19; and a reference electrode.

5 32. The wearable BHB sensor of embodiment 31, wherein the sensing reagent is on or adjacent to the sensing electrode.

33. The wearable BHB sensor of embodiment 31, wherein the sensing reagent is dispensed to be in contact with the sensing electrode at the time of use.

34. The wearable BHB sensor of embodiment 31, wherein the wearable
10 BHB sensor is a continuous sensor, a continual sensor, or an on-demand sensor.

35. The wearable BHB sensor of any one of embodiments 31 – 34, wherein the sensing reagent composition further comprises a co-factor, a mediator, an adjuvant, a carrier, and/or an excipient.

36. The wearable BHB sensor of any one of embodiments 31 – 35, wherein
15 the wearable BHB sensor is configured to continuously measure a BHB concentration of a subject and output a data stream.

37. The wearable BHB sensor of any one of embodiments 31 – 36, wherein the wearable BHB sensor is a needle-based sensor, a micro-needle-based sensor, a reverse iontophoretic sensor, a sweat-based sensor, or an implantable sensor.

20 38. The wearable BHB sensor of embodiment 37, wherein the wearable BHB sensor is located in body tissues.

39. The wearable BHB sensor of any of embodiments 31 – 38, wherein the wearable BHB sensor is configured to measure a BHB concentration in a body fluid.

40. The wearable BHB sensor of embodiment 39, wherein the body fluid
25 comprises one or more of whole blood, serum, plasma, a blood fraction other than serum or plasma, lymph, cerebrospinal fluid (CSF), interstitial fluid (ISF), intracellular fluid, transcellular fluid, saliva, tears, sweat, vaginal discharge, milk, mucus, chyme, pus, bile, semen, urine, amniotic fluid, synovial fluid, peritoneal fluid, pericardial fluid, peritoneum, glandular secretions, exudate, contents of cysts, and/or ascites.

30 41. The wearable BHB sensor of any of embodiments 31 – 40, wherein the wearable BHB sensor is an in-body wearable BHB sensor or an on-body wearable BHB sensor.

42. A system for detecting, measuring, and/or monitoring BHB concentration, comprising: the wearable BHB sensor of anyone of embodiments 31

– 41, wherein the wearable BHB sensor is configured to continuously, continually, or on-demand measure a BHB concentration of a subject and output a data stream; and a device connected to the wearable BHB sensor, wherein the device comprises: a processor configured to process the data stream from the wearable BHB sensor;
5 and an interface configured to display and/or communicate measured BHB concentration values.

43. A method of detecting, measuring, and/or monitoring BHB concentration, comprising: obtaining a body fluid from a subject; subjecting the body fluid to the wearable BHB sensor of any one of embodiments 31 – 41; determining a
10 single point BHB concentration in the body fluid; and displaying and/or communicating the single point BHB concentration on an interface.

44. A method of improving health and/or wellness in a subject in need thereof comprising using a non-wearable BHB sensor, device, or apparatus of any one of embodiments 20 – 30.

15 45. A method of improving health and/or wellness in a subject in need thereof comprising using a test kit, wherein the test kit utilizes the enzyme of any one of embodiments 1 – 19.

46. A method aiding weight loss in a subject in need thereof comprising using a non-wearable BHB sensor, device, or apparatus of any one of embodiments
20 20 – 30.

47. The method of any one of embodiments 44-46 used in conjunction with a food item or supplement comprising at least one sugar alcohol or low carbohydrate composition.

48. The method of any one of embodiments 44 – 47, wherein the food item
25 or supplement contain less than about 5 grams of carbohydrates, less than about 8 grams of carbohydrates, or less than about 10 grams of carbohydrates per unit.

49. The method of any one of embodiments 47 and 48 used in conjunction with a food item or supplement, wherein the food item or supplement comprises exogenous ketone supplement and/or ketogenic ingredients.

30 50. The method of embodiment 49, wherein the exogenous ketone supplement comprises ketone bodies and/or precursors of ketone bodies.

