DESCRIPTION OF THE INVENTION

[0070] The present invention provides engineered PAL polypeptides, mutants, biologically active fragments and analogues thereof, and pharmaceutical and industrial compositions comprising the same.

[0071] The invention provides engineered phenylalanine ammonia-lyase (PAL) polypeptides and compositions thereof, as well as polynucleotides encoding the engineered phenylalanine ammonia-lyase (PAL) polypeptides. In some embodiments, the engineered PAL polypeptides are optimized to provide enhanced catalytic activity, as well as reduced sensitivity to proteolysis and increased tolerance to acidic pH levels. In some embodiments the engineered PAL polypeptides are deimmunized. The invention also relates to the use of the compositions comprising the engineered PAL polypeptides for therapeutic and industrial purposes.

Abbreviations and Definitions:

[0072] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Generally, the nomenclature used herein and the laboratory procedures of cell culture, molecular genetics, microbiology, organic chemistry, analytical chemistry and nucleic acid chemistry described below are those well-known and commonly employed in the art. Such techniques are well-known and described in numerous texts and reference works well known to those of skill in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses. All patents, patent applications, articles and publications mentioned herein, both *supra* and *infra*, are hereby expressly incorporated herein by reference.

[0073] Although any suitable methods and materials similar or equivalent to those described herein find use in the practice of the present invention, some methods and materials are described herein. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary, depending upon the context they are used by those of skill in the art. Accordingly, the terms defined immediately below are more fully described by reference to the application as a whole. All patents, patent applications, articles and publications mentioned herein, both *supra* and *infra*, are hereby expressly incorporated herein by reference.

[0074] Also, as used herein, the singular "a", "an," and "the" include the plural references, unless the context clearly indicates otherwise.

[0075] Numeric ranges are inclusive of the numbers defining the range. Thus, every numerical range disclosed herein is intended to encompass every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein. It is also intended that every maximum (or minimum) numerical limitation disclosed herein includes every

CLAIMS

WHAT IS CLAIMED IS:

1. An engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity comprising: a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:4 or a functional fragment thereof; b) a polypeptide sequence comprising at least one amino acid residue difference as compared to SEQ ID NO:4 or the functional fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, v) reduced immunogenicity, or a combination of any of i), ii), iii), iv), or v), as compared to the reference sequence SEQ ID NO:4.

2. The engineered polypeptide of Claim 1 wherein the one or more amino acid positions are selected from 20, 24, 27, 39, 43, 45, 47, 54, 58, 59, 62, 70, 73, 80, 82, 91, 94, 98, 104, 105, 110, 112, 115, 117, 118, 119, 121, 123, 124, 125, 126, 127, 128, 129, 130, 131, 133, 134, 135, 139, 140, 141, 142, 143, 144, 145, 146, 147, 149, 150, 151, 153, 154, 156, 157, 158, 159, 172, 174, 175, 176, 177, 178, 180, 187, 191, 195, 199, 205, 206, 210, 212, 213, 214, 232, 240, 243, 245, 247, 248, 250, 256, 257, 266, 270, 275, 278, 279, 285, 286, 289, 290, 292, 304, 305, 307, 308, 309, 319, 321, 326, 331, 332, 334, 349, 353, 355, 364, 365, 369, 370, 371, 372, 374, 375, 377, 378, 379, 381, 382, 383, 384, 385, 387, 389, 394, 396, 399, 400, 403, 404, 407, 417, 418, 425, 431, 432, 433, 434, 435, 436, 437, 438, 439, 443, 446, 447, 453, 456, 459, 460, 461, 463, 471, 472, 473, 474, 475, 476, 477, 478, 479, 482, 483, 503, 507, 509, 521, 522, 524, 525, 528, 538, 546, 547, 551, 558, 560, 564, 565, and/or any combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:4.

3. The engineered polypeptide of any of the preceding Claims, wherein the amino acid residue of the reference sequence of SEQ ID NO:4 corresponds to A39, T54, G59, S73, A91, Y158, S180, K195, A112, R134, Q240, T243, I245, A256, L257, N270, N290, Y304, R305, H307, E308, I326, L349, D353, L364, A394, S399, N400, P404, L407, F443, N453, Y459, T460, T463, N474, E509, Q521, K522, T524, P528, S546, and/or P564.

