KETOREDUCTASE POLYPEPTIDES FOR THE STEREOSELECTIVE PRODUCTION OF (4*S*)-3-[(5*S*)-5-(4-FLUOROPHENYL)-5-HYDROXYPENTANOYL]-4-PHENYL-1,3-OXAZOLIDIN-2-ONE

1. TECHNICAL FIELD

[0001] The present disclosure relates to engineered polypeptides and uses of the polypeptides for preparing the intermediate (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one in a process for making Ezetimibe.

2. REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

[0002] The Sequence Listing concurrently submitted electronically under 37 C.F.R. § 1.821 via EFS-Web in a computer readable form (CRF) as file name CX2-025_ST25.txt is herein incorporated by reference. The electronic copy of the Sequence Listing was created on July 31, 2009, with a file size of 300 Kbytes. This Sequence Listing is identical except for minor formatting corrections to 376247_021USP1.txt (296 Kbytes) created August 28, 2008, which was incorporated by reference in the priority US provisional application 61/092,807.

3. BACKGROUND

[0003] Enzymes belonging to the ketoreductase (KRED) or carbonyl reductase class (EC1.1.1.184) are useful for the synthesis of optically active alcohols from the corresponding prostereoisomeric ketone substrates and by stereospecific reduction of corresponding racemic aldehyde and ketone substrates. KREDs typically convert a ketone or aldehyde substrate to the corresponding alcohol product, but may also catalyze the reverse reaction, oxidation of an alcohol substrate to the corresponding ketone/aldehyde product. The reduction of ketones and aldehydes and the oxidation of alcohols by enzymes such as KRED requires a co-factor, most commonly reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH), and nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) for the oxidation reaction. NADH and NADPH serve as electron donors, while NAD and NADP serve as electron acceptors. It is frequently observed that ketoreductases and alcohol dehydrogenases accept either the phosphorylated or the non-phosphorylated co-factor (in its oxidized and reduced state).

[0004] KRED enzymes can be found in a wide range of bacteria and yeasts (for reviews: Kraus and Waldman, Enzyme catalysis in organic synthesis Vols. 1&2.VCH Weinheim 1995; Faber, K., Biotransformations in organic chemistry, 4th Ed. Springer, Berlin Heidelberg New York. 2000; Hummel and Kula *Eur. J. Biochem.* 1989 184:1-13). Several KRED gene and enzyme sequences have been reported, *e.g.*, *Candida magnoliae* (Genbank Acc. No. JC7338; GI:11360538) *Candida*

parapsilosis (Genbank Acc. No. BAA24528.1; GI:2815409), *Sporobolomyces salmonicolor* (Genbank Acc. No. AF160799; GI:6539734).

[0005] In order to circumvent many chemical synthetic procedures for the production of key compounds, ketoreductases are being increasingly employed for the enzymatic conversion of different keto and aldehyde substrates to chiral alcohol products. These applications can employ whole cells expressing the ketoreductase for biocatalytic ketone reductions, or purified enzymes in those instances where presence of multiple ketoreductases in whole cells would adversely affect the stereopurity and yield of the desired product. For *in vitro* applications, a co-factor (NADH or NADPH) regenerating enzyme such as glucose dehydrogenase (GDH), formate dehydrogenase *etc.* is used in conjunction with the ketoreductase. Examples using ketoreductases to generate useful chemical compounds include asymmetric reduction of 4-chloroacetoacetate esters (Zhou, *J. Am. Chem. Soc.* 1983 105:5925-5926; Santaniello, *J. Chem. Res.* (S) 1984:132-133; U.S. Patent Nos. 5,559,030; U.S. Patent No. 5,700,670 and U.S. Patent No. 5,891,685), reduction of dioxocarboxylic acids (*e.g.*, U.S. Patent No. 6,399,339), reduction of tert-butyl (S) chloro-5-hydroxy-3-oxohexanoate (*e.g.,* U.S. Patent No. 6,645,746 and WO 01/40450), reduction pyrrolotriazine-based compounds (*e.g.*, U.S. application No. 2006/0286646); reduction of substituted acetophenones (*e.g.*, U.S. Patent No. 6,800,477); and reduction of ketothiolanes (WO 2005/054491).

[0006] It is desirable to identify other ketoreductase enzymes that can be used to carry out conversion of various keto substrates to its corresponding chiral alcohol products.

4. SUMMARY

[0007] The present disclosure provides ketoreductase polypeptides capable of reducing 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione ("the substrate") to (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one ("the product"), polynucleotides encoding such polypeptides, and methods for using the polypeptides. The ketoreductase polypeptides are also capable of reducing 1-(4-fluorophenyl)-3(R)-[3-oxo-3-(4-fluorophenyl)propyl)]-4(S)-(4-hydroxyphenyl)-2-azetidinone, to the corresponding stereoisomeric alcohol 1-(4-fluorophenyl)- 3(R)-[3(S)-hydroxy-3(4-fluoropheny l)-propy1)]-4(S)-(4-hydroxypheny1)-2-azetidinone.

[0008] In one embodiment, the ketoreductase polypeptides described herein have an amino acid sequence that has one or more amino acid differences as compared to a reference amino acid sequence of a wild-type ketoreductase or an engineered ketoreductase that result in an improved property of the enzyme for the defined keto substrate. Generally, the engineered ketoreductase polypeptides have an improved property as compared to the naturally-occurring wild-type ketoreductase enzymes obtained

from *Lactobacillus kefir* ("*L. kefir*"; SEQ ID NO:4), *Lactobacillus brevis* ("*L. brevis*"; SEQ ID NO:2), and *Lactobacillus minor* ("*L. minor*"; SEQ ID NO:158). In some embodiments, the polypeptides of the disclosure have an improved property as compared to another engineered polypeptide, such as SEQ ID NO: 8. Improvements in enzyme property include increases in enzyme activity, stereoselectivity, sterospecificity, thermostability, solvent stability, or reduced product inhibition. In the present disclosure, the ketoreductase polypeptides have at least the following amino acid sequence as compared to the amino acid sequence of SEQ ID NO:2, 4, or 158: the amino acid residue corresponding to X145 is a serine, and the amino acid residue corresponding to X190 is a cysteine. In some embodiments, as compared to the sequences of SEQ ID NO: 2, 4, or 158, the ketoreductase polypeptides have at least the following amino acid sequence differences: (1) the amino acid residue corresponding to X145 is a serine; the amino acid residue corresponding to residue X190 is a cysteine; and the amino acid residue corresponding to X96 is a glutamine. In some embodiments, as compared to the sequence of SEQ ID NO:2, 4, or 158, the ketoreductase polypeptides have at least the amino acid sequence as compared to the amino acid sequence of SEQ ID NO:2, 4, or 158: residue X145 is a serine; residue X190 is a cysteine, and residue X211 is an arginine.

[0009] In some embodiments, the ketoreductase polypeptides of the disclosure are improved as compared to SEQ ID NO:2, 4 or 158 with respect to their rate of enzymatic activity, *i.e.*, their rate of converting the substrate to the product. In some embodiments, the ketoreductase polypeptides are capable of converting the substrate to the product at a rate that is at least 1.5-times, 2-times, 3-times, 4-times, 5-times, 10-times, 25-times, 50-times, 100-times, 150-times, 200-times, 400-times, 1000-times, 3000-times, 5000-times, 7000-times or more than 7000-times the rate exhibited by the enzyme of SEQ ID NO:2, 4 or 158.

[0010] In some embodiments, the ketoreductase polypeptide is capable of converting the substrate 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to the product (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one, at a rate that is improved over a reference polypeptide having the amino acid sequence of SEQ ID NO:8. In some embodiments, such ketoreductase polypeptides are also capable of converting the substrate to the product with a percent stereomeric excess of at least about 95%. In some embodiments, such ketoreductase polypeptides are also capable of converting the substrate to the product with a percent stereomeric excess of at least about 99%. Exemplary polypeptides with such properties include, but are not limited to, polypeptides which comprising amino acid sequences corresponding to SEQ ID NO: 42, 44, 46, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[0011] In some embodiments, the ketoreductase polypeptide is capable of converting the substrate 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to the product (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one, with a percent stereomeric excess of at least about 99% and at a rate that is at least about 5 times or more improved over a reference polypeptide having the amino acid sequence of SEQ ID NO:8. Exemplary polypeptides with such properties include, but are not limited to, polypeptides which comprise an amino acid sequence corresponding to SEQ ID NO: 42, 44, 46, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[0012] In some embodiments, the ketoreductase polypeptide is capable of converting the substrate 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to the product (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one, with a percent stereomeric excess of at least about 99% and at a rate that is at least about 3000 to about 7000 times improved over a reference polypeptide having the amino acid sequence of SEQ ID NO:8. Exemplary polypeptides with such properties include, but are not limited to, polypeptides which comprise an amino acid sequence corresponding to SEQ ID NO: 44, 46, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 72, 74, 78, 80, 82, 84, and 86.

[0013] In some embodiments, the ketoreductase polypeptide is capable of converting the substrate 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to the product (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one, with a percent stereomeric excess of at least about 99% and at a rate that is at least 7000 times improved over a reference polypeptide having the amino acid sequence of SEQ ID NO:8. Exemplary polypeptides with such properties include, but are not limited to, polypeptides which comprise amino acid sequences corresponding to SEQ ID NO: 102, 108, 120, 122, 124, and 126.

[0014] In some embodiments, the ketoreductase polypeptide is capable of converting at least about 95% of the substrate to the product in less than about 24 hours when carried out with greater than about 100 g/L of substrate and less than about 5 g/L of the polypeptide. Exemplary polypeptides that have this capability include, but are not limited to, polypeptides which comprise amino acid sequences corresponding to SEQ ID NO: 102, 108, 120, 122, 124, and 126.

[0015] In some embodiments, the ketoreductase polypeptide is highly stereoselective, wherein the polypeptide can reduce the substrate to the product in greater than about 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% stereomeric excess. Exemplary ketoreductase polypeptides with high stereoselectivity include, but are not limited to, the polypeptides comprising the amino acid sequences corresponding to SEQ ID NO: 42, 44, 46, 50, 52, 54, 56, 58, 60, 62, 64, 66,

68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[0016] In some embodiments, an improved ketoreductase polypeptide comprises an amino acid sequence that corresponds to the sequence formulas of SEQ ID NO:161, 162 or 163 (or a region thereof, such as residues 90-211). SEQ ID NO:162 is based on the amino acid sequence of the *Lactobacillus kefir* ketoreductase of SEQ ID NO:4. The sequence formula of SEQ ID NO:161 is based on the amino acid sequence of the *Lactobacillus brevis* ketoreductase (SEQ ID NO:2). The sequence formula of SEQ ID NO:163 is based on the amino acid sequence of the *Lactobacillus minor* ketoreductase (SEQ ID NO:158). SEQ ID NO:161, 162 or 163 specify that residue X145 is a polar residue and residue X190 is cysteine. In one aspect, there is provided an engineered polypeptide having ketoreductase activity, wherein the polypeptide comprises the amino acid reference sequence of SEQ ID NO: 162, wherein the residue corresponding to X145 is an asparagine, glutamine, leucine, phenylalanine, or threonine residue.

[0017] In some embodiments, an improved ketoreductase polypeptide of the disclosure is based on the sequence formulas of SEQ ID NO:161, 162 or 163 and can comprise an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the reference sequence of SEQ ID NO:128, 130, or 160, with the proviso that the ketoreductase amino acid sequence has at the residue corresponding to residue X145 a serine and at the amino acid residue corresponding to X190 a cysteine. In some embodiments, the ketoreductase polypeptides can have one or more amino acid residue differences as compared to SEQ ID NO:128, 130, or 160. These differences can be amino acid insertions, deletions, substitutions, or any combination of such changes. In some embodiments, the amino acid sequence differences can comprise non-conservative, conservative, as well as a combination of non-conservative and conservative amino acid substitutions. Various amino acid residue positions where such changes can be made are described herein.

[0018] In some embodiments, an improved ketoreductase polypeptide is based on the sequence formulas of SEQ ID NO:161, 162 or 163 and can comprise a region having an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a region or domain thereof, such as residues 90-211 of the reference sequence of SEQ ID NO:128, 130, or 160, with the proviso that the ketoreductase polypeptide amino acid sequence has at the residue corresponding to residue X145 a serine and at the amino acid residue corresponding to X190 a cysteine. In some embodiments, the amino acid sequence differences can comprise non-conservative, conservative, as well as a combination of non-conservative and conservative amino acid substitutions. Various amino acid residue positions where such changes can be made in the defined region are described herein.

[0019] In some embodiments, an improved ketoreductase comprises an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or

99% identical to the amino acid sequence corresponding to SEQ ID NO: 8, 42, 44, 46, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126., wherein the improved ketoreductase polypeptide amino acid sequence includes any one set of the specified amino acid substitution combinations presented in Tables 3 and 4. In some embodiments, these ketoreductase polypeptides can have mutations at other amino acid residues.

[0020] The present disclosure provides polynucleotides encoding the engineered ketoreductases described herein or polynucleotides that hybridize to such polynucleotides under highly stringent conditions. The polynucleotide can include promoters and other regulatory elements useful for expression of the encoded engineered ketoreductase, and can utilize codons optimized for specific desired expression systems. In some embodiments, the polynucleotides encode a ketoreductase polypeptides having at least the following amino acid sequence as compared to the amino acid sequence of SEQ ID NO:2, 4, or 158: the amino acid residue corresponding to X145 is a serine, and the amino acid residue corresponding to X190 is a cysteine. Exemplary polynucleotides include, but are not limited to, a polynucleotide sequence of SEQ ID NO: 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, and 125.

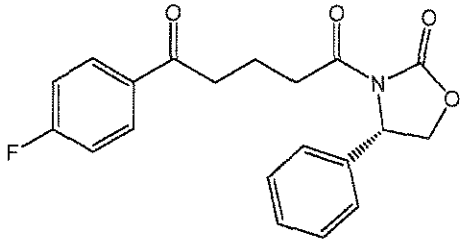
[0021] The present disclosure provides host cells comprising the polynucleotides and/or expression vectors described herein. The host cells may be *L. kefir* or *L. brevis*, or they may be a different organism, and as *E. coli*. The host cells can be used for the expression and isolation of the engineered ketoreductase enzymes described herein, or, alternatively, they can be used directly for the conversion of the substrate to the stereoisomeric product.

[0022] Whether carrying out the method with whole cells, cell extracts or purified ketoreductase enzymes, a single ketoreductase enzyme may be used or, alternatively, mixtures of two or more ketoreductase enzymes may be used.

[0022a] In another aspect, there is provided a method for reducing a carbonyl group of a substrate compound to its corresponding alcohol group, the method comprising contacting the substrate compound with an engineered polypeptide described herein in the presence of a NADH or NADPH cofactor, wherein the substrate is a ketone.

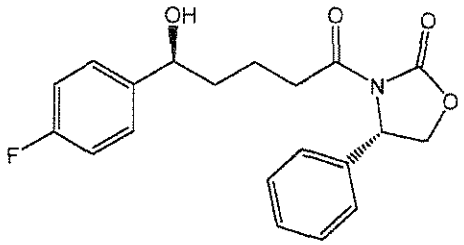
[0023] The ketoreductase enzymes described herein are capable of catalyzing the reduction reaction of the keto group in the compound of structural formula (**I**), 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione ("the substrate"),

(I)



to the corresponding stereoisomeric alcohol product of structural formula (II), (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one ("the product"):

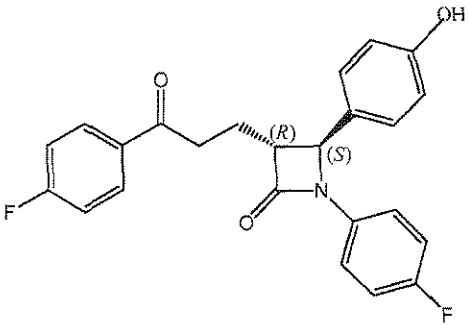
(II)



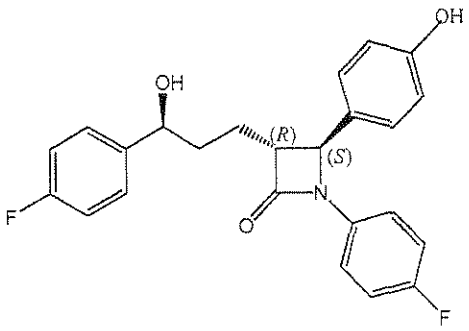
[0024] In some embodiments, the method for reducing or converting the substrate having the structural formula (I) to the corresponding product of structural formula (II) comprises contacting or incubating the substrate with a ketoreductase polypeptide disclosed herein under reaction conditions suitable for reducing or converting the substrate to the product.

[0025] In some embodiment, the ketoreductase enzymes described herein are also capable of catalyzing the reduction reaction of the keto group in the compound of structural formula (III), 1-(4-fluorophenyl)-3(R)-[3-oxo-3-(4-fluorophenyl)propyl)]-4(S)-(4-hydroxyphenyl)-2-azetidinone,

(III)



to the corresponding stereoisomeric alcohol product of structural formula (IV), 1-(4-fluorophenyl)-3(R)-[3(S)-hydroxy-3(4-fluorophenyl)-propyl)]-4(S)-(4-hydroxyphenyl)-2-azetidinone,

(IV) 

[0026] In some embodiments, the method for reducing the substrate having the structural formula (III) to the corresponding product of structural formula (IV) comprises contacting or incubating the compound of formula (III) with a ketoreductase polypeptide disclosed herein under reaction conditions suitable for reducing or converting the substrate of formula (III) to the product of formula (IV).

[0027] In some embodiments of this method for reducing the substrate to the product, the substrate is reduced to the product in greater than about 99% stereomeric excess, wherein the ketoreductase polypeptide comprises a sequence that corresponds to SEQ ID NO: SEQ ID NO: 42, 44, 46, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[0028] In some embodiments of this method for reducing the substrate to the product, at least about 95% of the substrate is converted to the product in less than about 24 hours when carried out with greater than about 100 g/L of substrate and less than about 5 g/L of the polypeptide, wherein the polypeptide comprises an amino acid sequence corresponding to SEQ ID NO: 102, 108, 120, 122, 124, or 126.

5. BRIEF DESCRIPTION OF THE FIGURES

[0029] FIG.1 illustrates the role of ketoreductases (KRED) in the conversion of the substrate compound of formula (I) to the corresponding product of formula (II). This reduction uses a KRED of the invention and a co-factor such as NADPH. A glucose dehydrogenase (GDH) is used to covert/recycle $NADP^+$ to NADPH. Glucose is converted to gluconic acid, which in turn is converted to its sodium salt (sodium gluconate) with the addition of sodium hydroxide.

6. DETAILED DESCRIPTION

6.1 Definitions

[0030] As used herein, the following terms are intended to have the following meanings.

[0031] "Ketoreductase" and "KRED" are used interchangeably herein to refer to a polypeptide having an enzymatic capability of reducing a carbonyl group to its corresponding alcohol. More specifically, the ketoreductase polypeptides of the invention are capable of stereoselectively reducing the compound of formula (**I**), *supra* to the corresponding product of formula (**II**), *supra*. The polypeptide typically utilizes a cofactor reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the reducing agent. Ketoreductases as used herein include naturally occurring (wild type) ketoreductases as well as non-naturally occurring engineered polypeptides generated by human manipulation.

[0032] "Coding sequence" refers to that portion of a nucleic acid (*e.g.*, a gene) that encodes an amino acid sequence of a protein.

[0033] "Naturally-occurring" or "wild-type" refers to the form found in nature. For example, a naturally occurring or wild-type polypeptide or polynucleotide sequence is a sequence present in an organism that can be isolated from a source in nature and which has not been intentionally modified by human manipulation.

[0034] "Recombinant" when used with reference to, *e.g.*, a cell, nucleic acid, or polypeptide, refers to a material, or a material corresponding to the natural or native form of the material, that has been modified in a manner that would not otherwise exist in nature, or is identical thereto but produced or derived from synthetic materials and/or by manipulation using recombinant techniques. Non-limiting examples include, among others, recombinant cells expressing genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise expressed at a different level.

[0035] "Percentage of sequence identity" and "percentage homology" are used interchangeably herein to refer to comparisons among polynucleotides and polypeptides, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage may be calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Alternatively, the percentage may be calculated by determining the number of positions at which either the identical nucleic acid base or amino acid residue occurs in both sequences or a nucleic acid base or amino acid residue is aligned with a gap to yield the number of matched positions, dividing the number of matched positions by the total number

of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Those of skill in the art appreciate that there are many established algorithms available to align two sequences. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman, 1981, *Adv. Appl. Math.* 2:482, by the homology alignment algorithm of Needleman and Wunsch, 1970, *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman, 1988, *Proc. Natl. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the GCG Wisconsin Software Package), or by visual inspection (see generally, *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1995 Supplement) (Ausubel)). Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, 1990, *J. Mol. Biol.* 215: 403-410 and Altschul *et al.*, 1977, *Nucleic Acids Res.* 3389-3402, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as, the neighborhood word score threshold (Altschul *et al*, supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, 1989, *Proc Natl Acad Sci USA* 89:10915). Exemplary determination of sequence alignment and % sequence identity can employ the BESTFIT or GAP programs in the GCG Wisconsin Software package (Accelrys, Madison WI), using default parameters provided.

[0036] "Reference sequence" refers to a defined sequence used as a basis for a sequence comparison. A reference sequence may be a subset of a larger sequence, for example, a segment of a full-length gene or polypeptide sequence. Generally, a reference sequence is at least 20 nucleotide or amino acid residues in length, at least 25 residues in length, at least 50 residues in length, or the full length of the nucleic acid or polypeptide. Since two polynucleotides or polypeptides may each (1) comprise a sequence (*i.e.*, a portion of the complete sequence) that is similar between the two sequences, and (2) may further comprise a sequence that is divergent between the two sequences, sequence comparisons between two (or more) polynucleotides or polypeptide are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity.

[0037] "Comparison window" refers to a conceptual segment of at least about 20 contiguous nucleotide positions or amino acids residues wherein a sequence may be compared to a reference sequence of at least 20 contiguous nucleotides or amino acids and wherein the portion of the sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The comparison window can be longer than 20 contiguous residues, and includes, optionally 30, 40, 50, 100, or longer windows.

[0038] "Substantial identity" refers to a polynucleotide or polypeptide sequence that has at least 80 percent sequence identity, at least 85 percent identity and 89 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 residue positions, frequently over a window of at least 30-50 residues, wherein the percentage of sequence identity is calculated by comparing the reference sequence to a sequence that includes deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. In specific embodiments applied to polypeptides, the term "substantial identity" means that two polypeptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 89 percent sequence identity, at least 95 percent sequence identity or more (*e.g.*, 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

[0039] "Corresponding to", "reference to" or "relative to" when used in the context of the numbering of a given amino acid or polynucleotide sequence refers to the numbering of the residues of a specified reference sequence when the given amino acid or polynucleotide sequence is compared to the reference sequence. In other words, the residue number or residue position of a given polymer is designated with respect to the reference sequence rather than by the actual numerical position of the

residue within the given amino acid or polynucleotide sequence. For example, a given amino acid sequence, such as that of an engineered ketoreductase, can be aligned to a reference sequence by introducing gaps to optimize residue matches between the two sequences. In these cases, although the gaps are present, the numbering of the residue in the given amino acid or polynucleotide sequence is made with respect to the reference sequence to which it has been aligned.

[0040] "Stereoselectivity" refers to the preferential formation in a chemical or enzymatic reaction of one stereoisomer over another. Stereoselectivity can be partial, where the formation of one stereoisomer is favored over the other, or it may be complete where only one stereoisomer is formed. When the stereoisomers are enantiomers, the stereoselectivity is referred to as enantioselectivity, the fraction (typically reported as a percentage) of one enantiomer in the sum of both. It is commonly alternatively reported in the art (typically as a percentage) as the enantiomeric excess (e.e.) calculated therefrom according to the formula [major enantiomer – minor enantiomer]/[major enantiomer + minor enantiomer]. Where the stereoisomers are diastereoisomers, the stereoselectivity is referred to as diastereoselectivity, the fraction (typically reported as a percentage) of one diastereomer in a mixture of two diasteromers, commonly alternatively reported as the diastereomeric excess (d.e.). Enantiomeric excess and diastereomeric excess are types of stereomeric excess.

[0041] "Highly stereoselective": refers to a ketoreductase polypeptide that is capable of converting or reducing the substrate to the corresponding (*S*)-product with at least about 99% stereomeric excess.

