

**ENGINEERED
PHENYLALANINE AMMONIA LYASE POLYPEPTIDES**

[0001] The present application claims priority US Prov. Pat. Appln. Ser. No. 62/565,555 filed September 29, 2017, and US Prov. Pat. Appln. Ser. No. 62/458, 232, filed February 13, 2017, both of which are incorporated by reference in their entireties for all purposes.

**REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER
PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE**

[0002] The Sequence Listing written in file CX7-159USP1_ST25.TXT, created on February 10, 2017, with a size of 44 kilobytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference.

FIELD OF THE INVENTION

[0003] The present 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 storage at elevated temperatures. In some embodiments the engineered PAL polypeptides contain fewer phenylalanine residues. The invention also relates to the use of the compositions comprising the engineered PAL polypeptides for therapeutic and industrial purposes.

BACKGROUND OF THE INVENTION

[0004] Phenylalanine ammonia lyase (PAL) along with histidine ammonia lyase (HAL) and tyrosine ammonia lyase (TAL) are members of the aromatic amino acid lyase family (EC 4.3.1.23-1.25 and 4.3.1.3). More specifically the enzymes having PAL activity (EC 4.3.1.23-1.25 and previously classified as EC 4.3.1.5) catalyze the nonoxidative deamination of L-phenylalanine into (E)-cinnamic acid. PAL is a non-mammalian enzyme that is widely distributed in plants and has also been identified in fungi and a limited number of bacteria.

[0005] PAL enzymes may be used as a therapeutic protein for the treatment of the metabolic disorder, phenylalanine hydroxylase deficiency (PAHD), better known as phenylketonuria (PKU). PKU is an autosomal metabolic genetic disorder in which the hepatic enzyme phenylalanine hydroxylase (PAH) or one or more of the enzymes involved in the synthesis or recycling of the co-factor tetrahydrobiopterin, is partially functional or non-functional due to a mutation in the corresponding genes. This lack of functionality results in elevated levels of phenylalanine in the bloodstream. Depending on the type of mutation the concentration of phenylalanine in the blood stream of PKU patients is typically >360 μ M. The phenylalanine is converted into phenylpyruvate (phenylketone) and other derivatives. In humans, if

PKU is not treated early, high levels of phenylalanine and some of its breakdown products can cause significant medical problems including intellectual disability, microcephaly and seizures. Numerous studies have focused on the use of PAL in the treatment of PKU by enzyme substitution (Ambrus et al., *Science* 201:837–839 [1978]; Bourget et al., *Appl. Biochem. Biotechnol.*, 10:57–59 [1984]; and Sarkissian et al., *Proc. Natl. Acad. Sci. USA* 96:2339–2344 [1999]).

SUMMARY OF THE INVENTION

[0006] The present 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 storage at elevated temperatures. In some embodiments the engineered PAL polypeptides contain fewer phenylalanine residues. The invention also relates to the use of the compositions comprising the engineered PAL polypeptides for therapeutic and industrial purposes. In some embodiments, the present invention is directed to engineered phenylalanine ammonia lyase (PAL) polypeptides and biologically active fragments and analogs thereof having improved properties such as increased storage stability and/or reduced sensitivity to proteolysis.

[0007] The present invention is directed to engineered PAL polypeptides and biologically active fragments and analogs thereof having improved properties when compared to a wild-type PAL enzyme or a reference PAL polypeptide under essentially the same conditions. The invention is further directed to methods of using the engineered PAL polypeptides and biologically active fragments and analogs thereof in therapeutic and/or industrial compositions and methods of using such compositions for therapeutic and/or industrial purposes.

[0008] The present invention provides engineered polypeptides comprising amino acid sequences having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to at least one of SEQ ID NO:4, 6, 8, 10, and/or 12, wherein the amino acid positions of the amino acid sequences are numbered with reference to the amino acid sequence of SEQ ID NO:2 or another amino acid sequence designated as a reference sequence. In some embodiments, the present invention provides engineered polypeptides comprising amino acid sequences having at least 85% or more sequence identity to at least one of SEQ ID NO:4, 6, 8, 10, and/or 12, wherein the amino acid positions of the amino acid sequences are numbered with reference to the amino acid sequence of SEQ ID NO:2 or another amino acid sequence designated as a reference sequence. In some embodiments, the present invention provides engineered polypeptides comprising amino acid sequences having at least 90% or more sequence identity to at least one of SEQ ID NO:4, 6, 8, 10, and/or 12, wherein the amino acid positions of the amino acid sequences are numbered with reference to the amino acid sequence of SEQ ID NO:2 or another amino acid sequence designated as a reference sequence. In

some embodiments, the present invention provides engineered polypeptides comprising amino acid sequences having at least 95% or more sequence identity to at least one of SEQ ID NO:4, 6, 8, 10, and/or 12, wherein the amino acid positions of the amino acid sequences are numbered with reference to the amino acid sequence of SEQ ID NO:2 or another amino acid sequence designated as a reference sequence. In some embodiments, the present invention provides engineered polypeptides comprising amino acid sequences having at least 98% or more sequence identity to at least one of SEQ ID NO:4, 6, 8, 10, and/or 12, wherein the amino acid positions of the amino acid sequences are numbered with reference to the amino acid sequence of SEQ ID NO:2 or another amino acid sequence designated as a reference sequence. In some embodiments, the present invention provides engineered polypeptides comprising amino acid sequences comprising at least one of SEQ ID NO:4, 6, 8, 10, and/or 12, wherein the amino acid positions of the amino acid sequences are numbered with reference to the amino acid sequence of SEQ ID NO:2 or another amino acid sequence designated as a reference sequence.