51. The method of embodiment 50, wherein the ketone bodies and/or precursors of ketone bodies comprise one or more of acetone, acetoacetic acid, beta-hydroxybutyrate (BHB), beta-ketopentanoate, beta-hydroxypentanoate, 1,3-

butanediol, and medium chain triglycerides (MCT) containing fatty acids with hydrocarbon side chains in the length of 6-12 carbons.

52. The method of embodiment 51, wherein the ketone bodies and/or precursors of ketone bodies are in the form of salts and/or esters.

5 53. The method of embodiment 51, wherein the MCT comprises one or more of the following: caproic acid (C6), caprylic acid (C8), capric acid (C10), and lauric acid (C12).

INCORPORATION BY REFERENCE

10 [0147] All references, articles, publications, patents, patent publications, and patent applications cited herein within the above text and/or cited below are incorporated by reference in their entireties for all purposes. All literature and similar materials cited in this application, including patents, patent applications, articles, books, treatises, and Internet web pages are expressly incorporated by reference in their entirety for
15 any purpose. When definitions of terms in incorporated references appear to differ from the definitions provided in this application, the definition provided in this application shall control.

[0148] However, mention of any reference, article, publication, patent, patent publication, and patent application cited herein is not, and should not be taken as
20 acknowledgment or any form of suggestion that they constitute valid prior art or form part of the common general knowledge in any country in the world.

CLAIMS

1. An enzyme with oxidase activity, wherein said enzyme is engineered to exhibit improved beta-hydroxybutyrate (BHB) activity.
5
2. The enzyme of claim 1, wherein said enzyme is capable of oxidizing beta-hydroxybutyrate (BHB) to produce 3-oxobutanoate.
3. The enzyme of any one of claims 1 or 2, wherein the amino acid
10 sequence is derived from an oxidase of the EC 1.1.3.6 family.
4. The enzyme of any one of claims 1 – 3, wherein the oxidase of the EC 1.1.3.6 family is a cholesterol oxidase.
- 15 5. The enzyme of any one of claims 1 – 4, wherein the enzyme is non-naturally occurring and includes one or more amino acid substitutions, deletions, or truncations relative to a native or wild-type enzyme.
6. The enzyme of any one of claims 1 – 5, wherein the amino acid
20 sequence of the enzyme comprises one or more amino acid substitutions corresponding to N137G, Y235Q, and/or A455Y of SEQ ID NO: 8.
7. The enzyme of any one of claims 1 – 6, wherein the amino acid
25 sequence of the enzyme comprises an N-terminal truncation of at most 3 amino acids, at most 5 amino acids, at most 10 amino acids, at most 15 amino acids, at most 20 amino acids, at most 25 amino acids, at most 26 amino acids, at most 27 amino acids, at most 28 amino acids, at most 29 amino acids, at most 30 amino acids, or at most 35 amino acids in one or more of SEQ ID NO: 8, 24, or 40 – 43.
- 30 8. The enzyme of any one of claims 1 – 6, wherein the amino acid sequence of the enzyme comprises an N-terminal truncation corresponding to amino acid positions 2-32 of SEQ ID NO: 8.

9. The enzyme of any one of claims 1 – 8 comprising an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.6%, at least 99.7%, or at least 99.8% identical to one of SEQ ID NOs: 8, 24, 40, 41, 42, or 43.

5

10. The enzyme of any one of claims 1 – 8 comprising the amino acid sequence of one of SEQ ID NOs: 8, 24, 40, 41, 42, or 43, wherein said amino acid sequence comprises one, at most two, at most three, at most four, at most five, at most six, at most seven, at most eight, at most nine, or at most ten amino acid substitutions.

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11. The enzyme of claim 10, wherein the amino acid substitutions are made only to the portions of the protein which are responsible for binding and/or propagating BHB activity.

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12. The enzyme of any one of claims 10 or 11 wherein the amino acid substitution(s) are conservative substitutions.

13. The enzyme of anyone of claims 1 – 11, wherein the enzyme exhibits increased BHB activity as measured by peroxide strips, acetoacetic strips, or liquid chromatography–mass spectrometry (LCMS).

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14. The enzyme of claim 13, wherein the enzyme exhibits increased BHB activity as measured by liquid chromatography–mass spectrometry (LCMS).