[0042] "Stereospecificity" refers to the preferential conversion in a chemical or enzymatic reaction of one stereoisomer over another. Stereospecificity can be partial, where the conversion of one stereoisomer is favored over the other, or it may be complete where only one stereoisomer is converted.

[0043] "Chemoselectivity" refers to the preferential formation in a chemical or enzymatic reaction of one product over another.

[0044] "Improved enzyme property" refers to a ketoreductase polypeptide that exhibits an improvement in any enzyme property as compared to a reference ketoreductase. For the engineered ketoreductase polypeptides described herein, the comparison is generally made to the wild-type ketoreductase enzyme, although in some embodiments, the reference ketoreductase can be another improved engineered ketoreductase. Enzyme properties for which improvement is desirable include, but are not limited to, enzymatic activity (which can be expressed in terms of percent conversion of the substrate), thermal stability, pH activity profile, cofactor requirements, refractoriness to inhibitors (*e.g.*, product inhibition), stereospecificity, and stereoselectivity (including enantioselectivity).

[0045] "Increased enzymatic activity" refers to an improved property of the engineered ketoreductase polypeptides, which can be represented by an increase in specific activity (*e.g.*, product produced/time/weight protein) or an increase in percent conversion of the substrate to the product (*e.g.*, percent conversion of starting amount of substrate to product in a specified time period using a specified amount of KRED) as compared to the reference ketoreductase enzyme. Exemplary methods to determine enzyme activity are provided in the Examples. Any property relating to enzyme activity may be affected, including the classical enzyme properties of K_m, V_{max} or k_{cat}, changes of which can lead to increased enzymatic activity. Improvements in enzyme activity can be from about 1.5 times the enzymatic activity of the corresponding wild-type ketoreductase enzyme, to as much as 2 times. 5 times, 10 times, 20 times, 25 times, 50 times, 75 times, 100 times, 150 times, 200 times, 500 times, 1000, times, 3000 times, 5000 times, 7000 times or more enzymatic activity than the naturally occurring ketoreductase or another engineered ketoreductase from which the ketoreductase polypeptides were derived. In specific embodiments, the engineered ketoreductase enzyme exhibits improved enzymatic activity in the range of 150 to 3000 times, 3000 to 7000 times, or more than 7000 times greater than that of the parent ketoreductase enzyme. It is understood by the skilled artisan that the activity of any enzyme is diffusion limited such that the catalytic turnover rate cannot exceed the diffusion rate of the substrate, including any required cofactors. The theoretical maximum of the diffusion limit, or k_{cat}/K_m, is generally about 10^8 to 10^9 (M^{-1} s^{-1}). Hence, any improvements in the enzyme activity of the ketoreductase will have an upper limit related to the diffusion rate of the substrates acted on by the ketoreductase enzyme. Ketoreductase activity can be measured by any one of standard assays used for measuring ketoreductase, such as a decrease in absorbance or fluorescence of NADPH due to its oxidation with the concomitant reduction of a ketone to an alcohol, or by product produced in a coupled assay. Comparisons of enzyme activities are made using a defined preparation of enzyme, a defined assay under a set condition, and one or more defined substrates, as further described in detail herein. Generally, when lysates are compared, the numbers of cells and the amount of protein assayed are determined as well as use of identical expression systems and identical host cells to minimize variations in amount of enzyme produced by the host cells and present in the lysates.

[0046] "Conversion": refers to the enzymatic reduction of the substrate to the corresponding product. "Percent conversion" refers to the percent of the substrate that is reduced to the product within a period of time under specified conditions. Thus, the "enzymatic activity" or "activity" of a ketoreductase polypeptide can be expressed as "percent conversion" of the substrate to the product.

[0047] "Thermostable" refers to a ketoreductase polypeptide that maintains similar activity (more than 60% to 80% for example) after exposure to elevated temperatures (*e.g.* 40-80°C) for a period of time (*e.g.* 0.5-24 hrs) compared to the untreated enzyme.

[0048] "Solvent stable" refers to a ketoreductase polypeptide that maintains similar activity (more than *e.g.*, 60% to 80%) after exposure to varying concentrations (*e.g.*, 5-99%) of solvent (isopropyl alcohol, tetrahydrofuran, 2-methyltetrahydrofuran, acetone, toluene, butylacetate, methyl tert-butylether, *etc.*) for a period of time (*e.g.*, 0.5-24 hrs) compared to the untreated enzyme.

[0049] "pH stable" refers to a ketoreductase polypeptide that maintains similar activity (more than *e.g.*, 60% to 80%) after exposure to high or low pH (*e.g.*, 4.5-6 or 8 to 12) for a period of time (*e.g.*, 0.5-24 hrs) compared to the untreated enzyme.

[0050] "Thermo- and solvent stable" refers to a ketoreductase polypeptide that is both thermostable and solvent stable.

[0051] "Derived from" as used herein in the context of engineered ketoreductase enzymes, identifies the originating ketoreductase enzyme, and/or the gene encoding such ketoreductase enzyme, upon which the engineering was based. For example, the engineered ketoreductase enzyme of SEQ ID NO:158 was obtained by artificially evolving, over multiple generations the gene encoding the *Lactobacillus kefir* ketoreductase enzyme of SEQ ID NO:4. Thus, this engineered ketoreductase enzyme is "derived from" the wild-type ketoreductase of SEQ ID NO:4.

[0052] "Hydrophilic Amino Acid or Residue" refers to an amino acid or residue having a side chain exhibiting a hydrophobicity of less than zero according to the normalized consensus hydrophobicity scale of Eisenberg *et al.*, 1984, *J. Mol. Biol.* 179:125-142. Genetically encoded hydrophilic amino acids include L-Thr (T), L-Ser (S), L-His (H), L-Glu (E), L-Asn (N), L-Gln (Q), L-Asp (D), L-Lys (K) and L-Arg (R).

[0053] "Acidic Amino Acid or Residue" refers to a hydrophilic amino acid or residue having a side chain exhibiting a pK value of less than about 6 when the amino acid is included in a peptide or polypeptide. Acidic amino acids typically have negatively charged side chains at physiological pH due to loss of a hydrogen ion. Genetically encoded acidic amino acids include L-Glu (E) and L-Asp (D).

[0054] "Basic Amino Acid or Residue" refers to a hydrophilic amino acid or residue having a side chain exhibiting a pK value of greater than about 6 when the amino acid is included in a peptide or polypeptide. Basic amino acids typically have positively charged side chains at physiological pH due to association with hydronium ion. Genetically encoded basic amino acids include L-Arg (R) and L-Lys (K).

[0055] "Polar Amino Acid or Residue" refers to a hydrophilic amino acid or residue having a side chain that is uncharged at physiological pH, but which has at least one bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Genetically encoded polar amino acids include L-Asn (N), L-Gln (Q), L-Ser (S) and L-Thr (T).

[0056] "Hydrophobic Amino Acid or Residue" refers to an amino acid or residue having a side chain exhibiting a hydrophobicity of greater than zero according to the normalized consensus hydrophobicity scale of Eisenberg *et al.*, 1984, *J. Mol. Biol.* 179:125-142. Genetically encoded hydrophobic amino acids include L-Pro (P), L-Ile (I), L-Phe (F), L-Val (V), L-Leu (L), L-Trp (W), L-Met (M), L-Ala (A) and L-Tyr (Y).

[0057] "Aromatic Amino Acid or Residue" refers to a hydrophilic or hydrophobic amino acid or residue having a side chain that includes at least one aromatic or heteroaromatic ring. Genetically encoded aromatic amino acids include L-Phe (F), L-Tyr (Y) and L-Trp (W). Although owing to the pKa of its heteroaromatic nitrogen atom L-His (H) it is sometimes classified as a basic residue, or as an aromatic residue as its side chain includes a heteroaromatic ring, herein histidine is classified as a hydrophilic residue or as a "constrained residue" (see below).

[0058] "Constrained amino acid or residue" refers to an amino acid or residue that has a constrained geometry. Herein, constrained residues include L-pro (P) and L-his (H). Histidine has a constrained geometry because it has a relatively small imidazole ring. Proline has a constrained geometry because it also has a five membered ring.

[0059] "Non-polar Amino Acid or Residue" refers to a hydrophobic amino acid or residue having a side chain that is uncharged at physiological pH and which has bonds in which the pair of electrons shared in common by two atoms is generally held equally by each of the two atoms (*i.e.*, the side chain is not polar). Genetically encoded non-polar amino acids include L-Gly (G), L-Leu (L), L-Val (V), L-Ile (I), L-Met (M) and L-Ala (A).

[0060] "Aliphatic Amino Acid or Residue" refers to a hydrophobic amino acid or residue having an aliphatic hydrocarbon side chain. Genetically encoded aliphatic amino acids include L-Ala (A), L-Val (V), L-Leu (L) and L-Ile (I).

[0061] "Cysteine". The amino acid L-Cys (C) is unusual in that it can form disulfide bridges with other L-Cys (C) amino acids or other sulfanyl- or sulfhydryl-containing amino acids. The "cysteine-like residues" include cysteine and other amino acids that contain sulfhydryl moieties that are available for formation of disulfide bridges. The ability of L-Cys (C) (and other amino acids with -SH containing side chains) to exist in a peptide in either the reduced free -SH or oxidized disulfide-bridged form affects whether L-Cys (C) contributes net hydrophobic or hydrophilic character to a

peptide. While L-Cys (C) exhibits a hydrophobicity of 0.29 according to the normalized consensus scale of Eisenberg (Eisenberg *et al.*, 1984, *supra*), it is to be understood that for purposes of the present disclosure L-Cys (C) is categorized into its own unique group.

[0062] "Small Amino Acid or Residue" refers to an amino acid or residue having a side chain that is composed of a total three or fewer carbon and/or heteroatoms (excluding the α-carbon and hydrogens). The small amino acids or residues may be further categorized as aliphatic, non-polar, polar or acidic small amino acids or residues, in accordance with the above definitions. Genetically-encoded small amino acids include L-Ala (A), L-Val (V), L-Cys (C), L-Asn (N), L-Ser (S), L-Thr (T) and L-Asp (D).

[0063] "Hydroxyl-containing Amino Acid or Residue" refers to an amino acid containing a hydroxyl (-OH) moiety. Genetically-encoded hydroxyl-containing amino acids include L-Ser (S) L-Thr (T) and L-Tyr (Y).

[0064] "Conservative" amino acid substitutions or mutations refer to the interchangeability of residues having similar side chains, and thus typically involves substitution of the amino acid in the polypeptide with amino acids within the same or similar defined class of amino acids. However, as used herein, conservative mutations do not include substitutions from a hydrophilic to hydrophilic, hydrophobic to hydrophobic, hydroxyl-containing to hydroxyl-containing, or small to small residue, if the conservative mutation can instead be a substitution from an aliphatic to an aliphatic, non-polar to non-polar, polar to polar, acidic to acidic, basic to basic, aromatic to aromatic, or constrained to constrained residue. Further, as used herein, A, V, L, or I can be conservatively mutated to either another aliphatic residue or to another non-polar residue. Table 1 below shows exemplary conservative substitutions.

Table 1: Conservative Substitutions

Residue	Possible Conservative Mutations
A, L, V, I	Other aliphatic (A, L, V, I) Other non-polar (A, L, V, I, G, M)
G, M	Other non-polar (A, L, V, I, G, M)
D, E	Other acidic (D, E)
K, R	Other basic (K, R)
P, H	Other constrained (P, H)
N, Q, S, T	Other polar (N, Q, S, T)
Y, W, F	Other aromatic (Y, W, F)
C	None

[0065] "Non-conservative substitution" refers to substitution or mutation of an amino acid in the polypeptide with an amino acid with significantly differing side chain properties. Non-conservative

substitutions may use amino acids between, rather than within, the defined groups listed above. In one embodiment, a non-conservative mutation affects (a) the structure of the peptide backbone in the area of the substitution (*e.g.*, proline for glycine) (b) the charge or hydrophobicity, or (c) the bulk of the side chain.

[0066] "Deletion" refers to modification to the polypeptide by removal of one or more amino acids from the reference polypeptide. Deletions can comprise removal of 1 or more amino acids, 2 or more amino acids, 5 or more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids, up to 10% of the total number of amino acids, or up to 20% of the total number of amino acids making up the reference enzyme while retaining enzymatic activity and/or retaining the improved properties of an engineered ketoreductase enzyme. Deletions can be directed to the internal portions and/or terminal portions of the polypeptide. In various embodiments, the deletion can comprise a continuous segment or can be discontinuous.

[0067] "Insertion" refers to modification to the polypeptide by addition of one or more amino acids from the reference polypeptide. In some embodiments, the improved engineered ketoreductase enzymes comprise insertions of one or more amino acids to the naturally occurring ketoreductase polypeptide as well as insertions of one or more amino acids to other improved ketoreductase polypeptides. Insertions can be in the internal portions of the polypeptide, or to the carboxy or amino terminus. Insertions as used herein include fusion proteins as is known in the art. The insertion can be a contiguous segment of amino acids or separated by one or more of the amino acids in the naturally occurring polypeptide.

[0068] "Different from" or "differs from" with respect to a designated reference sequence refers to difference of a given amino acid or polynucleotide sequence when aligned to the reference sequence. Generally, the differences can be determined when the two sequences are optimally aligned. Differences include insertions, deletions, or substitutions of amino acid residues in comparison to the reference sequence.

[0069] "Fragment" as used herein refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion, but where the remaining amino acid sequence is identical to the corresponding positions in the sequence. Fragments can be at least 14 amino acids long, at least 20 amino acids long, at least 50 amino acids long or longer, and up to 70%, 80%, 90%, 95%, 98%, and 99% of the full-length ketoreductase polypeptide.

[0070] "Isolated polypeptide" refers to a polypeptide which is substantially separated from other contaminants that naturally accompany it, *e.g.*, protein, lipids, and polynucleotides. The term embraces polypeptides which have been removed or purified from their naturally-occurring environment or expression system (*e.g.*, host cell or *in vitro* synthesis). The improved ketoreductase

enzymes may be present within a cell, present in the cellular medium, or prepared in various forms, such as lysates or isolated preparations. As such, in some embodiments, the improved ketoreductase enzyme can be an isolated polypeptide.

[0071] "Substantially pure polypeptide" refers to a composition in which the polypeptide species is the predominant species present (*i.e.*, on a molar or weight basis it is more abundant than any other individual macromolecular species in the composition), and is generally a substantially purified composition when the object species comprises at least about 50 percent of the macromolecular species present by mole or % weight. Generally, a substantially pure ketoreductase composition will comprise about 60 % or more, about 70% or more, about 80% or more, about 90% or more, about 95% or more, and about 98% or more of all macromolecular species by mole or % weight present in the composition. In some embodiments, the object species is purified to essential homogeneity (*i.e.*, contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species. In some embodiments, the isolated improved ketoreductases polypeptide is a substantially pure polypeptide composition.

[0072] "Stringent hybridization" is used herein to refer to conditions under which nucleic acid hybrids are stable. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature (T_m) of the hybrids. In general, the stability of a hybrid is a function of ion strength, temperature, G/C content, and the presence of chaotropic agents. The T_m values for polynucleotides can be calculated using known methods for predicting melting temperatures (see, *e.g.*, Baldino *et al.*, *Methods Enzymology* 168:761-777; Bolton *et al.*, 1962, *Proc. Natl. Acad. Sci. USA* 48:1390; Bresslauer *et al.*, 1986, *Proc. Natl. Acad. Sci USA* 83:8893-8897; Freier *et al.*, 1986, *Proc. Natl. Acad. Sci USA* 83:9373-9377; Kierzek *et al.*, *Biochemistry* 25:7840-7846; Rychlik *et al.*, 1990, *Nucleic Acids Res* 18:6409-6412 (erratum, 1991, *Nucleic Acids Res* 19:698); Sambrook *et al.*, *supra*); Suggs *et al.*, 1981, In *Developmental Biology Using Purified Genes* (Brown *et al.*, eds.), pp. 683-693, Academic Press; and Wetmur, 1991, *Crit Rev Biochem Mol Biol* 26:227-259. All publications incorporate herein by reference). In some embodiments, the polynucleotide encodes the polypeptide disclosed herein and hybridizes under defined conditions, such as moderately stringent or highly stringent conditions, to the complement of a sequence encoding an engineered ketoreductase enzyme of the present disclosure.

[0073] "Hybridization stringency" relates to such washing conditions of nucleic acids. Generally, hybridization reactions are performed under conditions of lower stringency, followed by washes of varying but higher stringency. The term "moderately stringent hybridization" refers to conditions that

permit target-DNA to bind a complementary nucleic acid that has about 60% identity, preferably about 75% identity, about 85% identity to the target DNA; with greater than about 90% identity to target-polynucleotide. Exemplary moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5× Denhart's solution, 5×SSPE, 0.2% SDS at 42°C., followed by washing in 0.2×SSPE, 0.2% SDS, at 42°C. "High stringency hybridization" refers generally to conditions that are about 10°C or less from the thermal melting temperature T_m as determined under the solution condition for a defined polynucleotide sequence. In some embodiments, a high stringency condition refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65°C. (*i.e.*, if a hybrid is not stable in 0.018M NaCl at 65°C, it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in conditions equivalent to 50% formamide, 5× Denhart's solution, 5×SSPE, 0.2% SDS at 42°C, followed by washing in 0.1×SSPE, and 0.1% SDS at 65°C. Other high stringency hybridization conditions, as well as moderately stringent conditions, are described in the references cited above.

[0074] "Heterologous" polynucleotide refers to any polynucleotide that is introduced into a host cell by laboratory techniques, and includes polynucleotides that are removed from a host cell, subjected to laboratory manipulation, and then reintroduced into a host cell.

[0075] "Codon optimized" refers to changes in the codons of the polynucleotide encoding a protein to those preferentially used in a particular organism such that the encoded protein is efficiently expressed in the organism of interest. Although the genetic code is degenerate in that most amino acids are represented by several codons, called "synonyms" or "synonymous" codons, it is well known that codon usage by particular organisms is nonrandom and biased towards particular codon triplets. This codon usage bias may be higher in reference to a given gene, genes of common function or ancestral origin, highly expressed proteins versus low copy number proteins, and the aggregate protein coding regions of an organism's genome. In some embodiments, the polynucleotides encoding the ketoreductases enzymes may be codon optimized for optimal production from the host organism selected for expression.

[0076] "Preferred, optimal, high codon usage bias codons" refers interchangeably to codons that are used at higher frequency in the protein coding regions than other codons that code for the same amino acid. The preferred codons may be determined in relation to codon usage in a single gene, a set of genes of common function or origin, highly expressed genes, the codon frequency in the aggregate protein coding regions of the whole organism, codon frequency in the aggregate protein coding regions of related organisms, or combinations thereof. Codons whose frequency increases with the level of gene expression are typically optimal codons for expression. A variety of methods are known

for determining the codon frequency (*e.g.*, codon usage, relative synonymous codon usage) and codon preference in specific organisms, including multivariat analysis, for example, using cluster analysis or correspondence analysis, and the effective number of codons used in a gene (see GCG CodonPreference, Genetics Computer Group Wisconsin Package; CodonW, John Peden, University of Nottingham; McInerney, J. O, 1998, Bioinformatics 14:372-73; Stenico *et al.*, 1994, Nucleic Acids Res. 222437-46; Wright, F., 1990, Gene 87:23-29). Codon usage tables are available for a growing list of organisms (see for example, Wada *et al.*, 1992, *Nucleic Acids Res.* 20:2111-2118; Nakamura *et al.*, 2000, *Nucl. Acids Res.* 28:292; Duret, *et al.*, *supra*; Henaut and Danchin, "*Escherichia coli* and *Salmonella*," 1996, Neidhardt, *et al.* Eds., ASM Press, Washington D.C., p. 2047-2066. The data source for obtaining codon usage may rely on any available nucleotide sequence capable of coding for a protein. These data sets include nucleic acid sequences actually known to encode expressed proteins (*e.g.*, complete protein coding sequences-CDS), expressed sequence tags (ESTS), or predicted coding regions of genomic sequences (see for example, Mount, D., *Bioinformatics: Sequence and Genome Analysis*, Chapter 8, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001; Uberbacher, E. C., 1996, *Methods Enzymol.* 266:259-281; Tiwari *et al.*, 1997, *Comput. Appl. Biosci.* 13:263-270).

[0077] "Control sequence" is defined herein to include all components, which are necessary or advantageous for the expression of a polypeptide of the present disclosure. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

[0078] "Operably linked" is defined herein as a configuration in which a control sequence is appropriately placed at a position relative to the coding sequence of the DNA sequence such that the control sequence directs the expression of a polynucleotide and/or polypeptide.

[0079] "Promoter sequence" is a nucleic acid sequence that is recognized by a host cell for expression of the coding region. The control sequence may comprise an appropriate promoter sequence. The promoter sequence contains transcriptional control sequences, which mediate the expression of the polypeptide. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

[0080] "Cofactor regeneration system" refers to a set of reactants that participate in a reaction that reduces the oxidized form of the cofactor (*e.g.*, NADP+ to NADPH). Cofactors oxidized by the ketoreductase-catalyzed reduction of the keto substrate are regenerated in reduced form by the cofactor regeneration system. Cofactor regeneration systems comprise a stoichiometric reductant that is a source of reducing hydrogen equivalents and is capable of reducing the oxidized form of the cofactor. The cofactor regeneration system may further comprise a catalyst, for example an enzyme catalyst that catalyzes the reduction of the oxidized form of the cofactor by the reductant. Cofactor regeneration systems to regenerate NADH or NADPH from NAD+ or NADP+, respectively, are known in the art and may be used in the methods described herein.

6.2 Ketoreductase Enzymes

[0081] The present disclosure provides engineered ketoreductase ("KRED") enzymes that are capable of stereoselectively reducing or converting the substrate 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to the product (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one and having an improved property when compared with the naturally-occurring, wild-type KRED enzyme of *L. kefir* (SEQ ID NO:4), *L. brevis* (SEQ ID NO:2), or *L. minor* (SEQ ID NO: 158), or when compared with other engineered ketoreductase enzymes (*e.g.* that of SEQ ID NO:8).

[0082] The engineered ketoreductase ("KRED") enzymes are also capable of stereoselectively reducing or converting the compound 1-(4-fluorophenyl)-3(R)-[3-oxo-3-(4-fluorophenyl)propyl)]-4(S)-(4-hydroxyphenyl)-2-azetidinone to the corresponding stereoisomeric alcohol product 1-(4-fluorophenyl)- 3(R)-[3(S)-hydroxy-3(4-fluoropheny l)-propy1)]-4(S)-(4-hydroxypheny1)-2-azetidinone and having an improved property when compared with the naturally-occurring, wild-type KRED enzyme of *L. kefir* (SEQ ID NO:4), *L. brevis* (SEQ ID NO:2), or *L. minor* (SEQ ID NO:158) or when compared with other engineered ketoreductase enzymes (*e.g.* that of SEQ ID NO:8).

[0083] Enzyme properties for which improvement is desirable include, but are not limited to, enzymatic activity, thermal stability, pH activity profile, cofactor requirements, refractoriness to inhibitors (*e.g.*, product inhibition), sterospecificity, stereoselectivity, and solvent stability. The improvements can relate to a single enzyme property, such as enzymatic activity, or a combination of different enzyme properties, such as enzymatic activity and stereoselectivity. For the polypeptides described herein, the amino acid sequence of the ketoreductase polypeptides have the requirement that: (1) the amino acid residue corresponding to residue position 145 of SEQ ID NO:2, 4, or 158 is serine and (2) the amino acid residue corresponding to residue position 190 of SEQ ID NO:2, 4, or 158 is cysteine.

[0084] In some embodiments, as noted above, the engineered ketoreductase with improved enzyme activity is described with reference to *Lactobacillus kefir* ketoreductase of SEQ ID NO:4, *Lactobacillus brevis* ketoreductase of SEQ ID NO:2, or *Lactobacillus minor* of SEQ ID NO:158. The amino acid residue position is determined in both ketoreductases beginning from the initiating methionine (M) residue (*i.e.*, M represents residue position 1), although it will be understood by the skilled artisan that this initiating methionine residue may be removed by biological processing machinery, such as in a host cell or *in vitro* translation system, to generate a mature protein lacking the initiating methionine residue. The amino acid residue position at which a particular amino acid or amino acid change is present is sometimes describe in terms "X*n*" , or "position *n*", where *n* refers to the residue position. Where the amino acid residues at the same residue position differ between the ketoreductases, the different residues are denoted by an "/" with the arrangement being, for example, "kefir residue/brevis residue/minor." A substitution mutation, which is a replacement of an amino acid residue in a corresponding residue of a reference sequence, for example the wildtype ketoreductases of SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:158 with a different amino acid residue is denoted by the symbol "→".