4. The engineered polypeptide of any of the preceding Claims, wherein the amino acid residue difference as compared to SEQ ID NO:4 is selected from one or more of the following substitutions A39V, T54K, G59R, S73K, A112C, R134Q, A91V, Y158H, S180A, K195E, Q240R/W, T243I/L, I245L, A256G, L257W/A, N270K, N290G, Y304H, R305M, H307G/Q/M, E308Q, I326F, L349M, D353A/N, L364Q, A394V, S399N, N400K, P404A, L407V, F443H, N453G, Y459F,

T460G, T463N, N474Q, E509L, Q521K/S, K522Y/F/N, T524S,P528L, S546R, and P564 G/L/M, when optimally aligned with the polypeptide of SEQ ID NO:4.

5. The engineered polypeptide of any of Claims 1-4, wherein the amino acid residue difference is selected from a combination of one or more of A39, A91, Q240, A256, N290, Y304, R305, H307, D353, A394, S399, P404, L407, Q521, K522, and T524.

6. The engineered polypeptide of any of the preceding Claims, wherein the engineered polypeptide has at least about 90% sequence identity to SEQ ID NO:4; and an amino acid residue difference at position H307.

7. The engineered polypeptide of Claim 6, wherein the amino acid residue difference is H307G/Q/M.

8. The engineered polypeptide of any of the preceding Claims, wherein the improved property is selected from reduced sensitivity to proteolysis and/or increased tolerance to acidic pH.

9. The engineered polypeptide of any of the preceding Claims, wherein the reference sequence is a wild-type PAL derived from *Anabaena variabilis*.

10. An engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity comprising: a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:10 or a functional fragment thereof; b) a polypeptide sequence comprising at least one amino acid residue difference as compared to SEQ ID NO:10 or the functional fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, v) reduced immunogenicity, or a combination of any of i), ii), iii), iv), or v), as compared to the reference sequence SEQ ID NO:10.

11. An engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity comprising an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:10, and at least one amino acid residue difference as compared to SEQ ID NO:10, and that exhibits at least one improved property selected from enhanced catalytic activity, reduced sensitivity to proteolysis, increased tolerance to acidic pH, reduced aggregation, and/or reduced immunogenicity, as compared to SEQ ID NO:10.

12. The engineered polypeptide of Claim 10 and/or 11, wherein the amino acid residue

difference as compared to SEQ ID NO:10 is selected from one or more of the following substitutions or substitution sets: I27E/V39A; I27E/V39A/R43L/V105C/A153R/L214E/P266H/L278D/C503Q; I27E/V39A/R43L/L214E/A547D;
I27E/V39A/V105C/A112C/R134Q/L214E/L278D/C503Q/A547D/C565N;
I27E/V39A/V105C/A112C/R134Q/A153R/Q205T/L214E/P266H/L278D/C503Q/A551D;
I27E/V39A/V105C/A112C/Q205T/P210C/P266H/C503Q/A547D;
I27E/V39A/V105C/A112C/Q205T/P266H/I285E/C503Q/A551D;
I27E/V39A/V105C/A112C/L214E/I285E/C503Q/A547D;
I27E/V39A/V105C/S131N/R134Q/Q205T/L214E/C503Q/A547D/C565N;
I27E/V39A/V105C/R134Q/A153R/P210C/L278D/I285E/C503Q/A547D/A551D;
I27E/V39A/V105C/R134Q/Q205T/P210C/L278D/C503Q/A547D;
I27E/V39A/V105C/R134Q/Q205T/L214E;
I27E/V39A/V105C/R134Q/Q205T/L214E/A551D/C565N;
I27E/V39A/V105C/R134Q/Q205T/L278D/I285E/C503Q/A547D/A551D/C565N;
I27E/V39A/V105C/R134Q/P210C; I27E/V39A/V105C/R134Q/P210C/L214E;
I27E/V39A/V105C/R134Q/P210C/L214E/I285E/A547D;
I27E/V39A/V105C/R134Q/P210C/L214E/C503Q/A551D/C565N;
I27E/V39A/V105C/R134Q/L214E/L278D/A547D/A551D;
I27E/V39A/V105C/R134Q/L214E/I285E/C503Q/A547D/A551D;
I27E/V39A/V105C/R134Q/P266H/C503Q;
I27E/V39A/V105C/R134Q/P266H/C503Q/A547D/A551D;
I27E/V39A/V105C/R134Q/L278D/C503Q/C565N;
I27E/V39A/V105C/R134Q/L278D/I285E/C503Q; I27E/V39A/V105C/R134Q/L278D/A551D;
I27E/V39A/V105C/R134Q/I285E/A547D/A551D; I27E/V39A/V105C/R134Q/C503Q/A551D;
I27E/V39A/V105C/A153R/Q205T/L278D/C503Q/A547D/A551D;
I27E/V39A/V105C/A153R/L214E; I27E/V39A/V105C/A153R/I285E;
I27E/V39A/V105C/A153R/C503Q/A547D/C565N; I27E/V39A/V105C/A153R/A551D/C565N;
I27E/V39A/V105C/Q205T/P210C/L214E/L278D/A547D;
I27E/V39A/V105C/Q205T/P210C/L278D/C503Q;
I27E/V39A/V105C/Q205T/P210C/L278D/A547D;
I27E/V39A/V105C/Q205T/L214E/L278D/C503Q/A547D;
I27E/V39A/V105C/Q205T/L278D/C503Q/A547D;
I27E/V39A/V105C/P210C/I285E/C503Q/A547D/A551D/C565N;
I27E/V39A/V105C/P210C/L214E/P266H/L278D;
I27E/V39A/V105C/L214E/P266H/C503Q/A547D/C565N;
I27E/V39A/V105C/L214E/L278D/L309P/C503Q/A547D/A551D;
I27E/V39A/V105C/L278D/C503Q/A547D/C565N; I27E/V39A/V105C/I285E/A547D;