[0009] The present invention also provides engineered polypeptides comprising at least one substitution or substitution set at one or more amino acid positions selected from 16, 16/150, 44/56, 44/56/102/239/285/469/470/495, 44/239, 44/239/285/469/495, 44/239/285/470, 44/239/469/470, 44/239/470/546, 44/239/495, 44/239/495/546, 44/469/470, 102, 102/470, 162, 165, 188, 239/285, 239/285/469, 239/469/470/495, 264, 267, 285/469/470/495, 285/470, 285/470/495, 364, 455, 469/470, 472, and 482, wherein the amino acid positions are numbered with reference to SEQ ID NO:6. In some additional embodiments, the engineered polypeptides comprise at least one substitution at one or more amino acid positions selected from 16, 264, 364, 472, 482, and/or any combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:6. In some embodiments, the engineered polypeptides comprise at least one substitution or substitution set at one or more amino acid positions selected from 16, 16/150, 162, 188, 264, 267, 398, 434, 472, and 482, and/or any combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:6.

[0010] The present invention also provides engineered polypeptides comprising at least one substitution or substitution set at one or more amino acid positions selected from 44/47/204/209/285, 44/47/364/470/495, 44/54/56/204/239/285/364/495, 44/54/56/204/239/470/495, 44/54/285/470, 44/102/285/364, 44/204/209/285, 44/204/285/364/470/495, 44/209/285/460/495, 44/285/364, 47/54/209, 47/204/209/239/285/495, 47/204/285/364/495, 47/209/239/364, 47/239/285/364, 47/470, 54, 54/56, 54/56/204/209/470, 54/56/204/209/495, 54/56/204/495, 54/56/209/562, 54/56/285/364/470, 54/56/285/470, 54/165/204/209/239/285/470/495, 54/239/495, 54/285/470, 54/470, 56, 165, 204, 204/209/239/285/470/495, 204/209/364, 204/209/364/495, 204/239, 204/239/285, 204/364, 204/470, 209/285/364/470, 209/285/364/470/495, 209/364, 209/364/495, 209/470, 239/364, 285/364, 285/364/495, 364, 364/470, 470, and 495, and/or any combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:8.

[0011] The present invention also provides engineered polypeptides comprising at least one substitution or substitution set at one or more amino acid positions selected from 54/56/162/204/285, 54/56/162/204/398, 54/56/204/285, 54/56/204/398, 54/56/204/398/472, 54/56/285, 54/56/398, 54/56/398/472, 54/162/204/398, 54/162/398, 54/204/285/398/472, 54/204/398, 54/285/398, 54/285/398/472, 56/162/398, 56/204/285, 56/204/398, 56/204/398/460, 56/204/398/472, 56/285, 56/285/398/472, 56/398, 56/398/472, and 201/204/398, and/or any combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:12. In some embodiments, the engineered polypeptides comprise at least one substitution or substitution set at one or more amino acid positions selected from 16, 18, 39, 47, 54, 59, 73, 91, 209, 214, 285, 290, 305, 307, 364, 407, 450, 470, 503, 521, 524, and 565, and/or any combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:12.

[0012] The present invention also provides engineered polypeptides comprising at least one substitution or substitution set at one or more amino acid positions selected from 15/18/39/47/54/59/73/91/209/214/285/290/307/364/407/450/470/503/521/524/565, 16/18/19/214/407, 16/18/23/47/54/59/73/91/209/214/285/290/305/307/364/407/450/470/503/521/524/565, 16/18/39/43/44/59/209/503/565, 16/18/39/45/47/54/59/73/98/209/214/285/290/305/364/407/450/470/503/521/524/565, 16/18/39/47/49/54/59/73/209/285/290/305/307/364/450/470/503/521/524/565, 16/18/39/47/54/59/73/91/98/285/290/305/307/364/450/470/503/524/565, 16/18/39/47/54/59/73/91/209/214/285/290/305/307/313/364/450/503/521/524/565, 16/18/39/47/54/59/73/91/209/214/285/290/305/307/364/407/450/470/503/521/524, 16/18/39/47/54/59/73/91/209/214/285/290/305/307/364/407/450/470/503/521/524/565, 16/18/39/47/54/59/73/91/209/214/285/290/305/307/364/407/450/470/521/524/565, 16/18/39/47/54/59/73/91/209/214/285/290/305/307/364/407/450/503/521/524, 16/18/39/47/54/59/73/91/209/214/285/290/305/307/364/450/470/521/524/565, 16/18/39/47/54/59/73/91/209/214/285/290/305/364/407/450/503/521/524/565, 16/18/39/47/54/59/73/91/209/214/285/290/305/364/407/470/503/521/524, 16/18/39/47/54/59/73/91/209/214/285/290/307/364/407/450/470/503/524/565, 16/18/39/47/54/59/73/91/209/285/290/305/307/407/450/503/521/524/565, 16/18/39/47/54/59/73/91/209/285/290/307/364/407/470/503/521/524/565, 16/18/39/47/54/59/73/91/214/285/290/305/307/364/407/503, 16/18/39/47/54/59/73/91/285/290/305/307/364/407/450/470/503/521, 16/18/39/47/54/59/73/91/285/290/305/307/364/407/450/470/503/521/524/565, 16/18/39/47/54/59/73/91/285/290/305/364/407/470/503/521/524, 16/18/39/47/54/59/73/285/290/305/307/364/407/450/470/503/521/524/565, 16/18/39/47/54/59/91/98/209/285/290/305/307/364/407/450/470/503/521/524,

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 450/503, 450/524, 503, and 524, and/or any combinations thereof, wherein the amino acid positions are
 numbered with reference to SEQ ID NO:2.