[0085] Herein, mutations are sometimes described as a mutation "to a" type of amino acid. For example, residue 211 can be mutated "to a" basic residue. But the use of the phrase "to a" does not exclude mutations from one amino acid of a class to another amino acid of the same class. For example, residue 211 can be mutated from a lysine to an arginine.

[0086] The polynucleotide sequence encoding the naturally occurring ketoreductase of Lactobacillus kefir and Lactobacillus brevis (also referred to as "alcohol dehydrogenase" or "ADH"), and thus the corresponding amino acid sequences, are available from Genbank accession no. AAP94029 GI:33112056 for *Lactobacillus kefir*, Genbank accession no. CAD66648 GI:28400789 for *Lactobacillus brevis*, and U.S. Pat. Appl. No. 20040265978 or SEQ ID NO:158 for *Lactobacillus minor*.

[0087] In some embodiments, the ketoreductase polypeptides herein can have a number of modifications to the reference sequence (*e.g.*, naturally occurring polypeptide or an engineered polypeptide) to result in an improved ketoreductase property. In such embodiments, the number of modifications to the amino acid sequence can comprise one or more amino acids, 2 or more amino acids, 3 or more amino acids, 4 or more amino acids, 5 or more amino acids, 6 or more amino acids, 8 or more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids, up to 10% of the total number of amino acids, up to 20% of the total number of amino acids, or up to 30% of the total number of amino acids of the reference enzyme sequence. In some embodiments, the number of modifications to the naturally occurring polypeptide or an engineered polypeptide that

produces an improved ketoreductase property may comprise from about 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 modifications of the reference sequence. The modifications can comprise insertions, deletions, substitutions, or combinations thereof.

[0088] In some embodiments, the modifications comprise amino acid substitutions to the reference sequence. Substitutions that can produce an improved ketoreductase property may be at one or more amino acids, 2 or more amino acids, 3 or more amino acids, 4 or more amino acids, 5 or more amino acids, 6 or more amino acids, 8 or more amino acids, 10 or more amino acids, or 20 or more amino acids, up to 10% of the total number of amino acids, up to 20% of the total number of amino acids, or up to 30% of the total number of amino acids of the reference enzyme sequence. In some embodiments, the number of substitutions to the naturally occurring polypeptide or an engineered polypeptide that produces an improved ketoreductase property can comprise from about 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 amino acid substitutions of the reference sequence.

[0089] In some embodiments, the improved property, as compared to wild-type or another engineered polypeptide, of the ketoreductase polypeptide is with respect to an increase of its stereoselectivity *i.e.*, herein, an increase in the stereomeric excess of the product, for reducing or converting the substrate 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to the product (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one. In some embodiments, the improved property of the ketoreductase polypeptide is with respect to an increase in its ability to convert or reduce a greater percentage of the substrate to the product. In some embodiments, the improved property of the ketoreductase polypeptide is with respect to an increase in its rate of conversion of the substrate to the product. This improvement in enzymatic activity can be manifested by the ability to use less of the improved polypeptide as compared to the wild-type or other reference sequence (for example, SEQ ID NO:8) to reduce or convert the same amount of product. In some embodiments, the improved property of the ketoreductase polypeptide is with respect to its stability or thermostability. In some embodiments, the ketoreductase polypeptide has more than one improved property.

[0090] In some embodiments, the ketoreductase polypeptide of the disclosure is capable of converting the substrate 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to the product (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one, with a percent stereomeric excess of at least about 90% and at a rate that is improved over the amino acid sequence of SEQ ID NO:8. Exemplary polypeptides with such properties include, but are not limited to, polypeptides which comprise an amino acid sequence

corresponding to SEQ ID NO: 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126. Because the reference polypeptide having the amino acid sequence of SEQ ID NO:8 is capable of converting the substrate to the product at a rate (for example, 4% of 1 g/L substrate converted to product in 24 hours with about 5 g/L of the KRED) and with a steroselectivity (94% stereomeric excess) that is improved over wild-type (SEQ ID NO:4), the polypeptides herein that are improved over SEQ ID NO:8 are also improved over wild-type.

[0091] In some embodiments, the ketoreductase polypeptide is capable of converting the substrate 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to the product (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one, with a percent stereomeric excess of at least about 99% and at a rate that is at least about 5 times improved over a reference polypeptide having the amino acid sequence of SEQ ID NO: 8. Exemplary polypeptides with such properties include, but are not limited to, polypeptides which comprise an amino acid sequence corresponding to SEQ ID NO: 42, 44, 46, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[0092] In some embodiments, the ketoreductase polypeptide is capable of converting the substrate 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to the product (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one, with a percent stereomeric excess of at least about 99% and at a rate that is at least about 120 times or more improved over a reference polypeptide having the amino acid sequence of SEQ ID NO:8. Exemplary polypeptides with such properties include, but are not limited to, polypeptides which comprise an amino acid sequence corresponding to SEQ ID NO: 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[0093] In some embodiments, the ketoreductase polypeptide is capable of converting the substrate 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to the product (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one, with a percent stereomeric excess of at least about 99% and at a rate that is at least about 3000 times or more improved over a reference polypeptide having the amino acid sequence of SEQ ID NO:8. Exemplary polypeptides with such properties include, but are not limited to, polypeptides which comprise an amino acid sequence corresponding to SEQ ID NO: 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[0094] In some embodiments, the ketoreductase polypeptide is capable of converting the substrate 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to the product (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one, with a percent stereomeric excess of at least about 99% and at a rate that is at least about 7000 times or more improved over a reference polypeptide having the amino acid sequence of SEQ ID NO:8. Exemplary polypeptides with such properties include, but are not limited to, polypeptides which comprise an amino acid sequence corresponding to SEQ ID NO: 102, 108, 120, 122, 124, and 126.

[0095] In some embodiments, the ketoreductase polypeptides of the disclosure comprise highly stereoselective ketoreductase polypeptides that can reduce the substrate to the product in greater than about 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% stereomeric excess. Exemplary ketoreductase polypeptides with such high stereoselectivity include, but are not limited to, the polypeptides comprising the amino acid sequences corresponding to SEQ ID NO: 42, 44, 46, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[0100] Tables 2, 3 and 4 below provide a list of the SEQ ID NOs disclosed herein with associated activities. The sequences below are based on the wild-type *L. kefir* ketoreductase sequences (SEQ ID NO: 3 and 4) unless otherwise specified. In tables below, each row lists two SEQ ID NOs, where the odd number refers to the nucleotide sequence that codes for the amino acid sequence provided by the even number. The column listing the number of mutations (i.e., residue changes) refers to the number of amino acid substitutions as compared to the *L. kefir* KRED amino acid sequence of SEQ ID NO:4. Each Table is followed by a caption indicating the meaning of the symbols "+" "++" "+++" and "++++" in each context.

[0101] Table 2

Table 2: Activity of Various KREDs

SEQ ID NO:	Residue Changes Relative to SEQ ID NO:4	Number of Changes Relative to L Kefir	Conversion[a]	%DE[b]
5/6	Y190C;	1	+	-
7/8	G7S; R108H; G117S; E145S; N157T; Y190C; K211R; I223V	8	+++	++
9/10	F147L; Y190P; V196L	3	+	+

13/14	H40R; A94G; S96V; E145F; F147M; Y190P; V196L; L199W; I226V; Y249W	10	+	++
27/28	E145A; F147L; Y190C	3	+++	++
29/30	F147L; L153G; Y190P	3	+++	-
31/32	F147L; Y190P	2	++	-
33/34	E145S; F147L; Y190P	3	+++	+
35/36	E145Q; F147L; Y190A	3	++	-
37/38	F147L; Y190P; K211L	3	++	-
39/40	F147L; L153Q; Y190P	3	+++	-

a - + indicates <10% conversion of substrate to product; ++ indicates 10-60% conversion; +++ indicates >60% conversion

b - - indicates R selectivity; + indicates <50% *S*, *S* – diastereomeric product; ++ indicates >50% *S*, *S*-diastereomeric product

[0102] The following Table 3 and Table 4 show the activity profiles of various ketoreductases.

Table 3: List of Sequences and Corresponding Activity Improvement

SEQ ID NO	**Residue Changes**	**Number of Changes Relative to the *L. kefir* (SEQ ID NO:4)**	**% Conversion** [a]	**% DE** [b]
7/8	G7S; R108H; G117S; E145S; N157T; Y190C; K211R; I223V	8	+	+
11/12	A94G; S96V; E145L; L153T; Y190P; V196L; I226V; Y249W;	8	+	+

Table 3: List of Sequences and Corresponding Activity Improvement

SEQ ID NO	Residue Changes	Number of Changes Relative to the *L. kefir* (SEQ ID NO:4)	% Conversion [a]	% DE [b]
15/16	H40R; A94G; S96V; E145F; F147M; Y190P; V196L; M206F; I226V; Y249W	10	+	+
17/18	A94G; S96V; E145F; F147M; L153T; Y190P; V196L; I226V; Y249W	9	+	++
19/20	A94G; S96V; E145F; F147M; L153T; Y190P; L195M; V196L; L199Y; I226V; Y249W	11	+	++
21/22	A94G; S96V; E145F; F147M; T152S; L153T; Y190P; V196L; I226V; Y249W	10	+	++
23/24	A94G; S96V; E145F; F147M; T152S; Y190P; L195M; V196L; M206F; I226V; Y249W	11	+	++
25/26	H40R; A94G; S96V; E145F; F147M; L153T; Y190P; V196L; I226V; Y249W	10	+	++
41/42	G7S; A94G; R108H; G117S; E145S; N157T; Y190C; K211R; I223V	9	++	+

Table 3: List of Sequences and Corresponding Activity Improvement

SEQ ID NO	Residue Changes	Number of Changes Relative to the *L. kefir* (SEQ ID NO:4)	% Conversion [a]	% DE [b]
43/44	G7S; S96Q; R108H; G117S; E145S; N157T; Y190C; K211R; I223V	9	++	++
45/46	G7S; R108H; G117S; E145S; N157T; Y190C; L199D; K211R; I223V	9	++	++
47/48	G7S; R108H; G117S; E145S; N157T; Y190C; A202G; K211R; I223V	9	++	+
49/50	G7S; R108H; V113A; G117S; E145S; N157T; Y190C; L199D; K211R; I223V	10	++	++
51/52	G7S; R108H; G117S; E145S; T152K; N157T; Y190C; L199D; K211R; I223V	10	+++	++
53/54	G7S; R108H; G117S; E145S; T152M; N157T; Y190C; L199D; K211R; I223V	10	+++	++
55/56	G7S; A94S; S96Q; R108H; G117S; E145S; N157T; Y190C; L199D; K211R; I223V	11	+++	++
57/58	G7S; R108H; G117S;	10	+++	++

Table 3: List of Sequences and Corresponding Activity Improvement

SEQ ID NO	Residue Changes	Number of Changes Relative to the *L. kefir* (SEQ ID NO:4)	% Conversion [a]	% DE [b]
	E145S; N157T; Y190C; P194Q; L199D; K211R; I223V			
59/60	G7S; A94S; S96Q; R108H; G117S; E145S; T152K; N157T; Y190C; L199D; K211R; I223V	12	+++	++
61/62	G7S; A94S; S96Q; R108H; G117S; E145S; T152M; N157T; Y190C; L199D; K211R; I223V;	12	+++	++
63/64	G7S; R108H; G117S; E145S; F147L; T152M; N157T; Y190C; L199D; K211R; I223V;	11	+++	++
65/66	G7S; S96N; R108H; G117S; E145S; T152M; N157T; Y190C; L199D; K211R; I223V	11	+++	++
67/68	G7S; R108H; G117S; E145S; T152M; N157T; Y190C; P194R; L199D; K211R; I223V	11	+++	++
69/70	G7S; S96Q; R108H; G117S; E145S; T152M; N157T; Y190C; L199D; K211R; I223V;	11	+++	++

Table 3: List of Sequences and Corresponding Activity Improvement

SEQ ID NO	Residue Changes	Number of Changes Relative to the *L. kefir* (SEQ ID NO:4)	% Conversion [a]	% DE [b]
71/72	G7S; S96Q; R108H; G117S; E145S; T152M; N157T; Y190C; P194R; L199D; K211R; I223V	12	+++	++
73/74	G7S; S96T; R108H; G117S; E145S; T152M; N157T; Y190C; P194R; L199D; K211R; I223V	12	+++	++
75/76	G7S; D25T; D75N; S96Q; R108H; G117S; E145S; T152M; N157T; Y190C; P194R; L199D; K211R; I223V	14	++++	++
77/78	G7S; S96Q; R108H; G117S; E145S; T152M; Y190C; L199D; K211R; I223V	10	++++	++
79/80	G7S; H40R; S96Q; R108H; G117S; E145S; T152M; N157T; Y190C; L199D; K211R; I223V;	12	+++	++
81/82	G7S; D25T; V95L; S96Q; R108H; G117S; E145S; T152M; L176V; Y190C; D198E; L199D; K211R; I223V	14	++++	++
83/84	G7S; D25T; V95L;	14	++++	++

Table 3: List of Sequences and Corresponding Activity Improvement

SEQ ID NO	Residue Changes	Number of Changes Relative to the *L. kefir* (SEQ ID NO:4)	% Conversion [a]	% DE [b]
	S96Q; R108H; G117S; E145S; T152M; L176V; Y190C; D197E; L199D; K211R; I223V			
89/90	G7S; S96Q; E145S; T152M; Y190C; L199D; K211R	7	++++	++
93/94	G7S; G53D; S96Q; R108H; G117S; E145S; T152M; V163I; Y190C; L199D; K211R; I223V	12	++++	++

a. + indicates < 50 mg product/g enzyme; ++ 50-1000 mg product/g enzyme; +++ indicates > 1000 mg product/g enzyme

b. + indicates 90-99 % *S*, *S*-diastereomeric product; ++ indicates >99 % *S*, *S*-diastereomeric product

[0103] Table 4

Table 4: List of Sequences and Corresponding Activity Improvement

SEQ ID NO:	Residue Changes	Number of Changes Relative to the *L. kefir* (SEQ ID NO:4)	% Conversion [a]	% DE [b]
75/76	G7S; D25T; D75N; S96Q; R108H; G117S; E145S; T152M; N157T; Y190C; P194R; L199D;	14	++	

	K211R; I223V			
81/82	G7S; D25T; V95L; S96Q; R108H; G117S; E145S; T152M; L176V; Y190C; D198E; L199D; K211R; I223V	14	++	++
83/84	G7S; D25T; V95L; S96Q; R108H; G117S; E145S; T152M; L176V; Y190C; D197E; L199D; K211R; I223V;	14	++	++
85/86	G7S; D25T; V95M; S96Q; R108H; G117S; E145S; T152M; L176V; Y190C; P194R; L199D; K211R; I223V	14	++	++
87/88	G7S; S96Q; E145S; T152M; Y190C; L199D; K211R; I223V	8	++	++
89/90	G7S; S96Q; E145S; T152M; Y190C; L199D; K211R;	7	++	++
91/92	G7S;S96Q;R108H;E145S ;T152M; Y190C;L199D;K211R; I223V;	9	++	++
93/94	G7S; G53D; S96Q; R108H; G117S; E145S; T152M; V163I; Y190C; L199D; K211R; I223V	12	++	++
95/96	G7S; V95L; S96Q; R108H; G117S; E145S;	11	++	++

	T152M; Y190C; L199D; K211R; I223V;			
97/98	G7S; S96Q; R108N; G117S; E145S; T152M; Y190C; L199D; K211R; I223V	10	++	++
99/100	G7S; S96Q; D101G; R108H; G117S; E145S; F147L; T152M; Y190C; L199D; K211R; I223V	12	++	++
101/102	G7S; S96Q; R108H; L111M; G117S; E145S; T152M; Y190C; L199D; K211R; I223V	11	+++	++
103/104	G7S; S96Q; R108H; G117S; E145S; T152M; Y190C; L199D; K211R; I223V; T250I;	11	++	++
105/106	G7S; E29G; S96Q; D101N; R108H; G117S; E145S; T152M; Y190C; L199D; E200P; K211R; I223V;	13	++	++
107/108	G7S; L17Q; S96Q; R108H; G117S; E145S; T152M; Y190C; L199D; K211R; I223V	11	+++	++
109/110	G7S; S96Q; R108H; S112D; G117S; E145S; T152M; Y190C; D198G; L199D; K211R; I223V	12	++	++
111/112	G7S; S96Q; R108S;	10	++	++

	G117S; E145S; T152M; Y190C; L199D; K211R; I223V			
113/114	D3N; G7S; L17Q; D42G; S96Q; R108H; Q127R; E145S; T152M; L176V; Y190C; P194R; L199D; E200P; K211R; I223V	16	++	++
115/116	D3N; G7S; L17Q; L21F; S96Q; R108H; E145S; F147L; T152M; L176V; Y190C; L199D; K211R; I223V;	14	++	++
117/118	D3N; G7S; L17Q; E29A; D42G; S96Q; E105G; R108H; G117S; E145S; T152M; Y190C; D197V; D198K; L199D; E200P; K211R; I223V;	18	++	++
119/120	G7S; L17Q; D42G; S96Q; R108H; G117S; E145S; T152M; V163I; Y190C; D198K; L199D; K211R; I223V	14	+++	++
121/122	G7S; L17Q; E29A;S96Q; R108H;G117S; E145S; T152M; V163I; Y190C; D198K; L199D; E200P; K211R	14	+++	++
123/124	G7S; L17Q; D42G; S96Q; R108H; G117S; E145S; F147L; T152M; V163I; L176V; Y190C;	16	+++	++

	D198K; L199D; K211R; I223V			
125/126	G7S; L17Q; E29A; S96Q; R108H; G117S; E145S; F147L; T152M; V163I; L176V; Y190C; D198K; L199D; E200P; K211R; I223V	17	+++	++
a. + indicates < 1 g product/g enzyme/hr; ++ indicates 1 – 2.5 g product/g enzyme/hr; and +++ indicates > 2.5 g product/g enzyme/hr b. + indicates 90-99 % S, S-diastereomeric product; ++ indicates >99 % S, S-diastereomeric product				

[0104] In some embodiments, the ketoreductase polypeptides herein comprises an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical as compared a reference sequence comprising the sequence of SEQ ID NO:128, 130, or 160, with the proviso that the ketoreductase polypeptide comprises an amino acid sequence in which the amino acid residue corresponding to residue position 145 is a polar residue, and the amino acid residue corresponding to residue position 190 is a cysteine. The polypeptides of SEQ ID NO: 128, 130, and 160 are variants of the *L.brevis, L. kefir,* and *L. minor* ketoreductases, respectively, each having the sequence substitutions: E145S and Y190C. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence in which the amino acid residue corresponding to residue position 145 is serine, and the amino acid residue corresponding to position 190 is cysteine. In some embodiments, the ketoreductase polypeptides can have one or more residue differences at other amino acid residues as compared to the reference sequence. The differences can include substitutions, deletions, and insertions as compared to any of the reference sequences of SEQ ID NO:128, 130, or 160. The differences can be non-conservative substitutions, conservative substitutions, or a combination of non-conservative and conservative substitutions. In some embodiments, these ketoreductase polypeptides can have optionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 differences at other amino acid residues. In some embodiments, the number of differences with the reference sequence can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations as compared to the reference sequence.

[0105] In some embodiments, an improved ketoreductase polypeptide comprises an amino acid sequence based on the sequence formulas as laid out in SEQ ID NO:161, 162, or 163, or a region thereof, such as residues 90-211. The sequence formula of SEQ ID NO:161 is based on the amino acid sequence of the *Lactobacillus brevis* ketoreductase, as represented by SEQ ID NO:2. The sequence formula of SEQ ID NO:162 is based on the amino acid sequence of the *Lactobacillus kefir* ketoreductase, as represented by SEQ ID NO:4. The sequence formula of SEQ ID NO:163 is based on the amino acid sequence of the *Lactobacillus minor* ketoreductase, as represented by SEQ ID NO:158. In some embodiments, the ketoreductase polypeptide based on the sequence formulas of SEQ ID NO:161, 162, or 163 can comprise an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:128, 130, or 160, with the proviso that the ketoreductase polypeptide has an amino acid sequence in which the residue corresponding to X145 is a polar residue, particularly serine, and the amino acid residue corresponding to X190 is a cysteine.

[0106] In some embodiments, an improved ketoreductase polypeptide comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163 in which the amino acid sequence has the specified features for residues X145 and X190 as described herein, and wherein the polypeptide can further include one or more features selected from the following: residue corresponding to X3 is an acidic, a polar, or hydrophilic residue; residue corresponding to X7 is a non-polar or polar residue; residue corresponding to X17 is a non-polar, aliphatic or polar residue; residue corresponding to X21 is a non-polar, aromatic, or hydrophobic residue; residue corresponding to X25 is an acidic, non-polar or polar residue; residue corresponding to X29 is an acidic, aliphatic or non-polar residue; residue corresponding to X40 is a constrained, basic, or hydrophilic residue; residue corresponding to X42 is an acidic or a non-polar residue; residue corresponding to X53 is a non-polar or an acidic residue; residue corresponding to X75 is an acidic or polar residue; residue corresponding to X94 is a non-polar or a polar residue; residue corresponding to X95 is a non-polar or aliphatic residue; residue corresponding to X96 is a polar residue; residue corresponding to X101 is an acidic, non-polar, or a polar residue; residue corresponding to X105 is an acidic or non-polar residue; residue corresponding to X108 is a hydrophilic, polar or constrained residue; residue corresponding to X111 is a non-polar or aliphatic residue; residue corresponding to X112 is an acidic or polar residue; residue corresponding to X113 is a non-polar or aliphatic residue; residue corresponding to X117 is a non-polar or polar residue; residue corresponding to X127 is a basic or polar residue; residue corresponding to X147 is a non-polar, aromatic, or hydrophobic residue; residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue; residue corresponding to X157 is a polar residue; residue corresponding to X163 is a non-polar or aliphatic residue; residue corresponding to X176 is a non-polar or aliphatic residue; residue corresponding to X194 is a constrained, basic, or

polar residue; residue corresponding to X197 is a hydrophilic, acidic, basic, aliphatic or non-polar residue; residue corresponding to X198 is an acidic, basic, hydrophilic, or non-polar residue; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue; residue corresponding to X200 is an acidic or constrained residue; residue corresponding to X202 is a non-polar or aliphatic residue; residue corresponding to X206 is a non-polar, aromatic, or hydrophobic residue; residue corresponding to X211 is a basic residue; residue corresponding to X223 is a non-polar or aliphatic residue; and residue corresponding to X250 is a polar or a non-polar residue. In some embodiments, the polypeptides comprising an amino acid sequence that corresponds to the sequence formulas provided in SEQ ID NO:161, 162 or 163 (or region thereof) can have additionally one or more of the residues not specified by an X to be mutated. In some embodiments, the mutations can be 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 mutations at other amino acid residues not defined by X above. In some embodiments, the number of mutations can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 mutations at other amino acid residues. In some embodiments, the mutations comprise conservative mutations.

[0107] In some of the embodiments above, the ketoreductase polypeptides comprising an amino acid sequence that corresponds to the sequence formula as laid out in SEQ ID NO:161, 162 or 163 (or region thereof) can have one or more conservatively mutations as compared to the reference sequence of SEQ ID NO:128, 130, or 160. Exemplary conservative mutations include amino acid replacements such as, but not limited to: the replacement of residue corresponding to X95 (valine) with another non-polar amino acid, *e.g.*, alanine, leucine, isoleucine, glycine, or methionine; the replacement of residue corresponding to X96 (serine) with another polar amino acid, *e.g.*, asparagine, glutamine, or threonine; the replacement of residue corresponding to X111 (leucine) with another non-polar amino acid, *e.g.*, alanine, leucine, isoleucine, glycine, or methionine; the replacement of residue corresponding to X113 (valine) with another aliphatic amino acid, *e.g.*, alanine, leucine, or isoleucine; the replacement of residue corresponding to X157 (asparagine) with another polar amino acid, *e.g.*, glutamine, serine, or threonine; the replacement of residue corresponding to X163 (valine) with another aliphatic amino acid, *e.g.*, alanine, leucine, or isoleucine; the replacement of residue corresponding to X176 (leucine) with another aliphatic amino acid, *e.g.*,, alanine, valine, and isoleucine; the replacement of residue corresponding to X202 (alanine) with another non-polar amino acid, *e.g.*, alanine, leucine, isoleucine, glycine, or methionine; the replacement of residue corresponding to X211 (lysine) with another basic amino acid, *e.g.*, arginine; the replacement of residue corresponding to X223 (isoleucine) with another aliphatic amino acid, *e.g.*, alanine, leucine, or valine.