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15. The enzyme of claim 14, wherein the engineered enzyme exhibits about one, about two, about three, about four, about five, about ten, or about twenty times greater BHB activity than the corresponding non-modified wild-type when measured by LCMS.

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16. The enzyme of claim 14, wherein the enzyme comprises a sequence at least 90%, at least 95%, or at least 98% identical to SEQ ID NO: 24 and wherein SEQ ID NO: 24 exhibits increased BHB activity as measured by liquid chromatography–mass spectrometry (LCMS) as compared to SEQ ID NO: 8.

17. The enzyme of anyone of claims 1 - 2, wherein the beta-hydroxybutyrate is (R)-beta-hydroxybutyrate.

5 18. The enzyme of anyone of claims 1 - 2, wherein the beta-hydroxybutyrate is (S)-beta-hydroxybutyrate.

19. The enzyme of anyone of claims 1 - 2, wherein the beta-hydroxybutyrate is a mixture of (R)-beta-hydroxybutyrate and (S)-beta-hydroxybutyrate.
10

20. A non-wearable BHB sensor capable of detecting and/or measuring BHB concentration comprising utilizing the enzyme of any one of claims 1 – 19.

15 21. The non-wearable BHB sensor of claim 20, wherein the non-wearable BHB sensor is a test strip.

22. The test strip of claim 21, wherein said test strip comprises:
a substrate layer; and
20 one or more sensing reagents dispensed upon at least a portion of the substrate layer, wherein at least one of the sensing reagents comprise an enzyme of any one of claims 1 – 19.

23. The test strip of claim 21 or 22, wherein the at least one sensing reagents comprising a co-factor, a mediator, an adjuvant, a carrier, and/or an excipient.
25

24. The test strip of claim 23, wherein the co-factor comprises flavin adenine dinucleotide (FAD).
30

25. The test strip of any one of claims 23 – 24, wherein the mediator comprises one or more of ferricyanide, phenazine, phenothiazine, thionine, methylene green/blue, tetrathiafulvalene, quinone derivatives, ferrocene, organometallic osmium complexes, and/or organometallic ruthenium complexes.

26. The test strip of any one of claims 22 – 25, wherein a material of the substrate layer comprises one or more of a composite material, a fibrous material, a woven textile, a non-woven textile, a polymer, an adhesive, a film, a gel, PTFE, and
5 /or silicone.

27. The non-wearable BHB sensor of claim 20, wherein the non-wearable sensor is a fingertip sweat sensor.

10 28. The non-wearable BHB sensor of claim 20, wherein the non-wearable sensor can be used to detect or measure BHB in any body fluid.

29. The non-wearable BHB sensor of claim 28, wherein the body fluid can be obtained through non-invasive, minimally invasive, or invasive means, optionally
15 via finger stick, blood draw, spinal tap, sweat and/or saliva collection.

30. The non-wearable BHB sensor of claim 29, wherein the body fluid comprises one or more of whole blood, serum, plasma, a blood fraction other than serum or plasma, lymph, cerebrospinal fluid (CSF), interstitial fluid (ISF), intracellular
20 fluid, transcellular fluid, saliva, tears, sweat, vaginal discharge, milk, mucus, chyme, pus, bile, semen, urine, amniotic fluid, synovial fluid, peritoneal fluid, pericardial fluid, peritoneum, glandular secretions, exudate, contents of cysts, and/or ascites.

31. A wearable BHB sensor capable of detecting, measuring, and/or
25 monitoring BHB concentration, comprising:
a sensor, comprising a sensing electrode;
a sensing reagent, wherein the sensing reagent composition comprising the enzyme of any one of claims 1 – 19; and
a reference electrode.

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32. The wearable BHB sensor of claim 31, wherein the sensing reagent is on or adjacent to the sensing electrode.

33. The wearable BHB sensor of claim 31, wherein the sensing reagent is dispensed to be in contact with the sensing electrode at the time of use.

34. The wearable BHB sensor of claim 31, wherein the wearable BHB
5 sensor is a continuous sensor, a continual sensor, or an on-demand sensor.