[0013] In some embodiments, the engineered polypeptide is an *Anabaena variabilis* variant enzyme. In some additional embodiments, the engineered polypeptide of the present invention is more thermostable than wild-type *Anabaena variabilis* phenylalanine ammonia lyase. In some additional embodiments, the engineered polypeptide more resistant to proteolysis than wild-type *Anabaena variabilis* phenylalanine ammonia lyase. In yet some further embodiments, the engineered polypeptide is resistant to proteolysis by at least one digestive tract enzyme than wild-type *Anabaena variabilis* phenylalanine ammonia lyase. In still some further embodiments, the engineered polypeptide is more resistant to proteolysis by chymotrypsin, trypsin, carboxypeptidases, and/or elastases than wild-type *Anabaena variabilis* phenylalanine ammonia lyase. In yet some further embodiments, the engineered polypeptide is more acid stable than wild-type *Anabaena variabilis* phenylalanine ammonia lyase. In still additional embodiments, the engineered polypeptide is purified.

[0014] The present invention also provides engineered phenylalanine ammonia lyase polypeptides comprising amino acid sequences having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:2, 4, 6, 8, 10, and/or 12, or a functional fragment thereof. In some embodiments, the engineered phenylalanine ammonia lyase

polypeptides comprise amino acid sequences having at least 90% sequence identity to SEQ ID NO:2, 4, 6, 8, 10, and/or 12, or a functional fragment thereof. In some additional embodiments, the engineered phenylalanine ammonia lyase polypeptides comprise an amino acid sequences having at least about 95% sequence identity to SEQ ID NO:2, 4, 6, 8, 10, and/or 12, or a functional fragment thereof. In some further embodiments, the engineered phenylalanine ammonia lyase polypeptide is a variant phenylalanine ammonia lyase polypeptide provided in any of Table 6.1, Table 7.1, Table 8.1, Table 9.1, Table 10.1, Table 14.1, and/or Table 15.1.

[0015] In some additional embodiments, the engineered phenylalanine ammonia lyase polypeptide is an *Anabaena variabilis* variant enzyme. In some additional embodiments, the engineered phenylalanine ammonia lyase polypeptide of the present invention is more thermostable than wild-type *Anabaena variabilis* phenylalanine ammonia lyase. In some additional embodiments, the engineered phenylalanine ammonia lyase polypeptide more resistant to proteolysis than wild-type *Anabaena variabilis* phenylalanine ammonia lyase. In yet some further embodiments, the engineered phenylalanine ammonia lyase polypeptide is resistant to proteolysis by at least one digestive tract enzyme, than wild-type *Anabaena variabilis* phenylalanine ammonia lyase. In still some further embodiments, the engineered phenylalanine ammonia lyase polypeptide is more resistant to proteolysis by chymotrypsin, trypsin, carboxypeptidases, and/or elastases than wild-type *Anabaena variabilis* phenylalanine ammonia lyase. In some additional embodiments, the engineered phenylalanine ammonia lyase polypeptide is more acid stable than wild-type *Anabaena variabilis* phenylalanine ammonia lyase. In some further embodiments, the engineered phenylalanine ammonia lyase polypeptide is purified.

[0016] The present invention also provides engineered polynucleotide sequences encoding at least one engineered polypeptide and/or phenylalanine ammonia lyase polypeptide provided herein. In some embodiments, the engineered polynucleotide encodes at least one engineered polypeptide provided herein. In some additional embodiments, the engineered polynucleotide encodes at least one engineered phenylalanine ammonia lyase polypeptide provided herein. In some embodiments, the engineered polynucleotide encodes an engineered polypeptide provided herein. In some additional embodiments, the engineered polynucleotide encodes an engineered phenylalanine ammonia lyase polypeptide provided herein. In some embodiments, the engineered polynucleotide sequence is operably linked to a control sequence. In some additional embodiments, the engineered polynucleotide sequence is codon-optimized.

[0017] The present invention also provides expression vectors comprising at least one engineered polynucleotide sequence provided herein. In some embodiments, the expression vectors comprise an engineered polynucleotide sequence provided herein. In some additional embodiments, the expression vectors further comprise at least one control sequence. In some additional embodiments, the expression vectors further comprise a control sequence. In some further embodiments, the control sequence comprises a promoter. In some embodiments, the promoter is a heterologous promoter.

[0018] The present invention also provides host cells transformed with at least one polynucleotide sequence provided herein. In some embodiments, the host cells are transformed with a polynucleotide sequence provided herein. In some additional embodiments, the host cells are *E. coli* cell.

[0019] The present invention also provides methods of producing an engineered polypeptide in a host cell comprising culturing a host cell comprising at least one polynucleotide encoding at least one engineered polypeptide provided herein, and/or at least one polynucleotide sequence provided herein, and/or at least one expression vector provided herein, under suitable culture conditions, such that at least one engineered polypeptide is produced. In some embodiments, the present invention also provides methods of producing an engineered phenylalanine ammonia lyase polypeptide in a host cell comprising culturing a host cell comprising at least one polynucleotide encoding at least one engineered phenylalanine ammonia lyase polypeptide provided herein, and/or at least one polynucleotide sequence provided herein, and/or at least one expression vector provided herein, under suitable culture conditions, such that at least one engineered phenylalanine ammonia lyase polypeptide is produced. In some embodiments, the methods further comprise recovering at least one engineered polypeptide and/or phenylalanine ammonia lyase polypeptide provided herein from the culture and/or host cells. In some further embodiments, the methods further comprise the step of purifying at least one engineered polypeptide and/or phenylalanine ammonia lyase polypeptide provided herein.