[0108] In some embodiments, an improved ketoreductase polypeptide comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163 in which the amino acid sequence has the specified features for residues X145 and X190 as described herein, and wherein the polypeptide can further include one or more features selected from the following: residue corresponding to X3 is aspartic acid, glutamic acid, serine, threonine, asparagine, or glutamine, particularly asparagine; residue corresponding to X7 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly serine; residue corresponding to X17 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly glutamine; residue corresponding to X21 is glycine, methionine, alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, or tryptophan, particularly phenylalanine; residue corresponding to X25 is aspartic acid, glutamic acid, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, isoleucine, particularly threonine; residue corresponding to X29 is aspartic acid, glutamine acid, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine or alanine; residue corresponding to X40 is histidine, lysine, arginine, serine, threonine, asparagine, or glutamine, particularly arginine; residue corresponding to X42 is aspartic acid, glutamic acid, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine; residue corresponding to X53 is glycine, methionine, alanine, valine, leucine, isoleucine, aspartic acid, glutamic acid, particularly aspartic acid; residue corresponding to X75 is aspartic acid, glutamic acid, serine, threonine, asparagine, or glutamine, particularly arginine; residue corresponding to X94 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly asparagine, glycine, or serine; residue corresponding to X95 is a glycine, methionine, alanine, valine, leucine, or isoleucine, particularly leucine or methionine; residue corresponding to X96 is serine, threonine, asparagine, glutamine, particularly glutamine, asparagine, or threonine; residue; residue corresponding to X101 is aspartic acid, glutamic acid, serine, threonine, asparagine, glutamine, or glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine or asparagine; residue corresponding to X105 is glutamic acid, aspartic acid, glycine, methionine, alanine, valine, leucine, isoleucine, particularly glycine; residue corresponding to X108 arginine, lysine, serine, threonine, asparagine, glutamine, histidine, particularly histidine or serine; residue corresponding to X111 is glycine, methionine, alanine, valine, leucine, or isoleucine, particularly methionine; or aliphatic residue; residue corresponding to X112 is aspartic acid, glutamic acid, serine, threonine, asparagine, glutamine, particularly aspartic acid; residue corresponding to X113 is an glycine, methionine, alanine, valine, leucine, isoleucine, particularly alanine; residue corresponding to X117 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly serine; residue corresponding to X127 is lysine, arginine, serine, threonine, asparagine, or glutamine, particularly arginine; residue corresponding to X147 is glycine, methionine,

alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, tryptophan, particularly leucine; residue corresponding to X152 is glycine, methionine, valine, leucine, isoleucine, arginine, lysine, serine threonine, asparagine, or glutamine, particularly methionine or lysine; residue corresponding to X157 is a serine, threonine, asparagine, and glutamine, particularly threonine; residue corresponding to X163 is a glycine, methionine, alanine, valine, leucine, or isoleucine, particularly isoleucine; residue corresponding to X176 is glycine, methionine, alanine, valine, leucine, or isoleucine, particularly valine; residue corresponding to X194 is proline, arginine, lysine, serine, threonine, asparagine, glutamine, particularly arginine or glutamine; residue corresponding to X197 is aspartic acid, glutamic acid, arginine, lysine, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, isoleucine, particularly valine or glutamic acid; residue corresponding to X198 is aspartic acid, glutamic acid, arginine, lysine, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine, glutamic acid, or lysine; residue corresponding to X199 is an aspartic acid, glutamic acid, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly aspartic acid; residue corresponding to X200 is an aspartic acid, glutamic acid, or proline, particularly proline; residue corresponding to X202 is glycine, methionine, alanine, valine, leucine, isoleucine, particularly glycine; residue corresponding to X206 is a glycine, methionine, alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, tryptophan, particularly glycine; residue corresponding to X211 is a arginine or lysine; residue corresponding to X223 is glycine, methionine, alanine, valine, leucine, or isoleucine, particularly valine; and residue corresponding to X250 is serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, isoleucine, particularly isoleucine. In some embodiments, the polypeptides comprising an amino acid sequence that corresponds to the sequence formulas of SEQ ID NO:161, 162 or 163 (or region thereof) can have additionally one or more of the residues not specified by an X to be mutated as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the mutations can be 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 mutations at other amino acid residues not defined by X above. In some embodiments, the number of mutations can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 mutations at other amino acid residues. In some embodiments, the mutations comprise conservative mutations.

[0109] In some embodiments, an improved ketoreductase polypeptide comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163 in which the amino acid sequence has the specified features for residues X145 and X190 as described herein, and wherein the polypeptide can further include one or more or at least all of the features selected from the following: the residue corresponding to X7 is a non-polar or polar residue; residue corresponding to X108 is a hydrophilic, polar or constrained residue; residue corresponding to X117 is a non-polar or a polar

residue; residue corresponding to X152 is a non-polar, basic, or hydrophilic residue; and residue corresponding to X199 is an acidic, aliphatic, or non-polar residue. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0110] In some embodiments, an improved ketoreductase polypeptide comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163 in which the amino acid sequence has the specified features for residues X145 and X190 as described herein, and wherein the polypeptide can further include one or more or at least all of the features selected from the following: the residue corresponding to X7 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly serine; residue corresponding to X108 is arginine, lysine, serine, threonine, asparagine, glutamine, histidine, particularly histidine or serine; residue corresponding to X117 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly serine; residue corresponding to X152 is glycine, methionine, valine, leucine, isoleucine, arginine, lysine, serine threonine, asparagine, or glutamine, particularly methionine or lysine; and residue corresponding to X199 is aspartic acid, glutamic acid, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly aspartic acid. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0111] In some embodiments, an improved ketoreductase polypeptide comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163 in which the amino acid sequence has the specified features for residues X145 and X190 as described herein, and wherein the polypeptide can further include one or more or at least all of the features selected from the following: residue corresponding to X3 is an acidic, polar, or hydrophilic residue; residue corresponding to X17 is a non-polar, aliphatic or polar residue; residue corresponding to X25 is an acidic, non-polar or polar residue; residue corresponding to X42 is an acidic or non-polar residue; residue corresponding to X94 is a non-polar or a polar residue; residue corresponding to X194 is a constrained, basic, or polar residue; residue corresponding to X198 is an acidic, basic, hydrophilic, or non-polar residue; and residue corresponding to X200 is an acidic or a constrained residue. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0112] In some embodiments, an improved ketoreductase polypeptide comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163 in which the amino acid sequence has the specified features for residues X145 and X190 as described herein, and wherein the polypeptide can further include one or more or at least all of the features selected from the following: residue corresponding to X3 is aspartic acid, glutamic acid, serine, threonine, asparagine, or glutamine, particularly asparagine; residue corresponding to X17 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly glutamine; residue corresponding to X25 is aspartic acid, glutamic acid, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, isoleucine, particularly threonine; residue corresponding to X42 is aspartic acid, glutamic acid, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine; residue corresponding to X94 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly asparagine, glycine, or serine; residue corresponding to X194 is proline, arginine, lysine, serine, threonine, asparagine, glutamine, particularly arginine or glutamine; residue corresponding to X198 is aspartic acid, glutamic acid, arginine, lysine, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine,

or isoleucine, particularly glycine, glutamic acid, or lysine; residue corresponding to X200 is aspartic acid, glutamic acid, or proline, particularly proline. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0113] In some embodiments, an improved ketoreductase polypeptide comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163 in which the amino acid sequence has the specified features for residues X145 and X190 as described herein, and wherein the polypeptide can further include one or more or at least all of the features selected from the following: residue corresponding to X3 is an acidic, polar, or hydrophilic residue; residue corresponding to X7 is a non-polar or polar residue; residue corresponding to X17 is a non-polar, aliphatic or polar residue; residue corresponding to X25 is an acidic, non-polar or polar residue; residue corresponding to X42 is an acidic or non-polar residue; residue corresponding to X94 is a non-polar or a polar residue; residue corresponding to X108 is a hydrophilic, polar or constrained residue; residue corresponding to X117 is a non-polar or a polar residue; residue corresponding to X152 is a non-polar, basic, or hydrophilic residue; residue corresponding to X194 is a constrained, basic, or polar residue; residue corresponding to X198 is an acidic, basic, hydrophilic, or non-polar residue; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue; residue corresponding to X200 is an acidic or constrained residue. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0114] In some embodiments, an improved ketoreductase polypeptide comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163 in which the amino acid sequence has the specified features for residues X145 and X190 as described herein, and wherein the polypeptide can further include one or more or at least all of the features selected from the following: residue corresponding to X3 is aspartic acid, glutamic acid, serine, threonine, asparagine, or glutamine, particularly asparagine; residue corresponding to X7 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly serine; residue corresponding to X17 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly glutamine; residue corresponding to X25 is aspartic acid, glutamic acid, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, isoleucine, particularly threonine; residue corresponding to X42 is aspartic acid, glutamic acid, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine; residue corresponding to X94 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly asparagine, glycine, or serine; residue corresponding to X108 is arginine, lysine, serine, threonine, asparagine, glutamine, histidine, particularly histidine or serine; residue corresponding to X117 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly serine; residue corresponding to X152 is glycine, methionine, valine, leucine, isoleucine, arginine, lysine, serine threonine, asparagine, or glutamine, particularly methionine or lysine; residue corresponding to X194 is proline, arginine, lysine, serine, threonine, asparagine, glutamine, particularly arginine or glutamine; residue corresponding to X198 is aspartic acid, glutamic acid, arginine, lysine, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine; residue corresponding to X199 is an aspartic acid, glutamic acid, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly aspartic acid; residue corresponding to X200 is aspartic acid, glutamic acid, or proline, particularly proline. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0115] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X3 is an acidic, a polar, or hydrophilic residue, particularly asparagine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0116] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X7 is a non-polar or polar residue, particularly serine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0117] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid

residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X17 is a non-polar, aliphatic or polar residue, particularly glutamine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0118] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X21 is a non-polar, aromatic, or hydrophobic residue, particularly phenylalanine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0119] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X25 is an acidic, non-

polar or polar residue, particularly threonine or serine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0120] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X29 is an acidic, aliphatic or non-polar residue, particularly alanine or glycine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0121] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, have at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X40 is a constrained, basic, or hydrophilic residue, particularly arginine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40

residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0122] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X42 is an acidic or a non-polar residue, particularly glycine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0123] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X53 is a non-polar or an acidic residue, particularly aspartic acid. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or

about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0124] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X75 is an acidic or polar residue, particularly asparagine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0125] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X94 is a non-polar or a polar residue, particularly glycine, serine, or asparagine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the

amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0126] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162, or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and the amino acid residue corresponding to X95 is a non-polar or aliphatic residue, particularly leucine or methionine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0127] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:128, 130, or 160, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residues corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and the amino acid residue corresponding to X96 is a polar residue, particularly threonine, asparagine or glutamine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,

97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0128] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X101 is an acidic, non-polar, or a polar residue, particularly asparagine or glycine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0129] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X105 is an acidic or non-polar residue, particularly glycine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0130] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine, serine or asparagine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0131] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X111 is a non-polar or aliphatic residue, particularly methionine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0132] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as

residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X112 is an acidic or polar residue, particularly aspartic acid. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0133] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residues corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X113 is a non-polar or aliphatic residue, particularly alanine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0134] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X117 is a non-polar or

a polar residue, particularly serine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0135] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X127 is a basic or polar residue, particularly arginine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0136] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X147 is a non-polar, aliphatic, aromatic, or hydrophobic residue, particularly leucine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In

some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0137] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue, particularly, methionine or lysine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0138] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X157 is a polar residue, particularly threonine or serine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise

conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0139] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X163 is a non-polar or aliphatic residue, particularly isoleucine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0140] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X176 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least

85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0141] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X194 is a basic constrained, basic, or polar residue, particularly arginine or glutamine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0142] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X197 is a hydrophilic, acidic, basic, aliphatic or a non-polar residue, particularly glutamic acid or valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0143] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X198 is an acidic, basic, hydrophilic, or non-polar residue, particularly glycine, lysine, or glutamic acid. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0144] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, and particularly aspartic acid. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0145] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as

residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X200 is an acidic or a constrained residue, particularly proline. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0146] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X202 is a non-polar residue, and particularly glycine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0147] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X206 is a non-polar,

aromatic, or hydrophobic residue, and particularly glycine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0148] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, have at least the following features: amino acid residue corresponding to X145 is a serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X211 is a basic residue, particularly arginine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0149] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to

the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0150] In some embodiments, the improved ketoreductase polypeptides comprising an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or region thereof, such as residues 90-211, in which the amino acid sequence has at least the following features: amino acid residue corresponding to X145 is a polar residue, particularly serine; amino acid residue corresponding to X190 is a cysteine; and amino acid residue corresponding to X250 is a polar or a non-polar residue, particularly isoleucine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35 or about 1-40 residue differences at other amino acid residues as compared to the reference sequence of SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0151] In some embodiments, an improved ketoreductase comprises an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence corresponding to SEQ ID NO: 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, or 98 as listed in Tables 3 and 4, wherein the improved ketoreductase polypeptide amino acid sequence includes any one set of the specified amino acid substitution combinations presented in Tables 3 and 4. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 differences at other amino acid residues as compared to the reference sequence. In some embodiments, the number of differences can be 1, 2,

3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations.

[0152] In some embodiments, an improved ketoreductase comprises an amino acid sequence corresponding to SEQ ID NO: 8, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[0153] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine or serine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X157 is a polar residue, particularly threonine; residue corresponding to X190 is a cysteine; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:8. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 8.

[0154] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X94 is a non-polar or a polar residue, particularly serine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine, residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X157 is a polar residue, particularly threonine; residue corresponding to X190 is a cysteine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is

a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:42. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 42.

[0155] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X157 is a polar residue, particularly threonine; residue corresponding to X190 is cysteine; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:44. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 44.

[0156] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue

corresponding to X157 is a polar residue, particularly threonine; residue corresponding to X190 is a cysteine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:46. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 46.

[0157] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X108 is hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X157 is a polar residue, particularly threonine; residue corresponding to X190 is cysteine; residue corresponding to X202 is a is a non-polar residue or aliphatic residue, particularly glycine; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:48. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 48.

[0158] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to

211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar or basic residue, particularly methionine or lysine; residue corresponding to X157 is a polar residue, particularly threonine; residue corresponding to X190 is a cysteine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:52 or 54. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to 52 or 54.

[0159] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X94 is a non-polar or a polar residue, particularly serine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X157 is a polar residue, particularly threonine; residue corresponding to X190 is a cysteine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:56. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations.

In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:56.

[0160] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X157 is a polar residue, particularly threonine; residue corresponding to X190 is a cysteine; residue corresponding to X194 is a constrained, basic, or polar residue, particularly arginine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; and residue corresponding to X211 is a basic residue. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:58. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 58.

[0161] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X96 is a polar residue, particularly glutamine or threonine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly, histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue, particularly methionine; residue corresponding to X157 is a polar residue, particularly threonine; residue corresponding to X190 is a cysteine; residue corresponding to X194 is a constrained, basic, or polar residue, particularly arginine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is

a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:72 or 74. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 72 or 74.

[0162] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X25 is an acidic, non-polar or polar residue, particularly threonine; residue corresponding to X40 is a constrained, basic, or hydrophilic residue; residue corresponding to X75 is an acidic or polar residue, particularly asparagine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue, particularly methionine; residue corresponding to X157 is a polar residue, particularly threonine; residue corresponding to X190 is a cysteine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine, and residue X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:76. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: SEQ ID NO: 76.

[0163] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X25 is a acidic, non-polar or polar residue, particularly threonine; residue corresponding to X95 is a non-polar or aliphatic residue, particularly leucine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue, particularly methionine; residue corresponding to X176 is non-polar or aliphatic residue, particularly valine; residue corresponding to X190 is a cysteine; residue corresponding to X198 is an acidic, basic, hydrophilic, or non-polar residue, particularly glutamic acid; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:82. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 82.

[0164] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X25 is an acidic, non-polar or polar residue, particularly threonine; residue corresponding to X95 is a non-polar or aliphatic residue, particularly leucine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue, particularly methionine; residue corresponding to X176 is a non-polar or aliphatic residue, particularly valine; residue corresponding to X190 is a cysteine; residue

corresponding to X197 is a hydrophilic, acidic, basic, aliphatic or a non-polar residue, particularly valine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:84. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 84.

[0165] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X25 is a acidic, non-polar or polar residue, particularly threonine; residue corresponding to X95 is a non-polar or aliphatic residue, particularly methionine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue, particularly methionine; residue corresponding to X176 is a non-polar or aliphatic residue, particularly valine; residue corresponding to X190 is a cysteine; residue corresponding to X194 is a constrained, basic, or polar residue, particularly arginine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:86. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%,

86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: SEQ ID NO: 86.

[0166] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue, particularly methionine; residue corresponding to X190 is a cysteine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; and residue corresponding to X211 is a basic residue, particularly arginine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:90. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 90.

[0167] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X53 is a non-polar or an acidic residue, particularly aspartic acid; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to residue X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue, particularly methionine; residue corresponding to X163 is a non-polar or aliphatic residue, particularly isoleucine; residue corresponding to X190 is a cysteine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at

other residue positions as compared to a reference sequence of SEQ ID NO:94. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 94.

[0168] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X101 is acidic, non-polar, or a polar residue, particularly glycine; residue corresponding to X108 is hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X147 is non-polar, aliphatic, aromatic, or hydrophobic residue, particularly leucine; residue corresponding to X152 is non-polar, basic residue, particularly methionine; residue corresponding to X190 is cysteine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:100. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 100.

[0169] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine;

residue corresponding to X111 is a non-polar or aliphatic residue, particularly methionine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue, particularly methionine; residue corresponding to X190 is a cysteine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:102. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 102.

[0170] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue, particularly methionine; residue corresponding to X190 is a cysteine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine; and residue corresponding to X250 is a polar or non-polar residue, particularly isoleucine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:104. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino

acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:104.

[0171] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X29 is an acidic, aliphatic or non-polar residue, particularly glycine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X101 is an acidic, non-polar, or a polar residue, particularly asparagine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue, particularly methionine; residue corresponding to X190 is a cysteine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X200 is an acidic or a constrained residue, particularly proline; residue corresponding to X211 is a basic residue, particularly arginine, and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:106. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 106.

[0172] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X3 is an acidic, polar, or hydrophilic residue, particularly asparagine; residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X17 is a non-polar, aliphatic or polar residue, particularly glutamine; residue corresponding to X42 is an acidic or non-polar residue, particularly glycine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X127 is a basic or polar residue, particularly arginine; residue corresponding to

X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic residue, or hydrophilic residue, particularly methionine; residue corresponding to X176 is a non-polar or aliphatic residue, particularly valine; residue corresponding to X190 is a cysteine; residue corresponding to X194 is a constrained, basic, or polar residue, particularly arginine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X200 is an acidic or a constrained residue, particularly proline; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:114. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 114.

[0173] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X3 is an acidic, polar, or hydrophilic residue, particularly asparagine; residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X17 is a non-polar, aliphatic or polar residue, particularly glutamine; residue corresponding to X21 is a non-polar, aromatic, or hydrophobic residue, particularly phenylalanine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X147 is a non-polar, aliphatic, aromatic, or hydrophobic residue, particularly leucine; residue corresponding to X152 is a non-polar, basic, or hydrophilic residue, particularly methionine; residue corresponding to X176 is a non-polar or aliphatic residue, particularly valine; residue corresponding to X190 is a cysteine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:116. In some

embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:116.

[0174] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X3 is an acidic, polar, or hydrophilic residue, particularly asparagine; residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X17 is a non-polar, aliphatic or polar residue, particularly glutamine; residue corresponding to X29 is an acidic, aliphatic or non-polar residue, particularly alanine; residue corresponding to X42 is an acidic or non-polar residue, particularly glycine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X105 is an acidic or non-polar residue, particularly glycine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic, or hydrophilic residue, particularly methionine; residue corresponding to X190 is a cysteine; residue corresponding to X197 is a hydrophilic, acidic, basic, aliphatic or a non-polar residue, particularly valine; residue corresponding to X198 is an acidic, basic, hydrophilic, or non-polar residue, particularly lysine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X200 is an acidic or a constrained residue, particularly proline; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:118. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 118.

[0175] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X17 is a non-polar, aliphatic or polar residue, particularly glutamine; residue X29 is an acidic, aliphatic or non-polar residue, particularly alanine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is a polar residue, particularly serine; residue corresponding to X152 is a non-polar, basic, or hydrophilic residue, particularly methionine; residue corresponding to X163 is a non-polar or aliphatic residue, particularly isoleucine; residue corresponding to X190 is a cysteine; residue corresponding to X198 is an acidic, basic, hydrophilic, or non-polar residue, particularly lysine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid; residue corresponding to X200 is an acidic or a constrained residue, particularly proline; and residue corresponding to X211 is a basic residue, particularly arginine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:122. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:122.

[0176] In some embodiments, an improved ketoreductase comprises an amino acid sequence based on the sequence formula of SEQ ID NO:161, 162 or 163, or a region thereof, such as residues 90 to 211, and has at least the following features: residue corresponding to X7 is a non-polar or polar residue, particularly serine; residue corresponding to X17 is a non-polar, aliphatic or polar residue, particularly glutamine; residue corresponding to X29 is an acidic, aliphatic or non-polar residue, particularly alanine; residue corresponding to X96 is a polar residue, particularly glutamine; residue corresponding to X108 is a hydrophilic, polar or constrained residue, particularly histidine; residue corresponding to X117 is a non-polar or a polar residue, particularly serine; residue corresponding to X145 is polar residue, particularly serine; residue corresponding to X147 is a non-polar, aliphatic, aromatic, or hydrophobic residue, particularly leucine; residue corresponding to X152 is a non-polar, basic, or hydrophilic residue, particularly methionine; residue corresponding to X163 is a non-polar or aliphatic residue, particularly isoleucine; residue corresponding to X176 is a non-polar or aliphatic

residue, particularly valine; residue corresponding to X190 is a cysteine; residue corresponding to X198 is an acidic, basic, hydrophilic, or non-polar residue, particularly lysine; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue, particularly aspartic acid,; residue corresponding to X200 is an acidic or a constrained residue, particularly proline; residue corresponding to X211 is a basic residue, particularly arginine; and residue corresponding to X223 is a non-polar or aliphatic residue, particularly valine. In some embodiments, the ketoreductase polypeptides can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 residue differences at other residue positions as compared to a reference sequence of SEQ ID NO:126. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 residue differences at other amino acid residues. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 126.

[0177] In some embodiments, an improved ketoreductase comprises an amino acid sequence that has a region or domain corresponding to residues 90-211 of the sequence formula of SEQ ID NO:161, 162 or 163, in which the amino acid sequence of the domain has at least the following features: the amino acid residue corresponding to X145 is a polar residue, and the amino acid residue corresponding to X190 is a cysteine. In some embodiments, the improved ketoreductase has a region or domain that corresponds to residues 90-211 based on the sequence formula of SEQ ID NO:161, 162 or 163, in which the amino acid sequence of the domain has at least the following features: the amino acid residue corresponding to X145 is a serine, and the amino acid residue corresponding to X190 is a cysteine. In some embodiments, the region or domain corresponding to residues 90-211 can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, or 1-20 residue differences at other amino acid residues as compared to the corresponding domain of a reference sequence based on SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, or about 20 residue differences at other amino acid residues in the domain. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to the amino acid sequence corresponding to residues 90-211 of a reference sequence based on SEQ ID NO:128, 130, 0r 160 with the preceding features.