I27E/V39A/V105C/C503Q/A551D; I27E/V39A/V105C/C503Q/A547D/A551D/C565N;
I27E/V39A/A112C/R134Q/Q205T/P210C/L214E/A551D/C565N;
I27E/V39A/A112C/R134Q/L214E/P266H/A551D;
I27E/V39A/A112C/R134Q/L214E/C503Q/A547D; I27E/V39A/A112C/R134Q/P266H/I285E;
I27E/V39A/A112C/Q205T/L214E/P266H/C503Q/A551D/C565N;
I27E/V39A/A112C/Q205T/L278D/I285E; I27E/V39A/A112C/L214E;
I27E/V39A/A112C/L214E/L278D/C503Q/A547D/A551D; I27E/V39A/A112C/I285E;
I27E/V39A/A112C/A547D; I27E/V39A/R134Q;
I27E/V39A/R134Q/A153R/Q205T/L214E/P266H/C503Q;
I27E/V39A/R134Q/A153R/P210C/L214E/L278D/I285E/A547D/C565N;
I27E/V39A/R134Q/A153R/L214E/P266H/L278D/C503Q/A547D/C565N;
I27E/V39A/R134Q/A153G/L214E/P266H/I285E/C503Q/ A551D/ C565N;
I27E/V39A/R134Q/A153R/L214E/C503Q/A547D; I27E/V39A/R134Q/A153R/L278D;
I27E/V39A/R134Q/A153R/L278D/A547D/A551D; I27E/V39A/R134Q/A153R/A547D;
I27E/V39A/R134Q/Q205T/L214E/P266H/I285E/C503Q/A551D/C565N;
I27E/V39A/R134Q/Q205T/P266H/C503Q/A551D/C565N;
I27E/V39A/R134Q/P210C/L214E/C503Q; I27E/V39A/R134Q/P210C/C503Q/A551D;
I27E/V39A/R134Q/L214E/P266H/A551D;
I27E/V39A/R134Q/L278D/I285E/C503Q/A547D/A551D;
I27E/V39A/R134Q/L278D/C503Q/A547D; I27E/V39A/R134Q/C503Q/A547D;
I27E/V39A/R134Q/A547D/C565N; I27E/V39A/Q205T/L214E/C503Q/C565N;
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I27E/V39A/Q205T/C503Q/A547D/C565N; I27E/V39A/P210C/T212S;
I27E/V39A/P210C/L214E/L278D/C503Q/A551D; I27E/V39A/P210C/L214E/I285E/C503Q/A551D;
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I27E/V39A/L278D; I27E/V39A/L278D/A547D; I27E/V39A/L278D/I285E/C503Q/A547D;
I27E/V39A/L278D/C503Q/C565N; I27E/V39A/C503Q; I27E/G45D/Q205T/P266H/C565N;
I27E/V105C; I27E/V105C/R134Q/A153R/P210C/L214E/C503Q/A547D;
I27E/V105C/R134Q/A153R/I285E/A547D; I27E/V105C/R134Q/A153R/C503Q;
I27E/V105C/R134Q/Q205T/P210C/C503Q;
I27E/V105C/R134Q/Q205T/L214E/P266H/L278D/C503Q/C565N;
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I27E/V105C/R134Q/L214E/L278D/C503Q/A547D/A551D/C565N; I27E/V105C/Q205T;
I27E/V105C/Q205T/L214E/P266H; I27E/V105C/Q205T/L214E/P266H/A551D/C565N;
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I27E/A112C/R134Q/L278D/I285E/C503Q/A551D/C565N;
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I27E/R134Q/Q205T/I285E/C503Q/A551D; I27E/R134Q/Q205T/P266H/L278D/A547D;
I27E/R134Q/P210C; I27E/R134Q/L214E/C503Q; I27E/R134Q/L214E/C503Q/A547D;
I27E/R134Q/L214E/C503Q/A547D/A551D; I27E/R134Q/L214E/C503Q/C565N;
I27E/R134Q/L278D/I285E/A551D/C565N; I27E/R134Q/I285E/C503Q;
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I27E/Q205T; I27E/Q205T/L214E/L278D/I285E/C503Q/C565N;
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V39A/V105C/Q205T/C503Q; V39A/V105C/Q205T/A551D; V39A/V105C/P210C/A547D;
V39A/V105C/L214E/P266H/A547D/C565N; V39A/V105C/L214E/I285E/C503Q/A551D/C565N;
V39A/A112C/R134Q/Q205T/L214E/L278D; V39A/A112C/R134Q/L214E/C503Q/A547D/A551D;
V39A/A112C/A153R/Q205T/L278D/C503Q/A547D; V39A/R134Q;