[0020] The present invention also provides compositions comprising at least one engineered polypeptide provided herein. The present invention also provides compositions comprising at least one engineered phenylalanine ammonia lyase polypeptide provided herein. The present invention also provides compositions comprising an engineered phenylalanine ammonia lyase polypeptide provided herein.

[0021] The present invention also provides compositions comprising at least one engineered polynucleotide provided herein. In some embodiments, the compositions comprise an engineered polynucleotide provided herein. In some embodiments, the composition is a pharmaceutical composition. In some additional embodiments, the composition further comprises at least one pharmaceutically acceptable excipient and/or carrier. In some further embodiments, the composition is suitable for the treatment of phenylketonuria. In some additional embodiments, the composition is suitable for the treatment of elevated blood phenylalanine. In some embodiments, the composition is suitable for the treatment of hyperphenylalaninemia. In some additional embodiments, the composition is suitable for the treatment of tyrosinemia. In some further embodiments, the composition is suitable for the treatment of elevated blood tyrosine. In some embodiments, the composition is suitable for the treatment of hypertyrosinemia. In yet some additional embodiments, the composition is suitable for use in gene therapy. In some further embodiments, the composition is suitable for use in gene therapy to treat phenylketonuria, elevated blood phenylalanine, hyperphenylalaninemia, tyrosinemia, elevated blood tyrosinemia, and/or hypertyrosinemia. In yet some additional embodiments, the composition is

suitable for use in mRNA therapy. In some further embodiments, the composition is suitable for use in mRNA therapy to treat phenylketonuria, elevated blood phenylalanine, hyperphenylalaninemia, tyrosinemia, elevated blood tyrosinemia, and/or hypertyrosinemia. In some embodiments, the composition is suitable for oral administration to a human. In some additional embodiments, the composition is in the form of a pill, tablet, capsule, gelcap, liquid, or emulsion. In some further embodiments, the pill, tablet, capsule, or gelcap further comprises an enteric coating. In some alternative embodiments, the composition is suitable for parenteral injection into a human. In yet some additional embodiments, the composition is coadministered with at least one additional therapeutically effective compound. In some other embodiments, the composition comprises at least one additional therapeutically effective compound.

[0022] The present invention also provides methods for treating and/or preventing the symptoms of phenylketonuria in a subject, comprising providing a subject having phenylketonuria, and providing at least one composition provided herein to a subject. In some embodiments, the phenylalanine concentration in the blood of the subject is reduced upon providing the composition to the subject. In some additional embodiments, the cinnamic acid concentration in the blood of the subject is increased upon providing the composition to the subject. In some further embodiments, the symptoms of phenylketonuria in the subject are ameliorated. In yet some additional embodiments, the subject is able to eat a diet that is less restricted in its phenylalanine content than diets required by subjects who have not been provided at least one pharmaceutical composition comprising at least one engineered phenylalanine ammonia lyase provided herein. In some embodiments, the subject is an infant, while in some other embodiments, the subject is a child, and in some other embodiments, the subject is a young adult, and in yet some additional embodiments, the subject is an adult.

[0023] The present invention also provides for the use of any of the compositions provided herein. In some embodiments, the compositions comprise at least one engineered phenylalanine ammonia lyase. In some embodiments, the any of the compositions provided herein, alone or in any combination. It is not intended that the present invention be limited to any particular use.

DESCRIPTION OF THE DRAWINGS

[0024] Figure 1 is a graph showing the tolerance of AvPAL and Variant #126 to intestinal fluids without proteases. Residual PAL activity of wild-type AvPAL and Variant # 126 are shown after incubation in fasted state simulated intestinal fluids (FaSSIF), early fed state simulated intestinal fluids (FeSSIF early), middle fed state simulated intestinal fluids (FeSSIF middle), and late fed state simulated intestinal fluids (FeSSIF late) in the absence of trypsin and chymotrypsin.

[0025] Figure 2 is a graph showing the tolerance of AvPAL and Variant #126 to intestinal fluids with proteases. Residual PAL activity of AvPAL and Variant #126 are shown after incubation in fasted state

simulated intestinal fluids (FaSSIF), early fed state simulated intestinal fluids (FeSSIF early), middle fed state simulated intestinal fluids (FeSSIF middle), and late fed state simulated intestinal fluids (FeSSIF late) in the presence of trypsin and chymotrypsin.

[0026] Figure 3 is a graph showing the relative plasma phenylalanine levels in beagles for groups treated with various doses of Variant #126. Error bars are omitted for clarity (*: $p < 0.05$, **: $p < 0.01$).

[0027] Figure 4 is a graph showing the plasma CA levels in beagles for groups treated with various doses of Variant 126.

[0028] Figure 5 is a graph showing the AUC for cinnamic acid in beagles in plasma levels for groups treated with various doses of Variant 126. Statistical significance provided for each group vs. vehicle (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

[0029] Figure 6 is a graph showing the relative plasma phenylalanine (Phe) levels in cynomolgus monkeys for groups treated with various doses of Variant 126. Some groups were not pretreated with famotidine (-famo).

[0030] Figure 7 is a graph showing the AUC of relative phenylalanine (Phe) in plasma of cynomolgus monkeys for groups treated with various doses of Variant 126. The dashed line represents the average AUC for all doses Variant 126 (**: $p < 0.01$ for t-test between vehicle and all doses combined).

[0031] Figure 8A is a graph showing the plasma cinnamic acid (CA) levels in cynomolgus monkeys for groups pre-treated with famotidine and who received various doses of Variant 126.