[0178] In some embodiments, the ketoreductase polypeptides with a domain or region corresponding to residues 90-211 and having the specified features for residues X145 and X190 as described herein, can further include in the region or domain one or more features selected from the following: residue corresponding to X94 is a non-polar or a polar residue; residue corresponding to X95 is a non-polar or aliphatic residue; residue corresponding to X96 is a polar residue; residue corresponding to X101 is an acidic, non-polar, or a polar residue; residue corresponding to X105 is an acidic or non-polar residue; residue corresponding to X108 is a hydrophilic, polar or constrained residue; residue corresponding to X111 is a non-polar or aliphatic residue; residue corresponding to X112 is an acidic or polar residue; residue corresponding to X113 is a non-polar or aliphatic residue; residue corresponding to X117 is a non-polar or a polar residue; residue corresponding to X127 is a basic or polar residue; residue corresponding to X147 is a non-polar, aliphatic, aromatic, or hydrophobic residue; residue corresponding to X152 is a non-polar, basic, or hydrophilic residue; residue corresponding to X157 is a polar residue; residue corresponding to X163 is a non-polar or aliphatic residue; residue corresponding to X176 is a non-polar or aliphatic residue; residue corresponding to X194 is a constrained, basic, or polar residue; residue corresponding to X197 is a hydrophilic, acidic, basic, aliphatic or a non-polar residue; residue corresponding to X198 is an acidic, basic, hydrophilic, or non-polar residue; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue; residue corresponding to X200 is an acidic or constrained residue; residue corresponding to X202 is a non-polar residue; residue corresponding to X206 is a non-polar, aromatic, or hydrophobic residue; residue corresponding to X211 is a basic residue. In some embodiments, the region or domain corresponding to residues 90-211 can have additionally from about 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, or 1-20 residue differences at other amino acid residues as compared to the corresponding domain of a reference sequence based on SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, or about 20 residue differences at other amino acid residues in the domain. In some embodiments, the differences comprise conservative mutations.

[0179] In some embodiments, the ketoreductases polypeptides having a domain with an amino acid sequence corresponding to residues 90-211 of the sequence formula of SEQ ID NO:161, 162 or 163,, as described above, can have one or more conservative mutations as compared to the corresponding domain of SEQ ID NO:128, 130, or 160. Examples of such conservative mutations include amino acid replacements such as, but not limited to: the replacement of residue corresponding to X95 (valine) with another non-polar amino acid, *e.g.*, alanine, leucine, isoleucine, glycine, or methionine; the replacement of residue corresponding to X96 (serine) with another polar amino acid, *e.g.*, asparagine, glutamine, or threonine; the replacement of residue corresponding to X111 (leucine) with another non-polar amino acid, *e.g.*, alanine, leucine, isoleucine, glycine, or methionine; the

replacement of residue corresponding to X113 (valine) with another aliphatic amino acid, *e.g.*, alanine, leucine, or isoleucine; the replacement of residue corresponding to X157 (asparagine) with another polar amino acid, *e.g.*, glutamine, serine, or threonine; the replacement of residue corresponding to X163 (valine) with another aliphatic amino acid, *e.g.*, alanine, leucine, or isoleucine; the replacement of residue corresponding to X176 (leucine) with another aliphatic amino acid, *e.g.*, alanine, valine, and isoleucine; the replacement of residue corresponding to X202 (alanine) with another non-polar amino acid, *e.g.*, alanine, leucine, isoleucine, glycine, or methionine; and the replacement of residue corresponding to X211 (lysine) with another basic amino acid, *e.g.*, arginine.

[0180] In some embodiments, the ketoreductase polypeptides with a domain or region corresponding to residues 90-211 and having the specified features for residues X145 and X190 as described herein, can further include in the region or domain one or more features selected from the following: residue corresponding to X94 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly asparagine, glycine, or serine; residue corresponding to X95 is a glycine, methionine, alanine, valine, leucine, or isoleucine, particularly leucine or methionine; residue corresponding to X96 is serine, threonine, asparagine, glutamine, particularly glutamine, asparagine, or threonine; residue; residue corresponding to X101 is aspartic acid, glutamic acid, serine, threonine, asparagine, glutamine, or glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine or asparagine; residue corresponding to X105 is glutamic acid, aspartic acid, glycine, methionine, alanine, valine, leucine, isoleucine, particularly glycine; residue corresponding to X108 is arginine, lysine, serine, threonine, asparagine, glutamine, histidine, particularly histidine or serine; residue corresponding to X112 aspartic acid, glutamic acid, serine, threonine, asparagine, glutamine, particularly aspartic acid; residue corresponding to X113 is an glycine, methionine, alanine, valine, leucine, isoleucine, particularly alanine; residue corresponding to X117 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly serine; residue corresponding to X127 is lysine, arginine, serine, threonine, asparagine, or glutamine, particularly arginine; residue corresponding to X147 is glycine, methionine, alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, tryptophan, particularly leucine; residue corresponding to X152 is glycine, methionine, valine, leucine, isoleucine, arginine, lysine, serine threonine, asparagine, or glutamine, particularly methionine or lysine; residue corresponding to X157 is a serine, threonine, asparagine, and glutamine, particularly threonine; residue corresponding to X163 is a glycine, methionine, alanine, valine, leucine, or isoleucine, particularly isoleucine; residue corresponding to X176 is glycine, methionine, alanine, valine, leucine, or isoleucine, particularly valine; residue corresponding to X194 is proline, arginine, lysine, serine, threonine, asparagine, glutamine, particularly arginine or glutamine; residue corresponding to X197 is aspartic acid, glutamic acid, arginine, lysine, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine,

isoleucine, particularly valine or glutamic acid; residue corresponding to X198 is aspartic acid, glutamic acid, arginine, lysine, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine, glutamic acid, or lysine; residue corresponding to X199 is an aspartic acid, glutamic acid, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly aspartic acid; residue corresponding to X200 is aspartic acid, glutamic acid, or proline, particularly proline; residue corresponding to X202 is glycine, methionine, alanine, valine, leucine, isoleucine, particularly glycine; residue corresponding to X206 is a glycine, methionine, alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, tryptophan, particularly glycine; residue corresponding to X211 is a arginine or lysine. In some embodiments, the region or domain corresponding to residues 90-211 can have additionally from about 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, or 1-20 residue differences at other amino acid residues as compared to the corresponding domain of a reference sequence based on SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, or about 20 residue differences at other amino acid residues in the domain. In some embodiments, the differences comprise conservative mutations.

[0181] In some embodiments, the ketoreductase polypeptides with a domain or region corresponding to residues 90-211 and having the specified features for residues X145 and X190 as described herein, can further include in the region or domain one or more features selected from the following: residue corresponding to X108 is a hydrophilic, polar or constrained residue; residue corresponding to X117 is a non-polar or a polar residue; residue corresponding to X152 is a non-polar, basic, or hydrophilic residue; and residue corresponding to X199 is an acidic, aliphatic, or non-polar residue. In some embodiments, the region or domain corresponding to residues 90-211 can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, or 1-20 residue differences at other amino acid residues as compared to the domain of a reference sequence based on SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, or about 20 residue differences at other amino acid residues in the domain. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to the amino acid sequence corresponding to residues 90-211 of a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0182] In some embodiments, the ketoreductase polypeptides with a domain or region corresponding to residues 90-211 and having the specified features for residues X145 and X190 as described herein,

can further include in the region or domain one or more features selected from the following: residue corresponding to X108 is arginine, lysine, serine, threonine, asparagine, glutamine, histidine, particularly histidine or serine; residue corresponding to X117 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly serine; residue corresponding to X152 is glycine, methionine, valine, leucine, isoleucine, arginine, lysine, serine threonine, asparagine, or glutamine, particularly methionine or lysine; and residue corresponding to X199 is an aspartic acid, glutamic acid, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly aspartic acid. In some embodiments, the region or domain corresponding to residues 90-211 can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, or 1-20 residue differences at other amino acid residues as compared to the domain of a reference sequence based on SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, or about 20 residue differences at other amino acid residues in the domain. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to the amino acid sequence corresponding to residues 90-211 of a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0183] In some embodiments, the ketoreductase polypeptides with a domain or region corresponding to residues 90-211 and having the specified features for residues X145 and X190 as described herein, can further include in the region or domain one or more features selected from the following: residue corresponding to X94 is a non-polar or a polar residue; residue corresponding to X194 is a constrained, basic, or polar residue; residue corresponding to X198 is an acidic, basic, hydrophilic, or non-polar residue; and residue corresponding to X200 is an acidic or a constrained residue. In some embodiments, the region or domain corresponding to residues 90-211 can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, or 1-20 residue differences at other amino acid residues as compared to the domain of a reference sequence based on SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, or about 20 residue differences at other amino acid residues in the domain. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to the amino acid sequence corresponding to residues 90-211 of a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features. In some embodiments, the region or domain corresponding to residues 90-211 can have

additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, or 1-20 residue differences at other amino acid residues as compared to the domain of a reference sequence based on SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, or about 20 residue differences at other amino acid residues in the domain. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to the amino acid sequence corresponding to residues 90-211 of a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0184] In some embodiments, the ketoreductase polypeptides with a domain or region corresponding to residues 90-211 and having the specified features for residues X145 and X190 as described herein, can further include in the region or domain one or more features selected from the following: residue corresponding to X94 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly asparagine, glycine, or serine; residue corresponding to X194 is proline, arginine, lysine, serine, threonine, asparagine, glutamine, particularly arginine or glutamine; residue corresponding to X198 is aspartic acid, glutamic acid, arginine, lysine, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine, glutamic acid, or lysine; residue corresponding to X200 is an aspartic acid, glutamic acid, or proline, particularly proline. In some embodiments, the region or domain corresponding to residues 90-211 can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, or 1-20 residue differences at other amino acid residues as compared to the domain of a reference sequence based on SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, or about 20 residue differences at other amino acid residues in the domain. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to the amino acid sequence corresponding to residues 90-211 of a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0185] In some embodiments, the ketoreductase polypeptides with a domain or region corresponding to residues 90-211 and having the specified features for residues X145 and X190 as described herein, can further include in the region or domain one or more features selected from the following: residue corresponding to X94 is a non-polar or a polar residue; residue corresponding to X108 is a

hydrophilic, polar or constrained residue; residue corresponding to X117 is a non-polar or a polar residue; residue corresponding to X152 is a non-polar, basic, or hydrophilic residue; residue corresponding to X194 is a constrained, basic, or polar residue; residue corresponding to X198 is an acidic, basic, hydrophilic, or non-polar residue; residue corresponding to X199 is an acidic, aliphatic, or non-polar residue; residue corresponding to X200 is an acidic or constrained residue. In some embodiments, the region or domain corresponding to residues 90-211 can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, or 1-20 residue differences at other amino acid residues as compared to the domain of a reference sequence based on SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, or about 20 residue differences at other amino acid residues in the domain. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to the amino acid sequence corresponding to residues 90-211 of a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0186] In some embodiments, the ketoreductase polypeptides with a domain or region corresponding to residues 90-211 and having the specified features for residues X145 and X190 as described herein, can further include in the region or domain one or more features selected from the following: residue corresponding to X94 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly asparagine, glycine, or serine; residue corresponding to X108 is arginine, lysine, serine, threonine, asparagine, glutamine, histidine, particularly histidine or serine; residue corresponding to X117 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly serine; residue corresponding to X152 is glycine, methionine, valine, leucine, isoleucine, arginine, lysine, serine threonine, asparagine, or glutamine, particularly methionine or lysine; residue corresponding to X194 is proline, arginine, lysine, serine, threonine, asparagine, glutamine, particularly arginine or glutamine; residue corresponding to X198 is aspartic acid, glutamic acid, arginine, lysine, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine; residue corresponding to X199 is an aspartic acid, glutamic acid, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly aspartic acid; residue corresponding to X200 is aspartic acid, glutamic acid, or proline, particularly proline. In some embodiments, the region or domain corresponding to residues 90-211 can have additionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, or 1-20 residue differences at other amino acid residues as compared to the domain of a reference sequence based on SEQ ID NO:128, 130, or 160. In some embodiments, the number of differences

can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, or about 20 residue differences at other amino acid residues in the domain. In some embodiments, the differences comprise conservative mutations. In some embodiments, the ketoreductase polypeptide comprises an amino acid sequence with at least the preceding features, and wherein the amino acid sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity as compared to the amino acid sequence corresponding to residues 90-211 of a reference sequence based on SEQ ID NO:128, 130, or 160 with the preceding features.

[0187] In some embodiments, the ketoreductase polypeptide has a region that corresponds to residues 1-89 of the sequence formula of SEQ ID NO:161, 162 or 163, in which the amino acid sequence has one or more of the following features: residue corresponding to X3 is an acidic, polar, or hydrophilic residue; residue corresponding to X7 is a non-polar or polar residue; residue corresponding to X17 is a non-polar, aliphatic or polar residue; residue corresponding to X21 is a non-polar, aromatic, or hydrophobic residue; residue corresponding to X25 is an acidic, non-polar or polar residue; residue corresponding to X29 is an acidic, aliphatic or non-polar residue; residue corresponding to X40 is a constrained, basic, or hydrophilic residue; residue corresponding to X42 is an acidic or non-polar residue; residue corresponding to X53 is a non-polar or an acidic residue; residue corresponding to X75 is an acidic or polar residue.

[0188] In some embodiments, the ketoreductase polypeptide has a region that corresponds to residues 1-89 of the sequence formula of SEQ ID NO:161, 162 or 163, in which the amino sequence of the domain or region has one or more of the following features: residue corresponding to X3 is aspartic acid, glutamic acid, serine, threonine, asparagine, or glutamine, particularly asparagine; residue corresponding to X7 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly serine; residue corresponding to X17 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine, particularly glutamine; residue corresponding to X21 is glycine, methionine, alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, or tryptophan, particularly phenylalanine; residue corresponding to X25 is aspartic acid, glutamic acid, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, isoleucine, particularly threonine; residue corresponding to X29 is aspartic acid, glutamine acid, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine or alanine; residue corresponding to X40 is histidine, lysine, arginine, serine, threonine, asparagine, or glutamine, particularly arginine; residue corresponding to X42 is aspartic acid, glutamic acid, glycine, methionine, alanine, valine, leucine, or isoleucine, particularly glycine; residue corresponding to X53 is glycine, methionine, alanine, valine, leucine, isoleucine, aspartic acid, glutamic acid, particularly

aspartic acid; residue corresponding to X75 is aspartic acid, glutamic acid, serine, threonine, asparagine, or glutamine, particularly arginine.

[0189] In some embodiments, the ketoreductase polypeptide has a region that corresponds to residues 212-252 of the sequence formula of SEQ ID NO:161, 162 or 163, in which the amino acid sequence has one or more of the following features: residue corresponding to X223 is a non-polar or aliphatic residue; and residue corresponding to X250 is a polar or non-polar residue.

[0190] .In some embodiments, the ketoreductase polypeptide has a region that corresponds to residues 212-252 of the sequence formula of SEQ ID NO:161, 162 or 163, in which the amino acid sequence has one or more of the following features: residue corresponding to X223 is glycine, methionine, alanine, valine, leucine, or isoleucine, particularly valine; and residue corresponding to X250 is serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, isoleucine, particularly isoleucine.

[0191] In some embodiments, the ketoreductase polypeptides of the disclosure can comprise a having an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a region or domain of SEQ ID NO:128, 130, or 160, such as residues 90-211, with the proviso that the residue corresponding to X145 is serine and the residue corresponding to X190 is cysteine, and wherein the amino acid sequence can have additionally one or more of the following substitutions such that the polypeptide is further improved (*e.g.*, with respect to stereoselectivity, enzymatic activity, and/or thermostability) over the wild-type L. kefir ketoreductase or another engineered ketoreductase (such as SEQ ID NO:8): 3→N, 7→S, 17→Q, 21→F, 25→T, 29→A or G, 42→G, 53→D, 75→N, 95→L or M, 96→Q, 101→Q or G, 105→G, 108→H or S, 112→D, 117→S, 127→R, 147→L, 152→M, 157→T, 163→L or I, 167→V, 176→V, 194→R, 197→V or E, 198→K or E, 199→D, 200→P, 211→R, 223→V, and 250→I.

[0192] In some embodiments, the ketoreductase polypeptides of the disclosure can comprise a region having an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a region or domain of SEQ ID NO:128, 130, or 160, such as residues 90-211, wherein the amino acid sequence can have additionally one or more of the following substitutions such that the polypeptide is further improved (*e.g.*, with respect to stereoselectivity, enzymatic activity, and/or thermostability) over the wild-type L. kefir ketoreductase or another engineered ketoreductase (such as SEQ ID NO:8): 7→S, 17→Q, 96→Q, 108→H, 117→S, 152→M, 163→ I, 176→V, 198→K, 199→D, 211→R, and 223→V.

[0193] In some embodiments, the ketoreductases of the disclosure are subject to one or more of the following provisos: (1) specifically excluded are polypeptides with the specific sequences selected from SEQ ID NO: 8, 44, 46, 48, 164 and 165; (2) the amino acid sequence requires at residue

corresponding to X152 a basic or non-polar residue, particularly methionine or lysine; (3) the amino acid sequence requires at residue corresponding to X199 an acidic residue, particularly aspartic acid; and (4) the amino acid sequence requires at residue corresponding to X96 a glutamine.

[0194] In some embodiments, each of the improved engineered ketoreductase enzymes described herein can comprise deletions of the polypeptides described herein. Thus, for each and every embodiment of the ketoreductase polypeptides of the disclosure, the deletions can comprise one or more amino acids, 2 or more amino acids, 3 or more amino acids, 4 or more amino acids, 5 or more amino acids, 6 or more amino acids, 8 or more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids, up to 10% of the total number of amino acids, up to 10% of the total number of amino acids, up to 20% of the total number of amino acids, or up to 30% of the total number of amino acids of the ketoreductase polypeptides, as long as the functional activity of the ketoreductase activity is maintained. In some embodiments, the deletions can comprise, 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-25, 1-30, 1-35 or about 1-40 amino acids. In some embodiments, the deletions can comprise deletions of 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, or 1-20 amino acid residues. In some embodiments, the number of deletions can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 22, 24, 26, 30, 35 or about 40 amino acids. In some embodiments, the deletions can comprise deletions of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, or 20 amino acid residues.

[0195] As will be appreciated by the skilled art, the polypeptides described herein are not restricted to the genetically encoded amino acids. In addition to the genetically encoded amino acids, the polypeptides described herein may be comprised, either in whole or in part, of naturally-occurring and/or synthetic non-encoded amino acids. Certain commonly encountered non-encoded amino acids of which the polypeptides described herein may be comprised include, but are not limited to: the D-stereomers of the genetically-encoded amino acids; 2,3-diaminopropionic acid (Dpr); α-aminoisobutyric acid (Aib); ε-aminohexanoic acid (Aha); δ-aminovaleric acid (Ava); N-methylglycine or sarcosine (MeGly or Sar); ornithine (Orn); citrulline (Cit); t-butylalanine (Bua); t-butylglycine (Bug); N-methylisoleucine (MeIle); phenylglycine (Phg); cyclohexylalanine (Cha); norleucine (Nle); naphthylalanine (Nal); 2-chlorophenylalanine (Ocf); 3-chlorophenylalanine (Mcf); 4-chlorophenylalanine (Pcf); 2-fluorophenylalanine (Off); 3-fluorophenylalanine (Mff); 4-fluorophenylalanine (Pff); 2-bromophenylalanine (Obf); 3-bromophenylalanine (Mbf); 4-bromophenylalanine (Pbf); 2-methylphenylalanine (Omf); 3-methylphenylalanine (Mmf); 4-methylphenylalanine (Pmf); 2-nitrophenylalanine (Onf); 3-nitrophenylalanine (Mnf); 4-nitrophenylalanine (Pnf); 2-cyanophenylalanine (Ocf); 3-cyanophenylalanine (Mcf); 4-cyanophenylalanine (Pcf); 2-trifluoromethylphenylalanine (Otf); 3-trifluoromethylphenylalanine

(Mtf); 4-trifluoromethylphenylalanine (Ptf); 4-aminophenylalanine (Paf); 4-iodophenylalanine (Pif); 4-aminomethylphenylalanine (Pamf); 2,4-dichlorophenylalanine (Opef); 3,4-dichlorophenylalanine (Mpcf); 2,4-difluorophenylalanine (Opff); 3,4-difluorophenylalanine (Mpff); pyrid-2-ylalanine (2pAla); pyrid-3-ylalanine (3pAla); pyrid-4-ylalanine (4pAla); naphth-1-ylalanine (1nAla); naphth-2-ylalanine (2nAla); thiazolylalanine (taAla); benzothienylalanine (bAla); thienylalanine (tAla); furylalanine (fAla); homophenylalanine (hPhe); homotyrosine (hTyr); homotryptophan (hTrp); pentafluorophenylalanine (5ff); styrylkalanine (sAla); authrylalanine (aAla); 3,3-diphenylalanine (Dfa); 3-amino-5-phenypentanoic acid (Afp); penicillamine (Pen); 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic); β-2-thienylalanine (Thi); methionine sulfoxide (Mso); N(w)-nitroarginine (nArg); homolysine (hLys); phosphonomethylphenylalanine (pmPhe); phosphoserine (pSer); phosphothreonine (pThr); homoaspartic acid (hAsp); homoglutanic acid (hGlu); 1-aminocyclopent-(2 or 3)-ene-4 carboxylic acid; pipecolic acid (PA), azetidine-3-carboxylic acid (ACA); 1-aminocyclopentane-3-carboxylic acid; allylglycine (aOly); propargylglycine (pgGly); homoalanine (hAla); norvaline (nVal); homoleucine (hLeu), homovaline (hVal); homoisolencine (hIle); homoarginine (hArg); N-acetyl lysine (AcLys); 2,4-diaminobutyric acid (Dbu); 2,3-diaminobutyric acid (Dab); N-methylvaline (MeVal); homocysteine (hCys); homoserine (hSer); hydroxyproline (Hyp) and homoproline (hPro). Additional non-encoded amino acids of which the polypeptides described herein may be comprised will be apparent to those of skill in the art (see, *e.g.*, the various amino acids provided in Fasman, 1989, CRC Practical Handbook of Biochemistry and Molecular Biology, CRC Press, Boca Raton, FL, at pp. 3-70 and the references cited therein, all of which are incorporated by reference). These amino acids may be in either the L- or D-configuration.

[0196] Those of skill in the art will recognize that amino acids or residues bearing side chain protecting groups may also comprise the polypeptides described herein. Non-limiting examples of such protected amino acids, which in this case belong to the aromatic category, include (protecting groups listed in parentheses), but are not limited to: Arg(tos), Cys(methylbenzyl), Cys (nitropyridinesulfenyl), Glu(δ-benzylester), Gln(xanthyl), Asn(N-δ-xanthyl), His(bom), His(benzyl), His(tos), Lys(fmoc), Lys(tos), Ser(O-benzyl), Thr (O-benzyl) and Tyr(O-benzyl).

[0197] Non-encoding amino acids that are conformationally constrained of which the polypeptides described herein may be composed include, but are not limited to, N-methyl amino acids (L-configuration); 1-aminocyclopent-(2 or 3)-ene-4-carboxylic acid; pipecolic acid; azetidine-3-carboxylic acid; homoproline (hPro); and 1-aminocyclopentane-3-carboxylic acid.

[0198] As described above the various modifications introduced into the naturally occurring polypeptide to generate an engineered ketoreductase enzyme can be targeted to a specific property of the enzyme.

6.3 Polynucleotides Encoding Engineered Ketoreductases

[0199] In another aspect, the present disclosure provides polynucleotides encoding the engineered ketoreductase enzymes disclosed herein. The polynucleotides may be operatively linked to one or more heterologous regulatory sequences that control gene expression to create a recombinant polynucleotide capable of expressing the polypeptide. Expression constructs containing a heterologous polynucleotide encoding the engineered ketoreductase can be introduced into appropriate host cells to express the corresponding ketoreductase polypeptide.

[0200] Because of the knowledge of the codons corresponding to the various amino acids, availability of a protein sequence provides a description of all the polynucleotides capable of encoding the subject. The degeneracy of the genetic code, where the same amino acids are encoded by alternative or synonymous codons allows an extremely large number of nucleic acids to be made, all of which encode the improved ketoreductase enzymes disclosed herein. Thus, having identified a particular amino acid sequence, those skilled in the art could make any number of different nucleic acids by simply modifying the sequence of one or more codons in a way which does not change the amino acid sequence of the protein. In this regard, the present disclosure specifically contemplates each and every possible variation of polynucleotides that could be made by selecting combinations based on the possible codon choices, and all such variations are to be considered specifically disclosed for any polypeptide disclosed herein, including the amino acid sequences presented in Tables 3 and 4.

[0201] In some embodiments, the polynucleotides encode a ketoreductase polypeptides having at least the following features as compared to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:158: (1) the amino acid residue corresponding to residue X145 is a serine residue, and (2) the amino acid residue corresponding to residue X190 is a cysteine residue. In some embodiments, the polynucleotide comprises a nucleotide sequence encoding a ketoreductase polypeptide with an amino acid sequence that has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity to any of the reference engineered ketoreductase polypeptides described herein, where the ketoreductase polypeptide comprises an amino acid sequence that has at least the following features: an amino acid residue corresponding to residue position of 145 of SEQ ID NO:2, 4, or 158 is serine and the amino acid residue corresponding to residue residue position 190 of SEQ ID NO:2, 4 or 158 is cysteine.