V39A/R134Q/Q205T/L214E/C503Q/C565N; V39A/R134Q/P210C/L214E/A547D/C565N; V39A/A153R/C503Q/A547D; V39A/Q205T/L278D/A547D/A551D; V39A/P210C/L214E/L278D/I285E/C503Q/A551D; V39A/P266H; V39A/P275R/L278D/C503Q/A551D; V39A/C503Q; V39A/C503Q/A551D/C565N; V105C; V105C/A112C/R134Q/Q205T/L214E/Y492H/C503Q/A547D; V105C/R134Q/A153R/Q205T/L214E/C503Q; V105C/R134Q/Q205T/L214E/A547D; V105C/R134Q/Q205T/P266H/L278D; V105C/R134Q/L214E/P266H/I285E/C503Q/A551D/C565N; V105C/R134Q/L214E/L278D/C565N; V105C/R134Q/L214E/C503Q/A547D; V105C/R134Q/L214E/C503Q/A547D/A551D; V105C/R134Q/C503Q; V105C/R134Q/C503Q/A547D; V105C/R134Q/C503Q/A547D/C565N; V105C/A153R/Q205T/L214E/P266H/C503Q/A547D; V105C/A153R/Q205T/P266H/I285E/A547D/C565N; V105C/Q205T/P210C/L214E/C503Q/A547D; V105C/Q205T/L214E/L278D; V105C/Q205T/L214E/C503Q/A547D/A551D/C565N; V105C/Q205T/C503Q/A551D; V105C/L214E/P266H/L278D/A547D; V105C/L214E/L278D/C503Q/A547D/A551D; V105C/L214E/I285E; V105C/L214E/I285E/C503Q/A547D/A551D/C565N; V105C/L214E/I285E/A547D/C565N; V105C/L278D/C503Q/A551D; V105C/I285E; V105C/I285E/A547D; V105C/C503Q; V105C/A547D/A551D; A112C/R134Q/A153R/L214E/L278D/I285E/C503Q/A547D/A551D/C565N; A112C/R134Q/L214E/C503Q/A547D/A551D/C565N; A112C/L214E/L278D; A112C/L278D/C503Q/A547D; R134Q/Q205T/L214E/I285E/C503Q/A551D/C565N; R134Q/Q205T/C503Q; R134Q/P210C/L214E/L278D/C503Q/A547D/C565N; R134Q/P210C/L214E/C503Q/A547D/A551D; R134Q/L214E; R134Q/L214E/L278D/C503Q; R134Q/L214E/L278D/C503Q/A551D; R134Q/L214E/I285E/C503Q; R134Q/C503Q; R134Q/C503Q/A547D/A551D; A153R; Q205T/L214E/I285E/C503Q/A551D; Q205T/L214E/I285E/C503Q/C565N; Q205T/L214E/C503Q/A547D/C565N; Q205T/L278D/I285E/A547D/A551D; P210C/L214E; P210C/L214E/P266H; L214E/P266H; L214E/P266H/C503Q/A547D/A551D/C565N; L214E/C503Q/A547D; L214E/A547D; P266H/L278D/C503Q; P266H/C565N; L278D/A547D; C503Q; C503Q/A547D; C503Q/A547D/A551D/C565N; C503Q/A547D/C565N; C503Q/A551D; C503Q/A551D/C565N; A547D; and/or C565N.