[0032] Figure 8B is a graph showing the plasma cinnamic acid (CA) levels in cynomolgus monkeys for groups not pre-treated with famotidine and who received various doses of Variant 126.

[0033] Figure 9A is a graph showing the plasma cinnamic acid (CA) AUC over 0-4 hrs for groups of cynomolgus monkeys treated with various doses of Variant 126 in cynomolgus monkeys pre-treated with famotidine (top) or not (middle).

[0034] Figure 9B is a graph showing the plasma cinnamic acid (CA) AUC over 0-4 hrs for groups of cynomolgus monkeys treated with various doses of Variant 126 in cynomolgus monkeys not pre-treated with famotidine.

[0035] Figure 9C is a graph providing a direct comparison of the 20 and 200 mg/kg groups that were not pre-treated with famotidine or received famotidine 2 hrs prior to dosing (bottom) (*: $p < 0.05$, **: $p < 0.01$).

DESCRIPTION OF THE INVENTION

[0036] The present invention provides engineered PAL polypeptides, mutants, biologically active fragments and analogues thereof, and pharmaceutical and industrial compositions comprising the same.

[0037] 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 storage at elevated temperatures. In some embodiments the engineered PAL polypeptides contain fewer phenylalanine residues. The present invention also provides methods for the use of compositions comprising the engineered PAL polypeptides for therapeutic and industrial purposes.

[0038] One method of detoxifying phenylalanine in the blood stream is the use of injectable recombinant PAL and PAL variants modified by pegylation (PEG-PAL). Pegylation has been shown to improve enzyme half-life and reduce subject antigenic response (See e.g., WO 2008/153776, WO 2011/097335, and US Pat. No. 7,531,341). PAL variants useful in PEG-PAL compositions have been described as variants of wild-type *Nostoc punctiforme* (NpPAL); *Anabaena variabilis* (AvPAL) and *Rhodospiridium toruloides* (RtPAL). In particular, variants of wild-type AvPAL have been described wherein the cysteine residues at positions 64, 318, 503 and 565 have been substituted with serine (See e.g., US Pat Nos: 7,790,433; 7,560,263; and 7,537,923).

[0039] An alternative route of PAL administration as a means of reducing plasma concentration of L-phenylalanine in PKU subjects is a non-invasive formulation such as an oral formulation (Sarkissian et al., Proc. Natl. Acad. Sci. USA 96:2339–2344 [1999]). A key advantage of oral delivery of PAL is the reduced exposure of the enzyme to the immune system thereby minimizing the immune response which is observed with injectable PEG-PAL. However, a major limitation for the oral formulation of PAL is loss of enzyme activity in the stomach and intestinal lumen. In order to be effective and functional PAL must resist degradation by acidic pHs and proteases such as trypsin, chymotrypsin, carboxypeptidases and pepsin that normally degrade proteinaceous foods to oligopeptides and amino acids. In some previous studies (Sarkissian, *supra*) in order to achieve a significant effect for the oral administration of PAL, a large amount of the enzyme was required partly due to enzymatic degradation by proteases and partly due to relatively low specific activity at pH 7.0. Various means have been explored to suppress PAL degradation upon digestion (Kim et al., Molec. Therap., 10:220–224 [2004]; and Shah et al., Int. J. Pharmaceut., 356:61–68 [2008]).

[0040] One approach to increase the effectiveness of PAL under the harsh conditions of the digestive tract is to provide engineered PAL polypeptides that are tolerant to the inherent harsh conditions. Kang et al. used site directed mutagenesis of a chymotrypsin cleavage site and pegylation of surface lysines of an AvPAL to reduce proteolytic inactivation (See, Kang et al., Mol. Gen. Metabol., 99:4-9 [2010]). In these studies ten cleavage sites were specifically mutated and all but two of these resulting mutants (F18A and R94G) lost more than 50% of the original enzyme activity. None of the mutants showed increased activity and the F18A mutant showed a slight increase in trypsin resistance (Kang et al., *supra*). Further studies with PAL, while effective, generally have not resulted in a longer lived enzyme. Therefore, oral administration of PAL mutants previously described in the literature and derivatives thereof did not result in effective treatment of PKU.

[0041] Despite the progress made with various formulations of PAL there remains a need for PAL polypeptides having improved properties for oral administration. These improved properties include without limitation a greater half-life, increased catalytic activity, improved stability to the conditions in the digestive track as well as storage conditions, and reduced aggregation.

[0042] PAL enzymes may also find use as therapeutic proteins for the treatment of disorders of tyrosine metabolism, such as tyrosinemia Type I, tyrosinemia Type II, tyrosinemia Type III, and alkaptonuria. These disorders of tyrosine metabolism are autosomal metabolic genetic disorders in which one of the enzymes involved in the degradation of tyrosine is partially functional or non-functional due to a mutation in the corresponding gene. This lack of functionality results in elevated levels of tyrosine and other tyrosine-metabolites in the bloodstream. Because tyrosine is derived from phenylalanine, it is beneficial for patients with a disorder of tyrosine metabolism to limit phenylalanine and tyrosine intake. PAL enzymes are thus a potential treatment of disorders of tyrosine metabolism.

[0043] If patients with a disorder of tyrosine metabolism are not treated early, high levels of tyrosine and some of its breakdown products can cause significant medical problems including liver and kidney failure, liver cancer, intellectual disability, and even death.