[0202] In some embodiments, the polynucleotides encode the polypeptides described herein but have at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity at the nucleotide level to a reference polynucleotide encoding an engineered ketoreductase. In some embodiments, the reference polynucleotide is selected from

polynucleotide sequences represented by SEQ ID NO: 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, and 125.

[0203] In some embodiments, the polynucleotide can encode an improved ketoreductase comprising an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence corresponding to SEQ ID NO: 42, 44, 46, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126 as listed in Tables 3 and 4, wherein the improved ketoreductase polypeptide amino acid sequence includes any one set of the specified amino acid substitution combinations presented in Tables 3 and 4. In some embodiments, the polynucleotides encode an engineered ketoreductase polypeptide comprising an amino acid sequence selected from SEQ ID NO: 42, 44, 46, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[0204] In some embodiments, the polynucleotides are capable of hybridizing under highly stringent conditions to a polynucleotide comprising SEQ ID NO: 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, and 125, where the polynucleotides encode a functional ketoreductase carrying out the conversion of substrate to product as described herein.

[0205] In various embodiments, the codons are preferably selected to fit the host cell in which the protein is being produced. For example, preferred codons used in bacteria are used to express the gene in bacteria; preferred codons used in yeast are used for expression in yeast; and preferred codons used in mammals are used for expression in mammalian cells. By way of example, the polynucleotide of SEQ ID NO: 3 has been codon optimized for expression in E. coli, but otherwise encodes the naturally occurring ketoreductase of Lactobacillus kefir.

[0206] In certain embodiments, all codons need not be replaced to optimize the codon usage of the ketoreductases since the natural sequence will comprise preferred codons and because use of preferred codons may not be required for all amino acid residues. Consequently, codon optimized polynucleotides encoding the ketoreductase enzymes may contain preferred codons at about 40%, 50%, 60%, 70%, 80%, or greater than 90% of codon positions of the full length coding region.

[0207] In various embodiments, an isolated polynucleotide encoding an improved ketoreductase polypeptide may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the isolated polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides and

nucleic acid sequences utilizing recombinant DNA methods are well known in the art. Guidance is provided in Sambrook et al., 2001, Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press; and Current Protocols in Molecular Biology, Ausubel. F. ed., Greene Pub. Associates, 1998, updates to 2006.

[0208] For bacterial host cells, suitable promoters for directing transcription of the nucleic acid constructs of the present disclosure, include the promoters obtained from the E. coli lac operon, Streptomyces coelicolor agarase gene (dagA), Bacillus subtilis levansucrase gene (sacB), Bacillus licheniformis alpha-amylase gene (amyL), Bacillus stearothermophilus maltogenic amylase gene (amyM), Bacillus amyloliquefaciens alpha-amylase gene (amyQ), Bacillus licheniformis penicillinase gene (penP), Bacillus subtilis xylA and xylB genes, and prokaryotic beta-lactamase gene (Villa-Kamaroff et al., 1978, Proc. Natl Acad. Sci. USA 75: 3727-3731), as well as the tac promoter (DeBoer et al., 1983, Proc. Natl Acad. Sci. USA 80: 21-25).

[0209] For filamentous fungal host cells, suitable promoters for directing the transcription of the nucleic acid constructs of the present disclosure include promoters obtained from the genes for Aspergillus oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, Aspergillus niger neutral alpha-amylase, Aspergillus niger acid stable alpha-amylase, Aspergillus niger or Aspergillus awamori glucoamylase (glaA), Rhizomucor miehei lipase, Aspergillus oryzae alkaline protease, Aspergillus oryzae triose phosphate isomerase, Aspergillus nidulans acetamidase, and Fusarium oxysporum trypsin-like protease (WO 96/00787), as well as the NA2-tpi promoter (a hybrid of the promoters from the genes for Aspergillus niger neutral alpha-amylase and Aspergillus oryzae triose phosphate isomerase), and mutant, truncated, and hybrid promoters thereof.

[0210] In a yeast host, useful promoters can be from the genes for Saccharomyces cerevisiae enolase (ENO-1), Saccharomyces cerevisiae galactokinase (GAL1), Saccharomyces cerevisiae alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP), and Saccharomyces cerevisiae 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos et al., 1992, Yeast 8:423-488.

[0211] The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the polypeptide. Any terminator which is functional in the host cell of choice may be used in the present invention.

[0212] For example, exemplary transcription terminators for filamentous fungal host cells can be obtained from the genes for Aspergillus oryzae TAKA amylase, Aspergillus niger glucoamylase, Aspergillus nidulans anthranilate synthase, Aspergillus niger alpha-glucosidase, and Fusarium oxysporum trypsin-like protease.

[0213] Exemplary terminators for yeast host cells can be obtained from the genes for Saccharomyces cerevisiae enolase, Saccharomyces cerevisiae cytochrome C (CYC1), and Saccharomyces cerevisiae glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos et al., 1992, supra.

[0214] The control sequence may also be a suitable leader sequence, a nontranslated region of an mRNA that is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used. Exemplary leaders for filamentous fungal host cells are obtained from the genes for Aspergillus oryzae TAKA amylase and Aspergillus nidulans triose phosphate isomerase. Suitable leaders for yeast host cells are obtained from the genes for Saccharomyces cerevisiae enolase (ENO-1), Saccharomyces cerevisiae 3-phosphoglycerate kinase, Saccharomyces cerevisiae alpha-factor, and Saccharomyces cerevisiae alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

[0215] The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3' terminus of the nucleic acid sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence which is functional in the host cell of choice may be used in the present invention. Exemplary polyadenylation sequences for filamentous fungal host cells can be from the genes for Aspergillus oryzae TAKA amylase, Aspergillus niger glucoamylase, Aspergillus nidulans anthranilate synthase, Fusarium oxysporum trypsin-like protease, and Aspergillus niger alpha-glucosidase. Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, Mol Cell Bio 15:5983-5990.

[0216] The control sequence may also be a signal peptide coding region that codes for an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleic acid sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region that encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region that is foreign to the coding sequence. The foreign signal peptide coding region may be required where the coding sequence does not naturally contain a signal peptide coding region.

[0217] Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to enhance secretion of the polypeptide. However, any signal peptide coding region which directs the expressed polypeptide into the secretory pathway of a host cell of choice may be used in the present invention.

[0218] Effective signal peptide coding regions for bacterial host cells are the signal peptide coding regions obtained from the genes for Bacillus NClB 11837 maltogenic amylase, Bacillus stearothermophilus alpha-amylase, Bacillus licheniformis subtilisin, Bacillus licheniformis beta-lactamase, Bacillus stearothermophilus neutral proteases (nprT, nprS, nprM), and Bacillus subtilis prsA. Further signal peptides are described by Simonen and Palva, 1993, Microbiol Rev 57: 109-137.

[0219] Effective signal peptide coding regions for filamentous fungal host cells can be the signal peptide coding regions obtained from the genes for Aspergillus oryzae TAKA amylase, Aspergillus niger neutral amylase, Aspergillus niger glucoamylase, Rhizomucor miehei aspartic proteinase, Humicola insolens cellulase, and Humicola lanuginosa lipase.

[0220] Useful signal peptides for yeast host cells can be from the genes for Saccharomyces cerevisiae alpha-factor and Saccharomyces cerevisiae invertase. Other useful signal peptide coding regions are described by Romanos et al., 1992, supra.

[0221] The control sequence may also be a propeptide coding region that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from the genes for Bacillus subtilis alkaline protease (aprE), Bacillus subtilis neutral protease (nprT), Saccharomyces cerevisiae alpha-factor, Rhizomucor miehei aspartic proteinase, and Myceliophthora thermophila lactase (WO 95/33836).

[0222] Where both signal peptide and propeptide regions are present at the amino terminus of a polypeptide, the propeptide region is positioned next to the amino terminus of a polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

[0223] It may also be desirable to add regulatory sequences, which allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. In prokaryotic host cells, suitable regulatory sequences include the lac, tac, and trp operator systems. In yeast host cells, suitable regulatory systems include, as examples, the ADH2 system or GAL1 system. In filamentous fungi, suitable regulatory sequences include the TAKA alpha-amylase promoter, Aspergillus niger glucoamylase promoter, and Aspergillus oryzae glucoamylase promoter.

[0224] Other examples of regulatory sequences are those which allow for gene amplification. In eukaryotic systems, these include the dihydrofolate reductase gene, which is amplified in the presence

of methotrexate, and the metallothionein genes, which are amplified with heavy metals. In these cases, the nucleic acid sequence encoding the KRED polypeptide of the present invention would be operably linked with the regulatory sequence.

[0225] Thus, in another embodiment, the present disclosure is also directed to a recombinant expression vector comprising a polynucleotide encoding an engineered ketoreductase polypeptide or a variant thereof, and one or more expression regulating regions such as a promoter and a terminator, a replication origin, etc., depending on the type of hosts into which they are to be introduced. The various nucleic acid and control sequences described above may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the polypeptide at such sites. Alternatively, the nucleic acid sequence of the present disclosure may be expressed by inserting the nucleic acid sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

[0226] The recombinant expression vector may be any vector (*e.g.*, a plasmid or virus), which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the polynucleotide sequence. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids.

[0227] The expression vector may be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon may be used.

[0228] The expression vector of the present invention preferably contains one or more selectable markers, which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Examples of bacterial selectable markers are the dal genes from Bacillus subtilis or Bacillus licheniformis, or markers, which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol (Example 1) or tetracycline resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3.

[0229] Selectable markers for use in a filamentous fungal host cell include, but are not limited to, amdS (acetamidase), argB (ornithine carbamoyltransferase), bar (phosphinothricin acetyltransferase), hph (hygromycin phosphotransferase), niaD (nitrate reductase), pyrG (orotidine-5'-phosphate decarboxylase), sC (sulfate adenyltransferase), and trpC (anthranilate synthase), as well as equivalents thereof. Embodiments for use in an Aspergillus cell include the amdS and pyrG genes of Aspergillus nidulans or Aspergillus oryzae and the bar gene of Streptomyces hygroscopicus.

[0230] The expression vectors of the present invention preferably contain an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome. For integration into the host cell genome, the vector may rely on the nucleic acid sequence encoding the polypeptide or any other element of the vector for integration of the vector into the genome by homologous or nonhomologous recombination.

[0231] Alternatively, the expression vector may contain additional nucleic acid sequences for directing integration by homologous recombination into the genome of the host cell. The additional nucleic acid sequences enable the vector to be integrated into the host cell genome at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, preferably 400 to 10,000 base pairs, and most preferably 800 to 10,000 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleic acid sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

[0232] For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. Examples of bacterial origins of replication are P15A ori (as shown in the plasmid of FIG. 5) or the origins of replication of plasmids pBR322, pUC19, pACYCl77 (which plasmid has the P15A ori), or pACYC184 permitting replication in E. coli, and pUB110, pE194, pTA1060, or pAMβ1 permitting replication in Bacillus. Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6. The origin of replication may be one having a mutation which makes it's functioning temperature-sensitive in the host cell (see, *e.g.*, Ehrlich, 1978, Proc Natl Acad Sci. USA 75:1433).

[0233] More than one copy of a nucleic acid sequence of the present invention may be inserted into the host cell to increase production of the gene product. An increase in the copy number of the nucleic acid sequence can be obtained by integrating at least one additional copy of the sequence into

the host cell genome or by including an amplifiable selectable marker gene with the nucleic acid sequence where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the nucleic acid sequence, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

[0234] Many of the expression vectors for use in the present invention are commercially available. Suitable commercial expression vectors include p3xFLAGTM™ expression vectors from Sigma-Aldrich Chemicals, St. Louis MO., which includes a CMV promoter and hGH polyadenylation site for expression in mammalian host cells and a pBR322 origin of replication and ampicillin resistance markers for amplification in E. coli. Other suitable expression vectors are pBluescriptII SK(-) and pBK-CMV, which are commercially available from Stratagene, LaJolla CA, and plasmids which are derived from pBR322 (Gibco BRL), pUC (Gibco BRL), pREP4, pCEP4 (Invitrogen) or pPoly (Lathe et al., 1987, Gene 57:193-201).

6.4 Host Cells for Expression of Ketoreductase Polypeptides

[0235] In another aspect, the present disclosure provides a host cell comprising a polynucleotide encoding an improved ketoreductase polypeptide of the present disclosure, the polynucleotide being operatively linked to one or more control sequences for expression of the ketoreductase enzyme in the host cell. Host cells for use in expressing the KRED polypeptides encoded by the expression vectors of the present invention are well known in the art and include but are not limited to, bacterial cells, such as E. coli, Lactobacillus kefir, Lactobacillus brevis, Lactobacillus minor, Streptomyces and Salmonella typhimurium cells; fungal cells, such as yeast cells (*e.g.*, Saccharomyces cerevisiae or Pichia pastoris (ATCC Accession No. 201178)); insect cells such as Drosophila S2 and Spodoptera Sf9 cells; animal cells such as CHO, COS, BHK, 293, and Bowes melanoma cells; and plant cells. Appropriate culture mediums and growth conditions for the above-described host cells are well known in the art.

[0236] Polynucleotides for expression of the ketoreductase may be introduced into cells by various methods known in the art. Techniques include among others, electroporation, biolistic particle bombardment, liposome mediated transfection, calcium chloride transfection, and protoplast fusion. Various methods for introducing polynucleotides into cells will be apparent to the skilled artisan.

[0237] An exemplary host cell is Escherichia coli W3110. The expression vector was created by operatively linking a polynucleotide encoding an improved ketoreductase into the plasmid pCK110900 operatively linked to the lac promoter under control of the lacI repressor. The expression vector also contained the P15a origin of replication and the chloramphenicol resistance gene. Cells

containing the subject polynucleotide in Escherichia coli W3110 were isolated by subjecting the cells to chloramphenicol selection.

6.5 Methods of Generating Engineered Ketoreductase Polypeptides.

[0238] In some embodiments, to make the improved KRED polynucleotides and polypeptides of the present disclosure, the naturally-occurring ketoreductase enzyme that catalyzes the reduction reaction is obtained (or derived) from Lactobacillus kefir or Lactobacillus brevis. In some embodiments, the parent polynucleotide sequence is codon optimized to enhance expression of the ketoreductase in a specified host cell. As an illustration, the parental polynucleotide sequence encoding the wild-type KRED polypeptide of Lactobacillus kefir was constructed from oligonucleotides prepared based upon the known polypeptide sequence of Lactobacillus kefir KRED sequence available in Genbank database (Genbank accession no. AAP94029 GI:33112056). The parental polynucleotide sequence, designated as SEQ ID NO: 3, was codon optimized for expression in E. coli and the codon-optimized polynucleotide cloned into an expression vector, placing the expression of the ketoreductase gene under the control of the lac promoter and lacI repressor gene. Clones expressing the active ketoreductase in E. coli were identified and the genes sequenced to confirm their identity. The sequence designated (SEQ ID NO: 3) was the parent sequence utilized as the starting point for most experiments and library construction of engineered ketoreductases evolved from the Lactobacillus kefir ketoreductase.

[0239] The engineered ketoreductases can be obtained by subjecting the polynucleoticde encoding the naturally occurring ketoreductase to mutagenesis and/or directed evolution methods, as discussed above. An exemplary directed evolution technique is mutagenesis and/or DNA shuffling as described in Stemmer, 1994, Proc Natl Acad Sci USA 91:10747-10751; WO 95/22625; WO 97/0078; WO 97/35966; WO 98/27230; WO 00/42651; WO 01/75767 and U.S. Pat. 6,537,746. Other directed evolution procedures that can be used include, among others, staggered extension process (StEP), in vitro recombination (Zhao et al., 1998, Nat. Biotechnol. 16:258–261), mutagenic PCR (Caldwell et al., 1994, PCR Methods Appl. 3:S136-S140), and cassette mutagenesis (Black et al., 1996, Proc Natl Acad Sci USA 93:3525-3529).

[0240] The clones obtained following mutagenesis treatment are screened for engineered ketoreductases having a desired improved enzyme property. Measuring enzyme activity from the expression libraries can be performed using the standard biochemistry technique of monitoring the rate of decrease (via a decrease in absorbance or fluorescence) of NADH or NADPH concentration, as it is converted into NAD^+ or $NADP^+$. In this reaction, the NADH or NADPH is consumed (oxidized) by the ketoreductase as the ketoreductase reduces a ketone substrate to the corresponding hydroxyl group. The rate of decrease of NADH or NADPH concentration, as measured by the decrease in

absorbance or fluorescence, per unit time indicates the relative (enzymatic) activity of the KRED polypeptide in a fixed amount of the lysate (or a lyophilized powder made therefrom). Where the improved enzyme property desired is thermal stability, enzyme activity may be measured after subjecting the enzyme preparations to a defined temperature and measuring the amount of enzyme activity remaining after heat treatments. Clones containing a polynucleotide encoding a ketoreductase are then isolated, sequenced to identify the nucleotide sequence changes (if any), and used to express the enzyme in a host cell.

[0241] Where the sequence of the engineered polypeptide is known, the polynucleotides encoding the enzyme can be prepared by standard solid-phase methods, according to known synthetic methods. In some embodiments, fragments of up to about 100 bases can be individually synthesized, then joined (*e.g.*, by enzymatic or chemical litigation methods, or polymerase mediated methods) to form any desired continuous sequence. For example, polynucleotides and oligonucleotides of the invention can be prepared by chemical synthesis using, *e.g.*, the classical phosphoramidite method described by Beaucage et al., 1981, Tet Lett 22:1859-69, or the method described by Matthes et al., 1984, EMBO J. 3:801-05, *e.g.*, as it is typically practiced in automated synthetic methods. According to the phosphoramidite method, oligonucleotides are synthesized, *e.g.*, in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors. In addition, essentially any nucleic acid can be obtained from any of a variety of commercial sources, such as The Midland Certified Reagent Company, Midland, TX, The Great American Gene Company, Ramona, CA, ExpressGen Inc. Chicago, IL, Operon Technologies Inc., Alameda, CA, and many others.

[0242] Engineered ketoreductase enzymes expressed in a host cell can be recovered from the cells and or the culture medium using any one or more of the well known techniques for protein purification, including, among others, lysozyme treatment, sonication, filtration, salting-out, ultra-centrifugation, and chromatography. Suitable solutions for lysing and the high efficiency extraction of proteins from bacteria, such as E. coli, are commercially available under the trade name CelLytic B™ from Sigma-Aldrich of St. Louis MO.

[0243] Chromatographic techniques for isolation of the ketoreductase polypeptide include, among others, reverse phase chromatography high performance liquid chromatography, ion exchange chromatography, gel electrophoresis, and affinity chromatography. Conditions for purifying a particular enzyme will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity, molecular weight, molecular shape, etc., and will be apparent to those having skill in the art.

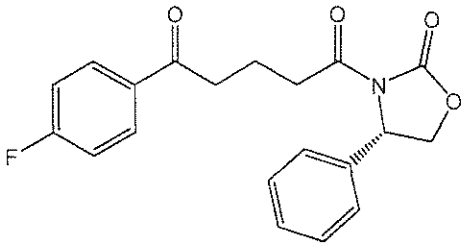
[0244] In some embodiments, affinity techniques may be used to isolate the improved ketoreductase enzymes. For affinity chromatography purification, any antibody which specifically binds the ketoreductase polypeptide may be used. For the production of antibodies, various host animals,

including but not limited to rabbits, mice, rats, etc., may be immunized by injection with a compound. The compound may be attached to a suitable carrier, such as BSA, by means of a side chain functional group or linkers attached to a side chain functional group. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacilli Calmette Guerin) and Corynebacterium parvum.

6.6 Methods of Using the Engineered Ketoreductase Enzymes and Compounds Prepared Therewith

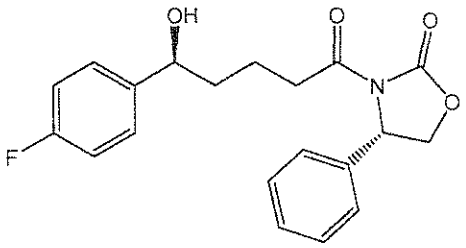
[0245] The ketoreductase enzymes described herein can catalyze the reduction of the substrate compound of structural formula (I) (5-((4S)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione:

(I)



to the corresponding stereosiomeric product of structural formula (II) ((4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one):

(II)

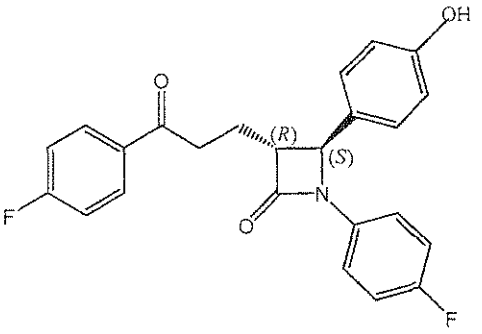


[0246] In some embodiments, the method for reducing the substrate having the chemical formula (I) to the corresponding product of formula (II) comprises contacting or incubating the substrate with a ketoreductase polypeptides disclosed herein under reaction conditions suitable for reducing or converting the substrate to the product compound. The product in an intermediate for the synthesis of Ezetimibe, an anti-hyperlipidemic drug for lowering cholesterol levels (U.S. Patent No. 5,767,115).

Thus, in a method for synthesizing Ezetimibe, the method can comprises a step in which the compound of formula (I) is converted to the compound of formula (II) using a ketoreductase polypeptide disclosed herein. In some embodiments, the product in greater than about 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% stereomeric excess over the corresponding (*R*) alcohol product.

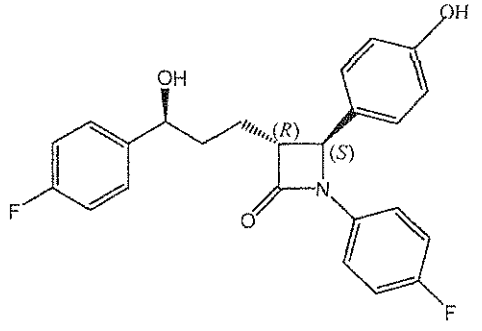
[0247] In some embodiments, the ketoreductase enzymes described herein are also capable of catalyzing the reduction reaction of the keto group in the compound of structural formula (III), 1-(4-fluorophenyl)-3(R)-[3-oxo-3-(4-fluorophenyl)propyl)]-4(S)-(4-hydroxyphenyl)-2-azetidinone,

(III)



to the corresponding stereoisomeric alcohol product of structural formula (**IV**), 1-(4-fluorophenyl)-3(R)-[3(S)-hydroxy-3(4-fluorophenyl)-propyl)]-4(S)-(4-hydroxyphenyl)-2-azetidinone (i.e., Ezetimibe):

(IV)



[0248] Thus, the present disclosure provides a method of synthesizing Ezetimibe, the method comprising contacting or incubating the compound of formula (III) with a ketoreductase polypeptide disclosed herein under reaction conditions suitable for reducing or converting the substrate compound of formula (III) to the production compound of formula (IV). Other compounds similar to the compounds of formula (I) and compounds of formula (III) are described in US Patent No. 5,767,115 (incorporated herein by reference).

[0249] In the method for reducing the compound of formula (I) to the compound of formula (II), or for reducing the compound of formula (III) to the compound of formula (IV), the ketoreductase polypeptides have, as compared to the wild-type *L. kefir*, *L. brevis*, *L. minor* KRED sequences of SEQ ID NO:4, 2, and 158, respectively, at least the following amino acid substitutions: (1) residue 145 is serine and (2) residue 190 is cysteine. Various embodiments of the ketoreductase polypeptides are described above. In some embodiments, as compared to the wild-type L. kefi, L. brevis, L. minor KRED sequences of SEQ ID NO:4, 2, and 158, the ketoreductase polypeptides have at least the following amino acid substitutions: (1) residue 145 is a serine residue, (2) residue 190 is a cysteine residue, and (3) residue 96 is a glutamine residue. In some embodiments, as compared to the wild-type L. kefi, L. brevis, L. minor KRED sequences of SEQ ID NO:4, 2, and 158, the ketoreductase polypeptides of the invention have at least the following amino acid substitutions: (1) residue X145 is a serine residue, (2) residue X190 is a cysteine residue, and (3) residue X211 is an arginine residue.