13. The engineered polypeptide of Claim 10 and/or 11, wherein the amino acid residue difference as compared to SEQ ID NO:10 is selected from one or more of the following substitutions or substitution sets: V80I/R134C/P564Q; V121C; A123G; A124G; M125L; L126I/T; L126M/R134L; L127A; A129G/L; N130Q; N130C/M370I R134W; M133R; R134I; R134N/G307C; G135C/S; and/or G135A/A394E.

14. The engineered polypeptide of Claim 10 and/or 11, wherein the amino acid residue difference as compared to SEQ ID NO:10 is selected from one or more of the following substitutions or substitution sets: G20S/I144L; R43S; L47M/I144L; L47M/R146E; L47M/M147G/A383E; L47M/P157C; Q58H/L143V; Q58K/P157D/G369C; A62S/M147V; S82I/G135C/P157F/W279L; R94C/I149E; T110I/I139R; L118M/L141H; A119E/T156H/A289D; I139M/V; R140D/G/M; R140N/A199E; R140E/A334S/A551D; L141K/Q/P/T; E142H/P/V; E142D/G371D; L143F/M; I144L/N/V; K145N/Q/R; K145G/P157T; R146H/L; R146W/D191Y; M147A; I149L/R; F150K/L/M; L151M; A153C/G; A153S/H250N; G154R; G154Y/L174M/Q321K/S456I/G483C; T156K/G483C; P157D/F/H/Y; Y158E; V159C/H/L/M; M247I; L319M; and/or Q389K.

15. The engineered polypeptide of Claim 10 and/or 11, wherein the amino acid residue difference as compared to SEQ ID NO:10 is selected from one or more of the following substitutions or substitution sets: P117T/Y176Q; V172I/C/L; L174M; S175G; Y176E/I/M/R/V; I177M/V; T178L/A477S; and/or S180C/T.

16. The engineered polypeptide of Claim 10 and/or 11, wherein the amino acid residue difference as compared to SEQ ID NO:10 is selected from one or more of the following substitutions or substitution sets: R43S/H374K; R43S/H374R; A112S/M370A/A507E; M147I/H374S; S187R/L381V; D191Y/H385N; A232S; Q240K/H374R; A256S/L381N; P275Q/M370S; P275T/H374R; Q332K/Y377M; A334S/H374Y; L349M; Q355K/H374S; M370G/I/S; G371H/N/Q/S; M372A/V; H374A/D/G/L/N/R/S/T; H374Q/P396Q; H374R/G417C; L375I; L375M; Y377C/I/N; Y378C/D/E/I/L/N/S; Y378F/P404Q; I379C/H/L/M/N; L381G/V; L381M/Q560K; L382C/H/I/M/S; A383S/V; K384R; H385C/G/N; H385M/P403H; H385S/P403H; D387S; L418M; G425V; A447S; S461G; and/or S525L.

17. The engineered polypeptide of Claim 10 and/or 11, wherein the amino acid residue difference as compared to SEQ ID NO:10 is selected from one or more of the following substitutions or substitution sets: A24S/F434M; A62S/T433N; S98I; L213M/S438L; Q240K/T433Y; S286R/Y435T; A289S/L431E; S331I; L431C/E/G/P/S/V; L432C/V; T433A/I/L/N/P/Q/R/S/V/W; F434C; Y435L; Y435Q/H446N; G436M; G436D/T; N437E/G/Q; N437T/L538M; S438C/F/M/R/T; I439C/F/L/V; and/or A477S.

18. The engineered polypeptide of Claim 10 and/or 11, wherein the amino acid residue difference as compared to SEQ ID NO:10 is selected from one or more of the following substitutions or substitution sets: A24E; Q58R/Y475H; A70S/N474E; L104M/V476L; A119E/G365A; L206M; P275Q; G276V; Q292H/A479G; Q355H/I478C; P404T/A477V; I471F/G/K/M/N/R/V/W; F472G; Q473H/K/M/R/S; Q473H/A507S; N474A/H/R/W; N474D/R490H; Y475C/F/L/Q; V476C/I/L;

I478N/S; A479G/S; F482C/L; G483C/H/S; G483A/S524I; G483R/G537C; and/or A558S.