[0044] In addition to therapeutic applications PAL enzymes may also be used in the industrial synthesis of L-phenylalanine and other substituted L-phenylalanine derivatives. These derivatives may then be used as pharmaceutical precursors (Gloge et al., Chem., 6: 3386-3390 [2000]; Bartsch et al., Prot. Eng. Des. Sel., 23:929-933 [2010]; and Turner, Curr. Opin. Chem. Biol., 234-240 [2011]). PAL enzymes may also be used in agricultural applications. PAL plays a significant role in biosynthesis of phenylpropanoids (such as flavonoids and lignin) in plants, fungi and bacteria and can act as a defense related enzyme (Bate et al., Proc. Natl. Acad. Sci. USA 91:7608-7612 [1994]). Modulation of PAL activity by using recombinant polypeptides having PAL activity could potentially lead to effective herbicides.

Abbreviations and Definitions:

[0045] 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.

[0046] 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.

[0047] Also, as used herein, the singular "a", "an," and "the" include the plural references, unless the context clearly indicates otherwise.

[0048] 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 lower (or higher) numerical limitation, as if such lower (or higher) numerical limitations were expressly written herein.

[0049] The term "about" means an acceptable error for a particular value. In some instances "about" means within 0.05%, 0.5%, 1.0%, or 2.0%, of a given value range. In some instances, "about" means within 1, 2, 3, or 4 standard deviations of a given value.

[0050] Furthermore, the headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the application as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the application as a whole. Nonetheless, in order to facilitate understanding of the invention, a number of terms are defined below.

[0051] Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

[0052] As used herein, the term "comprising" and its cognates are used in their inclusive sense (*i.e.*, equivalent to the term "including" and its corresponding cognates).

[0053] "EC" number refers to the Enzyme Nomenclature of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). The IUBMB biochemical classification is a numerical classification system for enzymes based on the chemical reactions they catalyze.

[0054] "ATCC" refers to the American Type Culture Collection whose biorepository collection includes genes and strains.

[0055] "NCBI" refers to National Center for Biological Information and the sequence databases provided therein.

[0056] As used herein, the term "phenylalanine ammonia lyase (PAL) polypeptide" refers to a class of enzymes within the aromatic amino acid lyase family (EC 4.3.1.23, EC 4.3.1.24 and EC4.3.1.25) which also includes histidine ammonia lyase, and tyrosine ammonia lyase. The PAL polypeptides are also sometimes referred to as phenylalanine/tyrosine ammonia lyases because some PAL enzymes may use

tyrosine as well as phenylalanine as a substrate. However, the AvPAL and variants disclosed and claimed herein do not use tyrosine as a substrate. PAL polypeptides catalyze the conversion of L-phenylalanine to trans-cinnamic acid and ammonia. PAL activity refers to the enzymatic activity of PAL polypeptides. In some preferred embodiments, a PAL enzyme also contains the cofactor 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO). This cofactor may be required for catalytic activity and is formed by cyclization and dehydration of a conserved active site Ala167-Ser168-Gly169 tripeptide segment.

[0057] “Protein,” “polypeptide,” and “peptide” are used interchangeably herein to denote a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

[0058] “Polynucleotide” is used herein to denote a polymer comprising at least two nucleotides where the nucleotides are either deoxyribonucleotides or ribonucleotides.

[0059] “Amino acids” are referred to herein by either their commonly known three-letter symbols or by the one-letter symbols recommended by IUPAC-IUB Biochemical Nomenclature Commission.

Nucleotides, likewise, may be referred to by their commonly accepted single letter codes.

[0060] The term “engineered,” “recombinant,” “non-naturally occurring,” and “variant,” when used with reference to a cell, a polynucleotide or a 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.

[0061] As used herein, “wild-type” and “naturally-occurring” refer to the form found in nature. For example a 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.

[0062] “Coding sequence” refers to that part of a nucleic acid (e.g., a gene) that encodes an amino acid sequence of a protein.

[0063] The term “percent (%) sequence identity” is used 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 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 (Smith and Waterman, *Adv. Appl. Math.*, 2:482 [1981]), by the homology alignment algorithm of Needleman and Wunsch (Needleman and Wunsch, *J. Mol. Biol.*, 48:443 [1970]), by the search for similarity method of Pearson and Lipman (Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444 [1988]), by computerized implementations of these algorithms (*e.g.*, GAP, BESTFIT, FASTA, and TFASTA in the GCG Wisconsin Software Package), or by visual inspection, as known in the art. Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity include, but are not limited to the BLAST and BLAST 2.0 algorithms (See *e.g.*, Altschul *et al.*, *J. Mol. Biol.*, 215: 403-410 [1990]; and Altschul *et al.*, *Nucleic Acids Res.*, 3389-3402 [1977]). 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 (See, 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 *e.g.*, Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 [1989]). 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.

[0064] “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, at least 100 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 or polypeptides over a “comparison window” to identify and compare local regions of sequence similarity. In some embodiments, a “reference sequence” can be based on a primary amino acid sequence, where the reference sequence is a sequence that can have one or more changes in the primary sequence. For instance, the phrase “reference sequence based on SEQ ID NO:4 having a valine at the residue corresponding to X39” refers to a reference sequence in which the corresponding residue at position X39 in SEQ ID NO:4 (*e.g.*, an alanine), has been changed to valine.

[0065] “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.

[0066] “Corresponding to”, “reference to,” and “relative to” when used in the context of the numbering of a given amino acid or polynucleotide sequence refer 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 PAL, 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.