[0250] As noted herein, in some embodiments, the ketoreductase polypeptides can comprise an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical as compared a reference sequence comprising the sequence of SEQ ID NO:128, 130, or 160, with the proviso that the polypeptide comprises an amino acid sequence in which the amino acid residue corresponding to residue X145 is a serine, and the amino acid residue corresponding to residue X190 is a cysteine. In some embodiments, these ketoreductase polypeptides can have one or more modifications to the amino acid sequence of SEQ ID NO:128, 130 or 160. The modifications can include substitutions, deletions, and insertions. The substitutions can be non-conservative substitutions, conservative substitutions, or a combination of non-conservative and conservative substitutions.

[0251] In some embodiments of the method for reducing the substrate to the product, the substrate is reduced to the product in greater than about 99% stereomeric excess, wherein the ketoreductase polypeptide comprises a sequence that corresponds to SEQ ID NO: SEQ ID NO: 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[0252] In another embodiment of this method for reducing the substrate to the product, at least about 95% of the substrate is converted to the product in less than about 24 hours when carried out with greater than about 100 g/L of substrate and less than about 5 g/L of the polypeptide, wherein the polypeptide comprises an amino acid sequence corresponding to SEQ ID NO:102, 108, 120, 122, 124, 126.

[0253] As is known by those of skill in the art, ketoreductase-catalyzed reduction reactions typically require a cofactor. Reduction reactions catalyzed by the engineered ketoreductase enzymes described

herein also typically require a cofactor, although many embodiments of the engineered ketoreductases require far less cofactor than reactions catalyzed with wild-type ketoreductase enzymes. As used herein, the term "cofactor" refers to a non-protein compound that operates in combination with a ketoreductase enzyme. Cofactors suitable for use with the engineered ketoreductase enzymes described herein include, but are not limited to, $NADP^+$ (nicotinamide adenine dinucleotide phosphate), NADPH (the reduced form of $NADP^+$), NAD^+ (nicotinamide adenine dinucleotide) and NADH (the reduced form of NAD^+). Generally, the reduced form of the cofactor is added to the reaction mixture. The reduced NAD(P)H form can be optionally regenerated from the oxidized $NAD(P)^+$ form using a cofactor regeneration system.

[0254] The term "cofactor regeneration system" refers to a set of reactants that participate in a reaction that reduces the oxidized form of the cofactor (*e.g.*, $NADP^+$ to NADPH). Cofactors oxidized by the ketoreductase-catalyzed reduction of the keto substrate are regenerated in reduced form by the cofactor regeneration system. Cofactor regeneration systems comprise a stoichiometric reductant that is a source of reducing hydrogen equivalents and is capable of reducing the oxidized form of the cofactor. The cofactor regeneration system may further comprise a catalyst, for example an enzyme catalyst that catalyzes the reduction of the oxidized form of the cofactor by the reductant. Cofactor regeneration systems to regenerate NADH or NADPH from NAD^+ or $NADP^+$, respectively, are known in the art and may be used in the methods described herein.

[0255] Suitable exemplary cofactor regeneration systems that may be employed include, but are not limited to, glucose and glucose dehydrogenase, formate and formate dehydrogenase, glucose-6-phosphate and glucose-6-phosphate dehydrogenase, a secondary (*e.g.*, isopropanol) alcohol and secondary alcohol dehydrogenase, phosphite and phosphite dehydrogenase, molecular hydrogen and hydrogenase, and the like. These systems may be used in combination with either $NADP^+$/NADPH or NAD^+/NADH as the cofactor. Electrochemical regeneration using hydrogenase may also be used as a cofactor regeneration system. See, *e.g.*, U.S. Pat. Nos. 5,538,867 and 6,495,023, both of which are incorporated herein by reference. Chemical cofactor regeneration systems comprising a metal catalyst and a reducing agent (for example, molecular hydrogen or formate) are also suitable. See, *e.g.*, PCT publication WO 2000/053731, which is incorporated herein by reference.

[0256] The terms "glucose dehydrogenase" and "GDH" are used interchangeably herein to refer to an NAD^+ or $NADP^+$-dependent enzyme that catalyzes the conversion of D-glucose and NAD^+ or $NADP^+$ to gluconic acid and NADH or NADPH, respectively. Equation (1), below, describes the glucose dehydrogenase-catalyzed reduction of NAD^+ or $NADP^+$ by glucose.

(1) glucose + $NAD(P)^+$ + H_2O $\xrightarrow{GDH}$ gluconic acid + NAD(P)H + H^+

[0257] Glucose dehydrogenases that are suitable for use in the practice of the methods described herein include both naturally occurring glucose dehydrogenases, as well as non-naturally occurring glucose dehydrogenases. Naturally occurring glucose dehydrogenase encoding genes have been reported in the literature. For example, the Bacillus subtilis 61297 GDH gene was expressed in E. coli and was reported to exhibit the same physicochemical properties as the enzyme produced in its native host (Vasantha et al., 1983, Proc. Natl. Acad. Sci. USA 80:785). The gene sequence of the B. subtilis GDH gene, which corresponds to Genbank Acc. No. M12276, was reported by Lampel et al., 1986, J. Bacteriol. 166:238-243, and in corrected form by Yamane et al., 1996, Microbiology 142:3047-3056 as Genbank Acc. No. D50453. Naturally occurring GDH genes also include those that encode the GDH from B. cereus ATCC 14579 (Nature, 2003, 423:87-91; Genbank Acc. No. AE017013) and B. megaterium (Eur. J. Biochem., 1988, 174:485-490, Genbank Acc. No. X12370; J. Ferment. Bioeng., 1990, 70:363-369, Genbank Acc. No. GI216270). Glucose dehydrogenases from Bacillus sp. are provided in PCT publication WO 2005/018579 as SEQ ID NOS: 10 and 12 (encoded by polynucleotide sequences corresponding to SEQ ID NOS: 9 and 11, respectively, of the PCT publication), the disclosure of which is incorporated herein by reference.

[0258] Non-naturally occurring glucose dehydrogenases may be generated using known methods, such as, for example, mutagenesis, directed evolution, and the like. GDH enzymes having suitable activity, whether naturally occurring or non-naturally occurring, may be readily identified using the assay described in Example 4 of PCT publication WO 2005/018579, the disclosure of which is incorporated herein by reference. Exemplary non-naturally occurring glucose dehydrogenases are provided in PCT publication WO 2005/018579 as SEQ ID NOS: 62, 64, 66, 68, 122, 124, and 126. The polynucleotide sequences that encode them are provided in PCT publication WO 2005/018579 as SEQ ID NOS: 61, 63, 65, 67, 121, 123, and 125, respectively. All of these sequences are incorporated herein by reference. Additional non-naturally occurring glucose dehydrogenases that are suitable for use in the ketoreductase-catalyzed reduction reactions disclosed herein are provided in U.S. application publication Nos. 2005/0095619 and 2005/0153417, the disclosures of which are incorporated herein by reference.

[0259] Glucose dehydrogenases employed in the ketoreductase-catalyzed reduction reactions described herein may exhibit an activity of at least about 10 μmol/min/mg and sometimes at least about 10^2 μmol/min/mg or about 10^3 μmol/min/mg, up to about 10^4 μmol/min/mg or higher in the assay described in Example 4 of PCT publication WO 2005/018579.

[0260] The ketoreductase-catalyzed reduction reactions described herein are generally carried out in a solvent. Suitable solvents include water, organic solvents (*e.g.*, ethyl acetate, butyl acetate, 1-octanol, heptane, octane, methyl t-butyl ether (MTBE), toluene, and the like), and ionic liquids (*e.g.*, 1-ethyl 4-methylimidazolium tetrafluoroborate, 1-butyl-3-methylimidazolium tetrafluoroborate, 1-butyl-3-methylimidazolium hexafluorophosphate, and the like). In some embodiments, aqueous solvents, including water and aqueous co-solvent systems, are used.

[0261] Exemplary aqueous co-solvent systems have water and one or more organic solvent. In general, an organic solvent component of an aqueous co-solvent system is selected such that it does not completely inactivate the ketoreductase enzyme. Appropriate co-solvent systems can be readily identified by measuring the enzymatic activity of the specified engineered ketoreductase enzyme with a defined substrate of interest in the candidate solvent system, utilizing an enzyme activity assay, such as those described herein.

[0262] The organic solvent component of an aqueous co-solvent system may be miscible with the aqueous component, providing a single liquid phase, or may be partly miscible or immiscible with the aqueous component, providing two liquid phases. Generally, when an aqueous co-solvent system is employed, it is selected to be biphasic, with water dispersed in an organic solvent, or vice-versa. Generally, when an aqueous co-solvent system is utilized, it is desirable to select an organic solvent that can be readily separated from the aqueous phase. In general, the ratio of water to organic solvent in the co-solvent system is typically in the range of from about 90:10 to about 10:90 (v/v) organic solvent to water, and between 80:20 and 20:80 (v/v) organic solvent to water. The co-solvent system may be pre-formed prior to addition to the reaction mixture, or it may be formed in situ in the reaction vessel.

[0263] The aqueous solvent (water or aqueous co-solvent system) may be pH-buffered or unbuffered. Generally, the reduction can be carried out at a pH of about 10 or below, usually in the range of from about 5 to about 10. In some embodiments, the reduction is carried out at a pH of about 9 or below, usually in the range of from about 5 to about 9. In some embodiments, the reduction is carried out at a pH of about 8 or below, often in the range of from about 5 to about 8, and usually in the range of from about 6 to about 8. The reduction may also be carried out at a pH of about 7.8 or below, or 7.5 or below. Alternatively, the reduction may be carried out a neutral pH, i.e., about 7.

[0264] During the course of the reduction reactions, the pH of the reaction mixture may change. The pH of the reaction mixture may be maintained at a desired pH or within a desired pH range by the addition of an acid or a base during the course of the reaction. Alternatively, the pH may be controlled by using an aqueous solvent that comprises a buffer. Suitable buffers to maintain desired

pH ranges are known in the art and include, for example, phosphate buffer, triethanolamine buffer, and the like. Combinations of buffering and acid or base addition may also be used.

[0265] When the glucose/glucose dehydrogenase cofactor regeneration system is employed, the co-production of gluconic acid (pKa=3.6), as represented in equation (1) causes the pH of the reaction mixture to drop if the resulting aqueous gluconic acid is not otherwise neutralized. The pH of the reaction mixture may be maintained at the desired level by standard buffering techniques, wherein the buffer neutralizes the gluconic acid up to the buffering capacity provided, or by the addition of a base concurrent with the course of the conversion. Combinations of buffering and base addition may also be used. Suitable buffers to maintain desired pH ranges are described above. Suitable bases for neutralization of gluconic acid are organic bases, for example amines, alkoxides and the like, and inorganic bases, for example, hydroxide salts (*e.g.*, NaOH), carbonate salts (*e.g.*, $NaHCO_3$), bicarbonate salts (*e.g.*, K_2CO_3), basic phosphate salts (*e.g.*, K_2HPO_4, Na_3PO_4), and the like. The addition of a base concurrent with the course of the conversion may be done manually while monitoring the reaction mixture pH or, more conveniently, by using an automatic titrator as a pH stat. A combination of partial buffering capacity and base addition can also be used for process control.

[0266] When base addition is employed to neutralize gluconic acid released during a ketoreductase-catalyzed reduction reaction, the progress of the conversion may be monitored by the amount of base added to maintain the pH. Typically, bases added to unbuffered or partially buffered reaction mixtures over the course of the reduction are added in aqueous solutions.

[0267] In some embodiments, the co-factor regenerating system can comprises a formate dehydrogenase. The terms "formate dehydrogenase" and "FDH" are used interchangeably herein to refer to an NAD^+ or $NADP^+$-dependent enzyme that catalyzes the conversion of formate and NAD^+ or $NADP^+$ to carbon dioxide and NADH or NADPH, respectively. Formate dehydrogenases that are suitable for use as cofactor regenerating systems in the ketoreductase-catalyzed reduction reactions described herein include both naturally occurring formate dehydrogenases, as well as non-naturally occurring formate dehydrogenases. Formate dehydrogenases include those corresponding to SEQ ID NOS: 70 (Pseudomonas sp.) and 72 (Candida boidinii) of PCT publication WO 2005/018579, which are encoded by polynucleotide sequences corresponding to SEQ ID NOS: 69 and 71, respectively, of PCT publication 2005/018579, the disclosures of which are incorporated herein by reference. Formate dehydrogenases employed in the methods described herein, whether naturally occurring or non-naturally occurring, may exhibit an activity of at least about 1 μmol/min/mg, sometimes at least about 10 μmol/min/mg, or at least about 10^2 μmol/min/mg, up to about 10^3 μmol/min/mg or higher, and can be readily screened for activity in the assay described in Example 4 of PCT publication WO 2005/018579.

[0268] As used herein, the term "formate" refers to formate anion (HCO_2^-), formic acid (HCO_2H), and mixtures thereof. Formate may be provided in the form of a salt, typically an alkali or ammonium salt (for example, HCO_2Na, $KHCO_2NH_4$, and the like), in the form of formic acid, typically aqueous formic acid, or mixtures thereof. Formic acid is a moderate acid. In aqueous solutions within several pH units of its pKa (pKa=3.7 in water) formate is present as both HCO_2^- and HCO_2H in equilibrium concentrations. At pH values above about pH 4, formate is predominantly present as HCO_2^-. When formate is provided as formic acid, the reaction mixture is typically buffered or made less acidic by adding a base to provide the desired pH, typically of about pH 5 or above. Suitable bases for neutralization of formic acid include, but are not limited to, organic bases, for example amines, alkoxides and the like, and inorganic bases, for example, hydroxide salts (*e.g.*, NaOH), carbonate salts (*e.g.*, $NaHCO_3$), bicarbonate salts (*e.g.*, K_2CO_3), basic phosphate salts (*e.g.*, K_2HPO_4, Na_3PO_4), and the like.

[0269] For pH values above about pH 5, at which formate is predominantly present as HCO_2^-, Equation (2) below, describes the formate dehydrogenase-catalyzed reduction of NAD^+ or $NADP^+$ by formate.

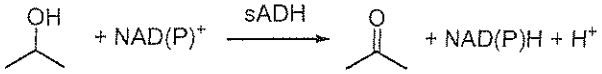
$$(2) \qquad HCO_2^- + NAD(P)^+ \xrightarrow{FDH} CO_2 + NAD(P)H$$

[0270] When formate and formate dehydrogenase are employed as the cofactor regeneration system, the pH of the reaction mixture may be maintained at the desired level by standard buffering techniques, wherein the buffer releases protons up to the buffering capacity provided, or by the addition of an acid concurrent with the course of the conversion. Suitable acids to add during the course of the reaction to maintain the pH include organic acids, for example carboxylic acids, sulfonic acids, phosphonic acids, and the like, mineral acids, for example hydrohalic acids (such as hydrochloric acid), sulfuric acid, phosphoric acid, and the like, acidic salts, for example dihydrogenphosphate salts (*e.g.*, KH_2PO_4), bisulfate salts (*e.g.*, $NaHSO_4$) and the like. Some embodiments utilize formic acid, whereby both the formate concentration and the pH of the solution are maintained.

[0271] When acid addition is employed to maintain the pH during a reduction reaction using the formate/formate dehydrogenase cofactor regeneration system, the progress of the conversion may be monitored by the amount of acid added to maintain the pH. Typically, acids added to unbuffered or partially buffered reaction mixtures over the course of conversion are added in aqueous solutions.

[0272] The terms "secondary alcohol dehydrogenase" and "sADH" are used interchangeably herein to refer to an NAD^+ or $NADP^+$-dependent enzyme that catalyzes the conversion of a secondary

alcohol and NAD^+ or $NADP^+$ to a ketone and NADH or NADPH, respectively. Equation (3), below, describes the reduction of NAD^+ or $NADP^+$ by a secondary alcohol, illustrated by isopropanol.

(3) 

[0273] Secondary alcohol dehydrogenases that are suitable for use as cofactor regenerating systems in the ketoreductase-catalyzed reduction reactions described herein include both naturally occurring secondary alcohol dehydrogenases , as well as non-naturally occurring secondary alcohol dehydrogenases. Naturally occurring secondary alcohol dehydrogenases include known alcohol dehydrogenases from, Thermoanerobium brockii, Rhodococcus etythropolis, Lactobacillus kefir, Lactobacillus minor and Lactobacillus brevis, and non-naturally occurring secondary alcohol dehydrogenases include engineered alcohol dehdyrogenases derived therefrom. Secondary alcohol dehydrogenases employed in the methods described herein, whether naturally occurring or non-naturally occurring, may exhibit an activity of at least about 1 μmol/min/mg, sometimes at least about 10 μmol/min/mg, or at least about 10^2 μmol/min/mg, up to about 10^3 μmol/min/mg or higher.

[0274] Suitable secondary alcohols include lower secondary alkanols and aryl-alkyl carbinols. Examples of lower secondary alcohols include isopropanol, 2-butanol, 3-methyl-2-butanol, 2-pentanol, 3-pentanol, 3,3-dimethyl-2-butanol, and the like. In one embodiment the secondary alcohol is isopropanol. Suitable aryl-akyl carbinols include unsubstituted and substituted 1-arylethanols.

[0275] In one embodiment, where oxidation of isopropanol to acetone is used for regeneration of NADH/NADPH, the reaction may be run at reduced pressure in such a manner that the acetone is removed from the reaction mixture.

[0276] When a secondary alcohol and secondary alcohol dehydrogenase are employed as the cofactor regeneration system, the resulting NAD^+ or $NADP^+$ is reduced by the coupled oxidation of the secondary alcohol to the ketone by the secondary alcohol dehydrogenase. Some engineered ketoreductases also have activity to dehydrogenate a secondary alcohol reductant. In some embodiments using secondary alcohol as reductant, the engineered ketoreductase and the secondary alcohol dehydrogenase are the same enzyme.

[0277] In carrying out embodiments of the ketoreductase-catalyzed reduction reactions described herein employing a cofactor regeneration system, either the oxidized or reduced form of the cofactor may be provided initially. As described above, the cofactor regeneration system converts oxidized cofactor to its reduced form, which is then utilized in the reduction of the ketoreductase substrate.

[0278] In some embodiments, cofactor regeneration systems are not used. For reduction reactions carried out without the use of a cofactor regenerating systems, the cofactor is added to the reaction mixture in reduced form.

[0279] In some embodiments, when the process is carried out using whole cells of the host organism, the whole cell may natively provide the cofactor. Alternatively or in combination, the cell may natively or recombinantly provide the glucose dehydrogenase.

[0280] In carrying out the stereoselective reduction reactions described herein, the engineered ketoreductase enzyme, and any enzymes comprising the optional cofactor regeneration system, may be added to the reaction mixture in the form of the purified enzymes, whole cells transformed with gene(s) encoding the enzymes, and/or cell extracts and/or lysates of such cells. The gene(s) encoding the engineered ketoreductase enzyme and the optional cofactor regeneration enzymes can be transformed into host cells separately or together into the same host cell. For example, in some embodiments one set of host cells can be transformed with gene(s) encoding the engineered ketoreductase enzyme and another set can be transformed with gene(s) encoding the cofactor regeneration enzymes. Both sets of transformed cells can be utilized together in the reaction mixture in the form of whole cells, or in the form of lysates or extracts derived therefrom. In other embodiments, a host cell can be transformed with gene(s) encoding both the engineered ketoreductase enzyme and the cofactor regeneration enzymes.

[0281] Whole cells transformed with gene(s) encoding the engineered ketoreductase enzyme and/or the optional cofactor regeneration enzymes, or cell extracts and/or lysates thereof, may be employed in a variety of different forms, including solid (*e.g.*, lyophilized, spray-dried, and the like) or semisolid (*e.g.*, a crude paste).

[0282] The cell extracts or cell lysates may be partially purified by precipitation (ammonium sulfate, polyethyleneimine, heat treatment or the like, followed by a desalting procedure prior to lyophilization (*e.g.*, ultrafiltration, dialysis, and the like). Any of the cell preparations may be stabilized by crosslinking using known crosslinking agents, such as, for example, glutaraldehyde or immobilization to a solid phase (*e.g.*, Eupergit C, and the like).

[0283] The solid reactants (*e.g.*, enzyme, salts, etc.) may be provided to the reaction in a variety of different forms, including powder (*e.g.*, lyophilized, spray dried, and the like), solution, emulsion, suspension, and the like. The reactants can be readily lyophilized or spray dried using methods and equipment that are known to those having ordinary skill in the art. For example, the protein solution can be frozen at -80°C in small aliquots, then added to a prechilled lyophilization chamber, followed by the application of a vacuum. After the removal of water from the samples, the temperature is

typically raised to 4°C for two hours before release of the vacuum and retrieval of the lyophilized samples.

[0284] The quantities of reactants used in the reduction reaction will generally vary depending on the quantities of product desired, and concomitantly the amount of ketoreductase substrate employed. The following guidelines can be used to determine the amounts of ketoreductase, cofactor, and optional cofactor regeneration system to use. Generally, keto substrates can be employed at a concentration of about 20 to 300 grams/liter using from about 50 mg to about 5 g of ketoreductase and about 10 mg to about 150 mg of cofactor. Those having ordinary skill in the art will readily understand how to vary these quantities to tailor them to the desired level of productivity and scale of production. Appropriate quantities of optional cofactor regeneration system may be readily determined by routine experimentation based on the amount of cofactor and/or ketoreductase utilized. In general, the reductant (*e.g.*, glucose, formate, and isopropanol) is utilized at levels above the equimolar level of ketoreductase substrate to achieve essentially complete or near complete conversion of the ketoreductase substrate.

[0285] The order of addition of reactants is not critical. The reactants may be added together at the same time to a solvent (*e.g.*, monophasic solvent, biphasic aqueous co-solvent system, and the like), or alternatively, some of the reactants may be added separately, and some together at different time points. For example, the cofactor regeneration system, cofactor, ketoreductase, and ketoreductase substrate may be added first to the solvent.

[0286] For improved mixing efficiency when an aqueous co-solvent system is used, the cofactor regeneration system, ketoreductase, and cofactor may be added and mixed into the aqueous phase first. The organic phase may then be added and mixed in, followed by addition of the ketoreductase substrate. Alternatively, the ketoreductase substrate may be premixed in the organic phase, prior to addition to the aqueous phase

[0287] Suitable conditions for carrying out the ketoreductase-catalyzed reduction reactions described herein include a wide variety of conditions which can be readily optimized by routine experimentation that includes, but is not limited to, contacting the engineered ketoreductase enzyme and substrate at an experimental pH and temperature and detecting product, for example, using the methods described in the Examples provided herein.

[0288] The ketoreductase catalyzed reduction is typically carried out at a temperature in the range of from about 15°C to about 75°C. For some embodiments, the reaction is carried out at a temperature in the range of from about 20°C to about 55°C. In still other embodiments, it is carried out at a temperature in the range of from about 20°C to about 45°C. The reaction may also be carried out under ambient conditions.

[0289] The reduction reaction is generally allowed to proceed until essentially complete, or near complete, reduction of substrate is obtained. Reduction of substrate to product can be monitored using known methods by detecting substrate and/or product. Suitable methods include gas chromatography, HPLC, and the like. Conversion yields of the alcohol reduction product generated in the reaction mixture are generally greater than about 50%, may also be greater than about 60%, may also be greater than about 70%, may also be greater than about 80%, may also be greater than 90%, and are often greater than about 97%.

7. EXAMPLES

[0290] Various features and embodiments of the disclosure are illustrated in the following representative examples, which are intended to be illustrative, and not limiting.

[0291] In the following descriptions, wherever glucose dehydrogenase (GDH) is used, it is GDH CDX901, obtainable from Julich Chiral Solutions, Jülich, Germany.

7.1 Example 1: Wild-type Ketoreductase Gene Acquisition and Construction of Expression Vectors.

[0292] Ketoreductase (KRED) encoding genes are designed for expression in E. coli based on the reported amino acid sequence of the ketoreductase and a codon optimization algorithm as described in Example 1 of US provisional application Serial No. 60/848,950, incorporated herein by reference. (Standard codon-optimization software also is reviewed in *e.g.*,"OPTIMIZER: a web server for optimizing the codon usage of DNA sequences," Puigbò et al., Nucleic Acids Res. 2007 Jul; 35(Web Server issue): W126-31. Epub 2007 Apr 16.) Genes are synthesized using oligonucleotides composed, *e.g.*, of 42 nucleotides and cloned into expression vector pCK110900 (depicted as FIG. 3 in United States Patent Application Publication 20060195947) under the control of a lac promoter. The expression vector also contains the P15a origin of replication and the chloramphenicol resistance gene. Resulting plasmids are transformed into E. coli W3110 using standard methods. Examples of codon-optimized genes and the encoding polypeptides as well are listed in Table 5. The activity of the wild-type ketoreductases is confirmed as described in US provisional application Serial No. 60/848,950.