19. The engineered polypeptide of Claim 10 and/or 11, wherein the amino acid residue difference as compared to SEQ ID NO:10 is selected from one or more of the following substitutions or substitution sets: V39A/K115E/M133R/C565N; V39A/M133R/F472G/C503Q/C565N; V39A/M133R/F472G/C565N; V39A/M133R/C503Q; V39A/M133R/C503Q/C565N; V39A/M147A/Y378E/C503Q/C565N; V39A/M147A/Y378E/C565N; V39A/M147A/L381G/F472G/C503Q/C565N; V39A/M147A/L381G/C503Q/C565N; V39A/M147A/F472G/C503Q/C565N; V39A/M147A/F472G/C565N; V39A/M147A/C565N; V39A/G248C/L381G/F472G/C503Q/C565N; V39A/Y378E/C503Q/C565N; V39A/Y378E/C565N; V39A/L381G; V39A/F472G/C503Q/C565N; V39A/C503Q/C565N; M133R/L381G/C565N; M133R/C503Q; Y378D/C503Q; Y378E/F472G/C503Q/C565N; L381G/F472GC503Q/C565N; and/or F472G/C503Q/C565N.

20. An engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity comprising: a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:26 or a functional fragment thereof; b) a polypeptide sequence comprising at least one amino acid residue difference as compared to SEQ ID NO:26 or the functional fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, v) reduced immunogenicity, or a combination of any of i), ii), iii), iv), or v), as compared to the reference sequence SEQ ID NO:26.

21. An engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity comprising an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:26, and at least one amino acid residue difference as compared to SEQ ID NO:26, and that exhibits at least one improved property selected from enhanced catalytic activity, reduced sensitivity to proteolysis, increased tolerance to acidic pH, reduced aggregation, and/or reduced immunogenicity, as compared to SEQ ID NO:26.

22. The engineered polypeptide of Claim 20 and/or 21, wherein the amino acid residue difference as compared to SEQ ID NO:26 is selected from one or more of the following substitutions or substitution sets: A24E/G381L; L127V; A129I/V; S131C/T; H132L/S; R134C/F/H/K; R134H/Y378E/G381L; R134H/Y378E/G381L/V388T; R134H/V388T; A136K; A289S; M372L; H374G/M/Q; G381A/C/F/I/L/M/N/Q/S/T; A383C/M; V388C/T; L431M; and/or L563M.

23. The engineered polypeptide of any of the preceding Claims, wherein the engineered

polypeptide has at least about 90% sequence identity to reference sequence SEQ ID NO:4.

24. The engineered polypeptide of Claim 23, wherein the engineered polypeptide has at least about 95% sequence identity to reference sequence SEQ ID NO:4.

25. An engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity comprising an amino acid sequence having at least about 90% sequence identity to SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and/or 26, or a functional fragment thereof.

26. The engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity of Claim 25, comprising an amino acid sequence having at least about 99% sequence identity to SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and/or 26, or a functional fragment thereof.

27. The engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity, wherein said engineered polypeptide is a variant PAL provided in any of Tables 2-1 through 2-5 and/or Tables 9-1 through 9-7.

28. The engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity of any of Claims 1-27, wherein said engineered polypeptide is an *Anabaena variabilis* enzyme.

29. The engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity of any of Claims 1-28, wherein said engineered polypeptide is thermostable.

30. The engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity of any of Claims 1-29, wherein said engineered polypeptide is resistant to proteolysis.

31. The engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity Claim 30, wherein said engineered polypeptide is resistant to proteolysis by at least one digestive tract enzyme.

32. The engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity Claim 31, wherein said engineered polypeptide is resistant to proteolysis by chymotrypsin, trypsin, carboxypeptidases, and/or elastases.

33. The engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity of any of Claims 1-32, wherein said engineered polypeptide is acid stable.

34. The engineered polypeptide of any of Claims 1-33, wherein said engineered polypeptide is deimmunized.

35. The deimmunized engineered polypeptide of Claim 34, wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and/or 26.

36. The engineered polypeptide of any of Claims 1-35, wherein said polypeptide is purified.

37. A polynucleotide sequence encoding at least one engineered polypeptide of any one of the preceding Claims 1-36.

38. The polynucleotide sequence of Claim 37, wherein said sequence is operably linked to a control sequence.

39. The polynucleotide sequence of Claim 37 and/or 38, wherein said polynucleotide is codon-optimized.

40. An expression vector comprising at least one polynucleotide sequence of any of Claims 37-39, and at least one control sequence.