[0067] “Amino acid difference” and “residue difference” refer to a difference in the amino acid residue at a position of a polypeptide sequence relative to the amino acid residue at a corresponding position in a reference sequence. The positions of amino acid differences generally are referred to herein as “X_n,” where n refers to the corresponding position in the reference sequence upon which the residue difference is based. For example, a “residue difference at position X91 as compared to SEQ ID NO:4” refers to a difference of the amino acid residue at the polypeptide position corresponding to position 91 of SEQ ID NO:4. Thus, if the reference polypeptide of SEQ ID NO:4 has a alanine at position 91, then a “residue

difference at position X91 as compared to SEQ ID NO:4” refers to an amino acid substitution of any residue other than alanine at the position of the polypeptide corresponding to position 91 of SEQ ID NO:4. In most instances herein, the specific amino acid residue difference at a position is indicated as “XnY” where “Xn” specified the corresponding residue and position of the reference polypeptide (as described above), and “Y” is the single letter identifier of the amino acid found in the engineered polypeptide (*i.e.*, the different residue than in the reference polypeptide). In some instances (*e.g.*, in the Tables in the Examples), the present disclosure also provides specific amino acid differences denoted by the conventional notation “AnB”, where A is the single letter identifier of the residue in the reference sequence, “n” is the number of the residue position in the reference sequence, and B is the single letter identifier of the residue substitution in the sequence of the engineered polypeptide. In some instances, a polypeptide of the present disclosure can include one or more amino acid residue differences relative to a reference sequence, which is indicated by a list of the specified positions where residue differences are present relative to the reference sequence. In some embodiments, where more than one amino acid can be used in a specific residue position of a polypeptide, the various amino acid residues that can be used are separated by a “/” (*e.g.*, X307G/X307Q or X307G/Q). The present disclosure includes engineered polypeptide sequences comprising one or more amino acid differences that include either/or both conservative and non-conservative amino acid substitutions.

[0068] The terms “amino acid substitution set” and “substitution set” refers to a group of amino acid substitutions within a polypeptide sequence. In some embodiments, substitution sets comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more amino acid substitutions. In some embodiments, a substitution set refers to the set of amino acid substitutions that is present in any of the variant AvPAL polypeptides listed in any of the Tables in the Examples.

[0069] “Conservative amino acid substitution” refers to a substitution of a residue with a different residue having a similar side chain, 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. By way of example and not limitation, an amino acid with an aliphatic side chain may be substituted with another aliphatic amino acid (*e.g.*, alanine, valine, leucine, and isoleucine); an amino acid with hydroxyl side chain is substituted with another amino acid with a hydroxyl side chain (*e.g.*, serine and threonine); an amino acid having aromatic side chains is substituted with another amino acid having an aromatic side chain (*e.g.*, phenylalanine, tyrosine, tryptophan, and histidine); an amino acid with a basic side chain is substituted with another amino acid with a basic side chain (*e.g.*, lysine and arginine); an amino acid with an acidic side chain is substituted with another amino acid with an acidic side chain (*e.g.*, aspartic acid or glutamic acid); and a hydrophobic or hydrophilic amino acid is replaced with another hydrophobic or hydrophilic amino acid, respectively. Exemplary conservative substitutions are provided in Table 1.

Table 1. Exemplary Conservative Amino Acid Substitutions	
Residue	Potential Conservative Substitutions
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)
N, Q, S, T	Other polar
H, Y, W, F	Other aromatic (H, Y, W, F)
C, P	Non-polar

[0070] “Non-conservative substitution” refers to substitution 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 and affect: (a) the structure of the peptide backbone in the area of the substitution (*e.g.*, proline for glycine); (b) the charge or hydrophobicity; and/or (c) the bulk of the side chain. By way of example and not limitation, exemplary non-conservative substitutions include an acidic amino acid substituted with a basic or aliphatic amino acid; an aromatic amino acid substituted with a small amino acid; and a hydrophilic amino acid substituted with a hydrophobic amino acid.

[0071] “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 transaminase 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.

[0072] “Insertion” refers to modification to the polypeptide by addition of one or more amino acids from the reference polypeptide. 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.

[0073] The terms “functional fragment” and “biologically active fragment” are used interchangeably herein, to refer to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion(s) and/or internal deletions, but where the remaining amino acid sequence is identical to the corresponding positions in the sequence to which it is being compared (e.g., a full length engineered PAL of the present invention) and that retains substantially all of the activity of the full-length polypeptide.

[0074] “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 recombinant PAL polypeptides 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 recombinant PAL polypeptides provided herein are isolated polypeptides.

[0075] “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 PAL 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 recombinant PAL polypeptides are substantially pure polypeptide compositions.

[0076] “Improved enzyme property” refers to an engineered PAL polypeptide that exhibits an improvement in any enzyme property as compared to a reference PAL polypeptide, such as a wild-type PAL polypeptide (e.g., AvPAL wild-type having SEQ ID NO:4) or another engineered PAL polypeptide. Improved properties include but are not limited to such properties as increased protein expression, increased thermoactivity, increased thermostability, increased pH activity, increased stability, increased enzymatic activity, increased substrate specificity and/or affinity, increased specific activity, increased resistance to substrate and/or end-product inhibition, increased chemical stability, improved chemoselectivity, improved solvent stability, increased tolerance to acidic pH, increased tolerance to proteolytic activity (*i.e.*, reduced sensitivity to proteolysis), reduced aggregation, increased solubility, reduced immunogenicity, and altered temperature profile.

[0077] The phrase “contains fewer phenylalanine residues” means that an engineered PAL polypeptide according to the invention will retain at least the activity, or stability when compared to a reference

PAL in a standard assay (e.g., as described in the Examples) after one or more phenylalanine residues have been removed from its polypeptide sequence via mutation to any other amino acid.