Table 5: Abbreviations, Source and Citations for Representative Ketoreductases

Ketoreductase	Microorganism from which enzyme was originally identified	Genbank Acc. No.	GI Number	Polynucleotide SEQ ID No	Polypeptide SEQ ID No or Source

Table 5: Abbreviations, Source and Citations for Representative Ketoreductases					
Ketoreductase	**Microorganism from which enzyme was originally identified**	**Genbank Acc. No.**	**GI Number**	**Polynucleotide SEQ ID No**	**Polypeptide SEQ ID No or Source**
ADH-CM	*Candida magnoliae*	AB036927.1	12657576	SEQ ID NO: 131	SEQ ID NO:132
YDL	*Saccharomyces cerevisiae*	NP_010159.1	6320079	SEQ ID NO:137	SEQ ID NO:138
ADH-LB	*Lactobacillus brevis*	1NXQ_A	30749782	SEQ ID NO:1	SEQ ID NO:2
ADH-RE	*Rhodococcus erythropolis*	AAN73270.1	34776951	SEQ ID NO:133	SEQ ID NO:134
YGL	*Saccharomyces cerevisiae*	NP_011476	6321399	SEQ ID NO:135	SEQ ID NO:136
YPR	*Saccharomyces cerevisiae*	NP_010656.1	6320576	SEQ ID NO:139	SEQ ID NO:140
GRE	*Saccharomyces cerevisiae*	NP_014490.1	6324421	SEQ ID NO:141	SEQ ID NO:142
ADH-LK	*Lactobacillus kefir*	AAP94029.1	33112056	SEQ ID NO:3	SEQ ID NO:4
ADH-SB	*Sporobolomyces salmonicolor*	Q9UUN9	30315955	SEQ ID NO:145	SEQ ID NO:146
ADH-SC	*Streptomyces coelicolor*	NP_631415.1	21225636	SEQ ID NO:143	SEQ ID NO:144
ADH-TB	*Thermoanaerobium brockii*	X64841.1	1771790	SEQ ID NO:153	SEQ ID NO:154
ADH-CP	*Candida parapsilosis*	BAA24528	2815409		Julich Chiral Solutions Cat. No. 03.11
DR-LB	*Lactobacillus brevis* diacetyl reductase	ABJ63353.1	116098204		Julich Chiral Solutions Cat. No. 8.1
ADH-HE	Horse liver	DEHOAL	625197	SEQ ID NO:155	SEQ ID NO:156

Table 5: Abbreviations, Source and Citations for Representative Ketoreductases					
Ketoreductase	**Microorganism from which enzyme was originally identified**	**Genbank Acc. No.**	**GI Number**	**Polynucleotide SEQ ID No**	**Polypeptide SEQ ID No or Source**
ADH-CB	*Candida boidinii*	CAD66648	28400789		Julich Chiral Solutions Cat. No. 02.10
LDH-LL	*Lactobacillus leichmannii*				Fluka Cat. No. 61306
ADH-AF	*Aspergillus flavus*	P41747	1168346	SEQ ID NO:147	SEQ ID NO:148
ADH-001	*Oenococcus oeni*	ZP_00318704.1	48864831	SEQ ID NO:149	SEQ ID NO:150
ADH-RU	*Ralstonia eutropha*	ZP_00202558.1	46131317	SEQ ID NO:151	SEQ ID NO:152
	Lactobacillus minor			SEQ ID NO:157	SEQ ID NO:158

[0293]

[0294] Polynucleotides encoding engineered ketoreductases of the present invention are likewise cloned into vector pCK110900 for expression in E. coli W3110.

7.2 Example 2: Production of Ketoreductase Powders; Shake Flask Procedure.

[0295] A single microbial colony of E. coli containing a plasmid with the ketoreductase gene of interest is inoculated into 50 ml Luria Bertani broth containing 30 μg/ml chloramphenicol and 1% glucose. Cells are grown overnight (at least 16 hrs) in an incubator at 30°C with shaking at 250 rpm. The culture is diluted into 250 ml Terrific Broth (12 g/L bacto-tryptone, 24g/L yeast extract, 4 ml/L glycerol, 65mM potassium phosphate, pH 7.0, 1mM MgSO4, 30 μg/ml chloramphenicol) in 1 liter flask to an optical density at 600 nm (OD600) of 0.2 and allowed to grow at 30°C. Expression of the ketoreductase gene is induced with 1mM IPTG when the OD600 of the culture is 0.6 to 0.8 and incubated overnight (at least 16 hrs). Cells are harvested by centrifugation (5000rpm, 15 min, and 4°C) and the supernatant discarded. The cell pellet is resuspended with an equal volume of cold (4°C) 100mM triethanolamine (chloride) buffer, pH 7.0 (including 2 mM MgSO4 in the case of ADH-LK and ADH-LB and engineered ketoreductases derived therefrom), and harvested by centrifugation as above. The washed cells are resuspended in two volumes of the cold triethanolamine (chloride)

buffer and passed through a French Press twice at 12000psi while maintaining the temperature at 4°C. Cell debris is removed by centrifugation (9000 rpm, 45 min., and 4°C). The clear lysate supernatant is collected and stored at -20°C. Lyophilization of frozen clear lysate provides a dry powder of crude ketoreductase enzyme.

7.3 Example 3: Production of Ketoreductases; Fermentation Procedure.

[0296] In an aerated agitated 15L fermenter, 6.0L of growth medium containing 0.88 g/L ammonium sulfate, 0.98 g/L of sodium citrate; 12.5 g/L of dipotassium hydrogen phosphate trihydrate, 6.25g/L of potassium dihydrogen phosphate, 6.2 g/L of Tastone-154 yeast extract, 0.083 g/L ferric ammonium citrate, and 8.3 ml/L of a trace element solution containing 2 g/L of calcium chloride dihydrate, 2.2 g/L of zinc sulfate septahydrate, 0.5 g/L manganese sulfate monohydrate, 1 g/L cuprous sulfate heptahydrate, 0.1 g/L ammonium molybdate tetrahydrate and 0.02 g/L sodium tetraborate decahydrate are brought to a temperature of 30°C. The fermenter is inoculated with a late exponential culture of E. coli W3110, containing a plasmid with the ketoreductase gene of interest, grown in a shake flask as described in Example 3 to a starting OD600 of 0.5 to 2.0. The fermenter is agitated at 500-1500 rpm and air is supplied to the fermentation vessel at 1.0-15.0 L/min to maintain dissolved oxygen level of 30% saturation or greater. The pH of the culture is controlled at 7.0 by addition of 20% v/v ammonium hydroxide. Growth of the culture is maintained by the addition of a feed solution containing 500g/L cerelose, 12 g/L ammonium chloride and 10.4 g/L magnesium sulfate heptahydrate. After the culture reached an OD600 of 50, expression of ketoreductase is induced by the addition of isopropyl-b-D-thiogalactoside (IPTG) to a final concentration of 1mM. The culture is grown for another 14 hours. The culture is then chilled to 4°C and maintained at 4°C until harvested. Cells are harvested by centrifugation at 5000G for 40 minutes in a Sorval RC12BP centrifuge at 4°C. Harvested cells are used directly in the following downstream recovery process or are stored at 4°C until such use.

[0297] The cell pellet is resuspended in 2 volumes of 100 mM triethanolamine (chloride) buffer, pH 6.8, at 4°C to each volume of wet cell paste. The intracellular ketoreductase is released from the cells by passing the suspension through a homogenizer fitted with a two-stage homogenizing valve assembly using a pressure of 12000 psig. The cell homogenate is cooled to 4°C immediately after disruption. A solution of 10% w/v polyethyleneimine, pH 7.2, is added to the lysate to a final concentration of 0.5% w/v and stirred for 30 minutes. The resulting suspension is clarified by centrifugation at 5000G in a standard laboratory centrifuge for 30 minutes. The clear supernatant is decanted and concentrated ten times using a cellulose ultrafiltration membrane with a molecular weight cut off of 30KD. The final concentrate is dispensed into shallow containers, frozen at –20°C and lyophilized to powder. The ketoreductase powder is stored at –20°C.

7.4 Example 4: Analytical methods for the conversion of 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one.

[0298] Analytical methods to determine conversion of (S)-1-(4-Fluoro-phenyl)-5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione and enantiomeric excess of (4S)-3-[(5S)-5-(4-Fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one.

[0299] Achiral HPLC method to determine conversion. Reduction (S)-1-(4-Fluoro-phenyl)-5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione to (4S)-3-[(5S)-5-(4-Fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one was determined using an Agilent 1100 HPLC equipped with an Agilent Zorbax Eclipse XDB column (7.5 cm length, 2.1 mm diameter), eluent: water/acetonitrile 50:50, flow 0.7 ml/min; column temperature 40°C). Retention times: (4S)-3-[(5S)-5-(4-Fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one: 1.3 min, (S)-1-(4-Fluoro-phenyl)-5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione: 2.2 min.

[0300] Chiral HPLC method to determine stereopurity of (4S)-3-[5-(4-Fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one. The stereomeric purity of (4S)-3-[5-(4-Fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one was determined using an Agilent 1100 HPLC equipped with a Chiralcel OD-H column (15 cm length, 2.1 mm diameter, eluent: hexane/ethanol 80:20, flow 1 ml/min). Retention times: (4R)-3-[(5S)-5-(4-Fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one: 6.64 min, (4S)-3-[(5S)-5-(4-Fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one:7.93 min, (S)-1-(4-Fluoro-phenyl)-5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione: 10.44 min.

7.5 Example 5: Evaluation of Wild-Type Ketoreductases for Reduction of 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione

[0301] KREDs described in Table 5 of Example 1 are screened using NADH and NADPH as co-factors and glucose dehydrogenase/glucose or isopropylalcohol ("IPA") as co-factor regeneration system. 100 µl of cell lysate was added to a deep well plate (Costar #3960) containing 25 µl 5 mg/ml Na-NADP (Oriental Yeast) and 2 mM $MgSO_4$ in 100 mM triethanolamine(chloride) (pH7.0), and 125 µl isopropyl alcohol containing 2 g/L (S)-1-(4-Fluoro-phenyl)-5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione. After sealing the plates with aluminum/polypropylene laminate heat seal tape (Velocity 11 (Menlo Park, CA), Cat# 06643-001), reactions were run for at least 16 hrs at ambient temperature. At the end of the reaction 1 ml acetonitrile (for reversed phase HPLC) or MTBE (for normal phase HPLC) was added per well. Plates were resealed, shaken for 20 minutes, and centrifuged (4000rpm, 10 min, 4°C). 200ul of the organic layer was transferred into a new shallow-well microtiter plate for analysis.

[0302] This example will demonstrate that wild-type ketoreductases have very little if any activity on 5-((4S)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione.

7.6 Example 6: Evaluation of ADH-LK Variants for Reduction of 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione

[0303] Several ADH-LK variants that had been generated are evaluated and found that an ADH-LK variant with SEQ ID NO:8 converted the substrate to the chiral (4S)-3-[(5S)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one product when evaluated under the conditions described in Example 5 and as listed in Table 6.

Table 6: Activity of an ADH-LK variant

SEQ ID NO	Number of mutations relative to ADH-LK	Activity
ADH-LK	0	0
8	8	~ 0.008 g/L.g_{enzyme}.day

[0304] This example shows that an ADH-LK variant containing G7S, R108H, G117S, E145S, N157T, Y190C, K112R, and I223V mutations converts 5-((4S)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione to (4S)-3-[(5S)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one with high stereoselectivity (94% stereomeric excess).

7.7 Example 7: High Throughput HPLC Assay for Ketoreductase Activity on 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione using isopropylalcohol for co-factor recycling.

[0305] Plasmid libraries obtained by directed evolution and containing evolved ketoreductase genes are transformed into E. coli and plated on Luria-Bertani (LB) broth containing 1% glucose and 30 µg/mL chloramphenicol (CAM). After incubation for at least 16 hrs at 30°C, colonies are picked using a Q-bot® robotic colony picker (Genetix USA, Inc., Beaverton, OR) into 96-well shallow well microtiter plates containing 180 µL Terrific broth (TB), 1% glucose, 30 µg/mL chloramphenicol (CAM), and 2 mM $MgSO_4$. Cells are grown overnight at 30°C with shaking at 200 rpm. 20 µL of this culture was then transferred into 96-deep well plates containing 350 µL Terrific broth (TB), 2 mM $MgSO_4$ and 30 µg/mL CAM. After incubation of deep-well plates at 30°C with shaking at 250 rpm for 2.5 to 3 hours (OD_{600} 0.6-0.8), recombinant gene expression by the cell cultures is induced by addition of isopropyl thiogalactoside (IPTG) to a final concentration of 1 mM. The plates are then incubated at 30°C with shaking at 250 rpm for 15-23 hrs.

[0306] 100 µl of cell lysate was added to a deep well plate (Costar #3960) containing 25 µl 5 mg/ml Na-NADP (Oriental Yeast) and 2 mM $MgSO_4$ in 100 mM triethanolamine(chloride) (pH7.0), and 125 µl isopropyl alcohol containing 2 g/L (S)-1-(4-Fluoro-phenyl)-5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione. After sealing the plates with aluminum/polypropylene laminate heat seal tape (Velocity 11 (Menlo Park, CA), Cat# 06643-001), reactions were run for at least 16 hrs at ambient temperature. At the end of the reaction 1 ml acetonitrile (for reversed phase HPLC) or MTBE (for normal phase HPLC) was added per well. Plates were resealed, shaken for 20 minutes, and centrifuged (4000rpm, 10 min, 4°C). 200ul of the organic layer was transferred into a new shallow-well microtiter plate for analysis as described in Example 4.

[0307] This example describes the method that was used to identify KRED variants improved for 5-((4S)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione reduction.

7.8 Example 8: Reduction of 5-((4*S*)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione by Engineered Ketoreductases Derived from ADH-LK.

[0308] Improved ADH-LK variants for the reduction of (S)-1-(4-Fluoro-phenyl)-5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione to (4S)-3-[(5S)-5-(4-Fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one were analyzed in small scale chemical reactions. In a glass vial with a teflon stirring bar, 500 mg (S)-1-(4-Fluoro-phenyl)-5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione, 100 mg KRED variant, 0.5 mg Na-NADP (Oriental Yeast), 2.5 ml isopropyl alcohol, and 2.5 ml 100 mM triethanolamine(chloride) buffer, pH 7.0, 2 mM $MgSO_4$ was mixed and stirred overnight at 25°C. Reaction samples were analyzed by the method of Example 4.

7.9 Example 9: Preparative scale production of (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one

[0309] Preparative scale production of (4S)-3-[(5S)-5-(4-Fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one using iPA for cofactor recycle. In a 1 liter round bottom flask thermostatted at 25 °C with Teflon stirring bar, 2.5 grams lyophilized KRED catalyst was dissolved in 200 ml 100 mM triethanolamine(chloride), pH 7.0, 2mM $MgSO_4$. After the enzyme was dissolved, 175 mg $NADP^+$ was added, followed by 5 grams of (S)-1-(4-Fluoro-phenyl)-5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione. 200 ml 2-propanol was added, resulting in the formation of a white precipitate. After stirring for 5 hours at 25 °C by which time the reaction was complete, the mixture was filtered through Celite to remove the insoluble protein fraction. Isopropanol was distilled off until about 200 ml solution remained. The aqueous layer was extracted twice with 200 ml ethyl acetate and the combined ethyl acetate layers were washed with saturated NaCl. The ethyl acetate layer was dried over Na_2SO_4 and after filtration, ethyl acetate was distilled off yielding ~5 g of the chiral alcohol 2 as

slightly yellow colored oil. The stereomeric purity (determined as described in Example 4) of (4S)-3-[(5S)-5-(4-Fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one was >99% (S,S).

[0310] Preparative scale production of (4S)-3-[(5S)-5-(4-Fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one using GDH and external pH control. A 2L resin kettle is equipped with a mechanical overhead stirrer, pH probe and a port for titrating aqueous 4N NaOH. The external titrator (Schott Titronic) is programmed to maintain the pH at 7.00 +/- 0.10

[0311] To the resin kettle is charged 5-((4S)-2-oxo-4-phenyl (1,3-oxazolidin-3-yl))-1-(4-fluorophenyl) pentane-1,5-dione (120 g) as a powder, followed by dextrose powder (91 g), toluene (200 ml), and buffer (750 ml of 0.02M potassium phosphate and 0.002 M Magnesium sulfate). The head plate is fitted and dogged down. All appropriate ports are closed except for the pH probe port which is fitted with the pH probe. The motor is then fitted to the coupling and stirring is initiated to a rate of 1200 rpm. The pH of the reaction mixture is measured and adjusted to 7.0 + 0.1. The temperature of the reaction mixture is brought to 30 + 1°C. While the reaction is brought to temperature, 0.4 g Na-NADP, 0.8 g GDH, and 2.00 g of lyophilized KRED were dissolved in 40 ml of de-ionized water. When the reactor temperature is in the appropriate range, the enzyme suspension is added in one portion while stirring. The titration program is started and the pH is maintained at 7.0 +/- 0.1 for the duration of the reaction by addition of 4N NaOH. The reaction is stirred at 30°C for 16 hr. The reactor is sampled periodically and checked for substrate conversion by HPLC as described in Example 4. Periodic sampling and analysis is continued until the conversion reaches 99% or better.

[0312] When the reaction is deemed to be over, stirring is stopped and the bi-phasic mixture is allowed to separate. Clear aqueous layer (240 ml) is removed from the bottom of the vessel as best as possible by syringe. 90 ml of this aqueous layer is added to 22 g Celite and set aside, the rest is discarded. Toluene (240 ml) is added to the reaction mixture, which is then stirred for 10 minutes and allowed to settle again for 30 minutes. Another portion of 180 ml of clear aqueous phase is removed by syringe and discarded. The stirring is restarted, followed by addition of the Celite and aqueous mixture that had been set aside. Stirring is continued for 10 minutes. The reaction mixture is filtered through an "M" sintered glass funnel to remove insoluble material (primarily denatured enzymes and Celite). The cake is filtered until almost dry. The reactor is rinsed with toluene (100 mL). The reactor rinse is added to the filter cake. The filter cake is tamped down, then washed with 100 ml more toluene and allowed to run dry. The biphasic filtrate is transferred to a separatory funnel and separated. Saturated aqueous ammonium sulfate (100 ml) is added to the organic layer and mixed lightly and allowed to separate. The lower (aqueous) layer is removed. The toluene is then washed twice with de-ionized water. After the final separation, the resulting wet toluene solution containing the product is charged to a 1 liter flask and stripped down under vacuum on a rotary evaporator.

While doing this, the heating bath is warmed to no more than 50°C and the vacuum is brought down from 110 mm (initially) to 2 mm. The resulting crude product is an oil that solidifies on standing within two days. Yield: 125 g.

[0313] All publications, patents, patent applications and other documents cited in this application are hereby incorporated by reference in their entireties for all purposes to the same extent as if each individual publication, patent, patent application or other document were individually indicated to be incorporated by reference for all purposes.

[0314] While various specific embodiments have been illustrated and described, it will be appreciated that various changes can be made without departing from the spirit and scope of the invention(s).

WHAT IS CLAIMED IS:

1. An engineered polypeptide having ketoreductase activity, wherein the polypeptide comprises the amino acid reference sequence of SEQ ID NO: 162, wherein the residue corresponding to X145 is an asparagine, glutamine, leucine, phenylalanine, or threonine residue.

2. The engineered polypeptide of claim 1, wherein the residue corresponding to X190 is a cysteine, proline, or alanine residue.

3. The engineered polypeptide of claim 2, wherein the amino acid sequence further includes one or more of the following:
residue corresponding to X3 is aspartic acid, glutamic acid, serine, threonine, asparagine, or glutamine;
residue corresponding to X7 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine;
residue corresponding to X17 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine;
residue corresponding to X40 is histidine, lysine, arginine, serine, threonine, asparagine, or glutamine;
residue corresponding to X94 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine;
residue corresponding to X96 is serine, threonine, asparagine, valine, or glutamine;
residue corresponding to X108 arginine, lysine, serine, threonine, asparagine, glutamine, histidine;
residue corresponding to X117 is glycine, methionine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, or glutamine;
residue corresponding to X127 is lysine, arginine, serine, threonine, asparagine, or glutamine;
residue corresponding to X147 is glycine, methionine, alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, or tryptophan;
residue corresponding to X152 is glycine, methionine, valine, leucine, or isoleucine, arginine, lysine, serine threonine, asparagine, or glutamine;
residue corresponding to X153 is threonine;
residue corresponding to X157 is a serine, threonine, asparagine, or glutamine;
residue corresponding to X163 is a glycine, methionine, alanine, valine, leucine, or isoleucine;
residue corresponding to X176 is glycine, methionine, alanine, valine, leucine, or isoleucine;
residue corresponding to X194 is proline, arginine, lysine, serine, threonine, asparagine, or glutamine;
residue corresponding to X196 is leucine;
residue corresponding to X198 is aspartic acid, glutamic acid, arginine, lysine, serine, threonine, asparagine, glutamine, glycine, methionine, alanine, valine, leucine, or isoleucine;
residue corresponding to X199 is an aspartic acid, glutamic acid, glycine, methionine, alanine, valine, leucine, or isoleucine;
residue corresponding to X211 is a arginine or lysine;
residue corresponding to X223 is glycine, methionine, alanine, valine, leucine, or isoleucine; or

residue corresponding to X226 is valine;
wherein the amino acid sequence can optionally have one or more residue differences at other amino acid residues as compared to the reference sequence.

4. The recombinant polypeptide of claim 3, wherein the amino acid sequence includes the following:
residue corresponding to X94 is alanine or glycine;
residue corresponding to X96 is serine or valine;
residue corresponding to X145 is glutamine, leucine, or phenylalanine;
residue corresponding to X147 is leucine or methionine;
residue corresponding to X190 is proline;
residue corresponding to X196 is leucine; and
residue corresponding to X226 is valine;
wherein the amino acid sequence can optionally have one or more residue differences at other amino acid residues as compared to the reference sequence.

5. The engineered polypeptide of claim 2, wherein the amino acid sequence further includes one or more of the following:
residue corresponding to X3 is asparagine;
residue corresponding to X7 is serine;
residue corresponding to X17 is glutamine;
residue corresponding to X21 is phenylalanine;
residue corresponding to X25 is threonine;
residue corresponding to X29 is glycine or alanine;
residue corresponding to X40 is arginine;
residue corresponding to X42 is glycine;
residue corresponding to X53 is aspartic acid;
residue corresponding to X75 is arginine;
residue corresponding to X94 is asparagine, glycine, or serine;
residue corresponding to X95 is leucine or methionine;
residue corresponding to X96 is glutamine, asparagine, or threonine;
residue corresponding to X101 is glycine or asparagine;
residue corresponding to X105 is glycine;
residue corresponding to X108 is histidine or serine;
residue corresponding to X112 is aspartic acid;
residue corresponding to X113 is alanine;
residue corresponding to X117 is serine;
residue corresponding to X127 is arginine;
residue corresponding to X147 is leucine;
residue corresponding to X152 is methionine or lysine;
residue corresponding to X157 is threonine;
residue corresponding to X163 is isoleucine;
residue corresponding to X176 is valine;

residue corresponding to X194 is arginine or glutamine;
residue corresponding to X197 is valine or glutamic acid;
residue corresponding to X198 is glycine, glutamic acid, or lysine;
residue corresponding to X199 is aspartic acid;
residue corresponding to X200 is proline;
residue corresponding to X202 is glycine;
residue corresponding to X206 is glycine;
residue corresponding to X211 is arginine or lysine;
residue corresponding to X223 is valine; or
residue corresponding to X250 is isoleucine;
wherein the amino acid sequence can optionally have one or more residue differences at other amino acid residues as compared to the reference sequence.

6. The recombinant polypeptide of claim 3, wherein the amino acid sequence includes one or more of the following:
residue corresponding to X7 is serine;
residue corresponding to X108 is histidine or serine;
residue corresponding to X117 is serine;
residue corresponding to X152 is methionine or lysine; or
residue corresponding to X199 is aspartic acid;
wherein the amino acid sequence can optionally have one or more residue differences at other amino acid residues as compared to the reference sequence.

7. A method for reducing a carbonyl group of a substrate compound to its corresponding alcohol group, the method comprising contacting the substrate compound with an engineered polypeptide of claim 1 in the presence of a NADH or NADPH cofactor, wherein the substrate is a ketone.