41. The expression vector of Claim 40, wherein said control sequence is a promoter.

42. The expression vector of Claim 41, wherein said promoter is a heterologous promoter.

43. A host cell transformed with at least one polynucleotide sequence of any of Claims 35-37, and/or the vector of any of Claims 40-42.

44. The host cell of Claim 43, wherein said host cell is *E. coli*.

45. A method of producing an engineered PAL polypeptide in a host cell comprising culturing a host cell comprising a at least one polynucleotide encoding at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity of any of Claims 1 to 36, and/ or at least one polynucleotide sequence of Claim 37 and/or 39, and/or at least one vector of any of Claim 40-42, under suitable culture conditions, such that at least one engineered PAL polypeptide is

produced.

46. The method of Claim 45, further comprising recovering at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) from the culture and/or host cells.

47. The method of Claim 46, further comprising the step of purifying said at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) produced.

48. A composition comprising at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity, of any of Claims 1 to 36.

49. The composition of Claim 48, wherein said composition is a pharmaceutical composition.

50. The pharmaceutical composition of Claim 49, further comprising at least one pharmaceutically acceptable excipient and/or carrier.

51. The composition of any of Claims 48-50, wherein said composition is suitable for the treatment of phenylketonuria.

52. The pharmaceutical composition of any of Claims 49-51, wherein said composition is suitable for oral administration to a human.

53. The pharmaceutical composition of any of Claims 49-52, wherein said composition is in the form of a pill, tablet, capsule, gelcap, liquid, or emulsion.

54. The pharmaceutical composition of Claim 53, wherein said pill, tablet, capsule, or gelcap further comprises an enteric coating.

55. The pharmaceutical composition of any of Claims 49-51, wherein said composition is suitable for parenteral injection into a human.

56. The pharmaceutical composition of any of Claims 49-55, wherein said composition is coadministered with at least one additional therapeutically effective compound.

57. The pharmaceutical composition of Claim 56, wherein said composition comprises at least one additional therapeutically effective compound.

58. A method for treating and/or preventing the symptoms of phenylketonuria in a subject, comprising providing a subject having phenylketonuria, and providing the pharmaceutical composition of any of Claims 49-57 to said subject.

59. The method of Claim 58, wherein said symptoms of phenylketonuria are ameliorated.

60. The method of Claim 58 and/or 59, wherein said subject is able to eat a diet that is less restricted in its methionine, phenylalanine and/or tyrosine content than diets required by subjects who have not been provided at least one pharmaceutical composition comprising at least one engineered polypeptide having phenylalanine ammonia lyase (PAL) activity as set forth in Claims 1-36.

61. The method of any of Claims 58-60, wherein said subject is an infant or child.

62. The method of any of Claims 58-60, wherein said subject is an adult or young adult.

63. Use of the compositions provided in any of Claims 48-57.

[0069a] Figure 7 provides a graph showing the results obtained for Example 5.

[0069b] Figure 8 provides a graph showing the results obtained for Example 8.

DESCRIPTION OF THE INVENTION

[0070] The present invention provides engineered PAL polypeptides, mutants, biologically active fragments and analogues thereof, and pharmaceutical and industrial compositions comprising the same.

[0071] The invention provides engineered phenylalanine ammonia-lyase (PAL) polypeptides and compositions thereof, as well as polynucleotides encoding the engineered phenylalanine ammonia-lyase (PAL) polypeptides. In some embodiments, the engineered PAL polypeptides are optimized to provide enhanced catalytic activity, as well as reduced sensitivity to proteolysis and increased tolerance to acidic pH levels. In some embodiments the engineered PAL polypeptides are deimmunized. The invention also relates to the use of the compositions comprising the engineered PAL polypeptides for therapeutic and industrial purposes.

Abbreviations and Definitions:

[0072] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Generally, the nomenclature used herein and the laboratory procedures of cell culture, molecular genetics, microbiology, organic chemistry, analytical chemistry and nucleic acid chemistry described below are those well-known and commonly employed in the art. Such techniques are well- known and described in numerous texts and reference works well known to those of skill in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses. All patents, patent applications, articles and publications mentioned herein, both *supra* and *infra*, are hereby expressly incorporated herein by reference.