35. The wearable BHB sensor of any one of claims 31 – 34, wherein the sensing reagent composition further comprises a co-factor, a mediator, an adjuvant, a carrier, and/or an excipient.
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36. The wearable BHB sensor of any one of claims 31 – 35, wherein the wearable BHB sensor is configured to continuously measure a BHB concentration of a subject and output a data stream.

37. The wearable BHB sensor of any one of claims 31 – 36, wherein the wearable BHB sensor is a needle-based sensor, a micro-needle-based sensor, a reverse iontophoretic sensor, a sweat-based sensor, or an implantable sensor.
15

38. The wearable BHB sensor of claim 37, wherein the wearable BHB
20 sensor is located in body tissues.

39. The wearable BHB sensor of any of claims 31 – 38, wherein the wearable BHB sensor is configured to measure a BHB concentration in a body fluid.

40. The wearable BHB sensor of claim 39, wherein the body fluid comprises one or more of whole blood, serum, plasma, a blood fraction other than serum or plasma, lymph, cerebrospinal fluid (CSF), interstitial fluid (ISF), intracellular fluid, transcellular fluid, saliva, tears, sweat, vaginal discharge, milk, mucus, chyme, pus, bile, semen, urine, amniotic fluid, synovial fluid, peritoneal fluid, pericardial fluid,
25 peritoneum, glandular secretions, exudate, contents of cysts, and/or ascites.
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41. The wearable BHB sensor of any of claims 31 – 40, wherein the wearable BHB sensor is an in-body wearable BHB sensor or an on-body wearable BHB sensor.

42. A system for detecting, measuring, and/or monitoring BHB concentration, comprising:

- the wearable BHB sensor of anyone of claims 31 – 41, wherein the wearable
5 BHB sensor is configured to continuously, continually, or on-demand measure a BHB concentration of a subject and output a data stream; and
a device connected to the wearable BHB sensor, wherein the device comprises:
a processor configured to process the data stream from the wearable BHB
sensor; and
10 an interface configured to display and/or communicate measured BHB concentration values.

43. A method of detecting, measuring, and/or monitoring BHB concentration, comprising:

- 15 obtaining a body fluid from a subject;
subjecting the body fluid to the wearable BHB sensor of any one of claims 31 – 41;
determining a single point BHB concentration in the body fluid; and
displaying and/or communicating the single point BHB concentration on an
20 interface.

44. A method of improving health and/or wellness in a subject in need thereof comprising using a non-wearable BHB sensor, device, or apparatus of any one of claims 20 – 30.

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45. A method of improving health and/or wellness in a subject in need thereof comprising using a test kit, wherein the test kit utilizes the enzyme of any one of claims 1 – 19.

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46. A method aiding weight loss in a subject in need thereof comprising using a non-wearable BHB sensor, device, or apparatus of any one of claims 20 – 30.

47. The method of any one of claims 44-46 used in conjunction with a food item or supplement comprising at least one sugar alcohol or low carbohydrate composition.

5 48. The method of anyone of claims 44 – 47, wherein the food item or supplement contain less than about 5 grams of carbohydrates, less than about 8 grams of carbohydrates, or less than about 10 grams of carbohydrates per unit.

10 49. The method of any one of claims 47 and 48 used in conjunction with a food item or supplement, wherein the food item or supplement comprises exogenous ketone supplement and/or ketogenic ingredients.

50. The method of claim 49, wherein the exogenous ketone supplement comprises ketone bodies and/or precursors of ketone bodies.

15 51. The method of claim 50, wherein the ketone bodies and/or precursors of ketone bodies comprise one or more of acetone, acetoacetic acid, beta-hydroxybutyrate (BHB), beta-ketopentanoate, beta-hydroxypentanoate, 1,3-butanediol, and medium chain triglycerides (MCT) containing fatty acids with hydrocarbon side chains in the length of 6-12 carbons.

52. The method of claim 51, wherein the ketone bodies and/or precursors of ketone bodies are in the form of salts and/or esters.

25 53. The method of claim 51, wherein the MCT comprises one or more of the following: caproic acid (C6), caprylic acid (C8), capric acid (C10), and lauric acid (C12).

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