[0073] Although any suitable methods and materials similar or equivalent to those described herein find use in the practice of the present invention, some methods and materials are described herein. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary, depending upon the context they are used by those of skill in the art. Accordingly, the terms defined immediately below are more fully described by reference to the application as a whole. All patents, patent applications, articles and publications mentioned herein, both *supra* and *infra*, are hereby expressly incorporated herein by reference.

[0074] Also, as used herein, the singular "a", "an," and "the" include the plural references, unless the context clearly indicates otherwise.

[0075] Numeric ranges are inclusive of the numbers defining the range. Thus, every numerical range disclosed herein is intended to encompass every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein. It is

also intended that every maximum (or minimum) numerical limitation disclosed herein includes every

The Claims defining the invention are as follows:

1. An engineered polypeptide comprising: a) an amino acid sequence having at least 90% sequence identity to reference sequence SEQ ID NO:4, where said amino acid sequence comprises an amino acid residue difference at position H307G/Q/M; and wherein said engineered polypeptide further comprises at least one or more of the following substitutions A39V, T54K, G59R, S73K, A112C, R134Q, A91V, Y158H, S180A, K195E, Q240R/W, T243EL, I245L, A256G, L257W/A, N270K, N290G, Y304H, R305M, E308Q, I326F, L349M, D353A/N, L364Q, A394V, S399N, N400K, P404A, L407V, F443H, N453G, Y459F, T460G, T463N, N474Q, E509L, Q521K/S, K522Y/F/N, T524S, P528L, S546R, and P564 G/L/M, when optimally aligned with the polypeptide of SEQ ID NO:4, and wherein said engineered polypeptide b) exhibits an improved property selected from i) reduced sensitivity to proteolysis, ii) increased tolerance to acidic pH, iii) reduced immunogenicity, or a combination of any of i), ii), or iii), as compared to the reference sequence SEQ ID NO:4.

2. The engineered polypeptide of Claim 1, wherein the improved property is selected from reduced sensitivity to proteolysis and/or increased tolerance to acidic pH.

3. The engineered polypeptide of Claim 1, wherein the engineered polypeptide has at least 90% sequence identity to reference SEQ ID NO:4.

4. The engineered polypeptide of Claim 1, wherein said engineered polypeptide is resistant to proteolysis, acid stable, and/or deimmunized.

5. The engineered polypeptide of Claim 4, wherein said engineered polypeptide is resistant to proteolysis by at least one digestive tract enzyme, wherein said engineered polypeptide is resistant to proteolysis by chymotrypsin, trypsin, carboxypeptidases, and/or elastases.

6. The engineered polypeptide of Claim 1, wherein said engineered polypeptide is deimmunized.

7. The deimmunized engineered polypeptide of Claim 6, wherein said polypeptide comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO:8.

8. The engineered polypeptide of Claim 1, wherein said polypeptide is purified.

9. A polynucleotide sequence encoding at least one engineered polypeptide of Claim 1.

10. The polynucleotide sequence of Claim 9, wherein said polynucleotide sequence is operably linked to a control sequence.

11. The polynucleotide sequence of Claim 9, wherein said polynucleotide sequence is codon-optimized.

12. An expression vector comprising at least one polynucleotide sequence of Claim 9 and at least one control sequence.

13. The expression vector of Claim 12, wherein said control sequence is a promoter.

14. A host cell transformed with at least expression vector of Claim 12.

15. A method of producing an engineered polypeptide in a host cell comprising culturing a host cell comprising at least one expression vector of Claim 12, under suitable culture conditions, such that at least one engineered PAL polypeptide is produced.

16. The method of Claim 15, further comprising recovering at least one engineered polypeptide from the culture and/or host cells.

17. The method of Claim 16, further comprising the step of purifying said at least one engineered polypeptide.

18. A composition comprising at least one engineered polypeptide of Claim 1.

19. The composition of Claim 18, wherein said composition is a pharmaceutical composition.

20. The composition of Claim 19, wherein said composition is suitable for the treatment of phenylketonuria.

21. The pharmaceutical composition of Claim 20, wherein said composition is suitable for oral administration to a human.

22. The pharmaceutical composition of Claim 21, wherein said composition is in the form of a pill, tablet, capsule, gelcap, liquid, or emulsion.

23. The pharmaceutical composition of Claim 22, wherein said pill, tablet, capsule, or gelcap further comprises an enteric coating.

24. The pharmaceutical composition of Claim 20, wherein said composition is suitable for parenteral injection into a human.