[0078] “Increased enzymatic activity” and “enhanced catalytic activity” refer to an improved property of the engineered PAL polypeptides, which can be represented by an increase in specific activity (e.g., product produced/time/weight protein) and/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 PAL) as compared to the reference PAL enzyme (e.g., wild-type AvPAL and/or another engineered AvPAL). 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 1.1 fold the enzymatic activity of the corresponding wild-type enzyme, to as much as 2-fold, 5-fold, 10-fold, 20-fold, 25-fold, 50-fold, 75-fold, 100-fold, 150-fold, 200-fold or more enzymatic activity than the naturally occurring PAL or another engineered PAL from which the PAL polypeptides were derived.

[0079] In some embodiments, the engineered PAL polypeptides have a k_{cat} of at least 0.1/sec, at least 0.2/sec, at least 0.3/sec, at least 0.5/sec, at least 1.0/sec, and in some preferred embodiments greater than 2.0/sec. In some embodiments, the K_m is in the range of about 1 μ M to about 5mM; in the range of about 5 μ M to about 2mM; in the range of about 10 μ M to about 2mM; or in the range of about 10 μ M to about 1mM. In some specific embodiments, the engineered PAL enzyme exhibits improved enzymatic activity in the range of 1.5 to 10 fold, 1.5 to 25 fold, 1.5 to 50 fold, 1.5 to 100 fold or greater, than that of the reference PAL enzyme. PAL activity can be measured by any standard assay known in the art (e.g., by monitoring changes in spectrophotometric properties of reactants or products). In some embodiments, the amount of products produced is measured by High-Performance Liquid Chromatography (HPLC) separation combined with UV absorbance or fluorescent detection directly or following o-phthalaldehyde (OPA) derivatization. In some embodiments, 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, in order to minimize variations in amount of enzyme produced by the host cells and present in the lysates.

[0080] “Physiological pH” as used herein means the pH range generally found in a subject’s (e.g., human) small intestine. There normally is a gradient pH from the pyloric valve to the large intestine, in the range of about 6.0 to 7.5.

[0081] The terms “proteolytic activity” and “proteolysis” used interchangeably herein refer to the breakdown of proteins into smaller polypeptides or amino acids. The breakdown of proteins is generally the result of hydrolysis of the peptide bond by protease (proteinase) enzymes. Protease enzymes include

but are not limited to pepsin, trypsin, chymotrypsin, elastase; carboxypeptidase A and B, and peptidases (e.g., amino peptidase, dipeptidase and enteropeptidase).

[0082] The phrases “reduced sensitivity to proteolysis,” “reduced proteolytic sensitivity” and “increased tolerance to proteolysis,” are used interchangeably herein mean that an engineered PAL polypeptide according to the invention has a higher enzyme activity compared to a reference PAL in a standard assay (e.g., as described in the Examples) after treatment with one or more proteases.

[0083] The phrase “increased storage stability at elevated temperature” means that an engineered PAL polypeptide according to the invention will retain more activity compared to a reference PAL in a standard assay (e.g., as described in the Examples) after it has been produced in a dried form, e.g. by lyophilization or spray-drying, and stored for a period of time ranging from a few days to multiple months at a temperature above room temperature (e.g., 30°C, 37°C, 45°C, 55°C, etc.).

[0084] “Aggregation” means clumping or precipitation of a PAL polypeptide. Aggregation can lead to inactivation of the enzyme. The term “reduced aggregation” means an engineered PAL polypeptide will be less prone to aggregation, as compared to a reference PAL. Methods for assessing aggregation are known in the art, including but not limited to the use of fluorescent microscopy with appropriate dyes (e.g., thioflavin T or Nile Red), dynamic light scattering, flow cytometry with appropriate dyes (e.g., Bodipy), filtration and analysis by SDS-PAGE, and/or Western blotting, fluorescent correlation spectroscopy, and electron microscopy. There are commercially available kits to assess aggregation (e.g., the ProteoStat[®] Protein Aggregation Assay kit [Enzo]).

[0085] “Conversion” refers to the enzymatic conversion (or biotransformation) of substrate(s) to the corresponding product(s). “Percent conversion” refers to the percent of the substrate that is converted to the product within a period of time under specified conditions. Thus, the “enzymatic activity” or “activity” of a PAL polypeptide can be expressed as “percent conversion” of the substrate to the product in a specific period of time.

[0086] “Hybridization stringency” relates to hybridization conditions, such as washing conditions, in the hybridization 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. Another high stringency condition is hybridizing in conditions equivalent to hybridizing in 5X SSC containing 0.1% (w:v) SDS at 65°C and washing in 0.1x SSC containing 0.1% SDS at 65°C. Other high stringency hybridization conditions, as well as moderately stringent conditions, are described in the references cited above.

[0087] “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 more efficiently expressed in that organism. 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 PAL enzymes are codon optimized for optimal production from the host organism selected for expression. “Control sequence” refers herein to include all components that are necessary or advantageous for the expression of a polynucleotide and/or 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, leaders, polyadenylation sequences, propeptide sequences, promoter sequences, signal peptide sequences, initiation sequences, and transcription terminators. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. In some embodiments, the control sequences are 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.

[0088] “Operably linked” is defined herein as a configuration in which a control sequence is appropriately placed (*i.e.*, in a functional relationship) at a position relative to a polynucleotide of interest such that the control sequence directs or regulates the expression of the polynucleotide encoding a polypeptide of interest.

[0089] “Promoter sequence” refers to a nucleic acid sequence that is recognized by a host cell for expression of a polynucleotide of interest, such as a coding sequence. The promoter sequence contains transcriptional control sequences that mediate the expression of a polynucleotide of interest. 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.

[0090] “Substrate” in the context of an enzymatic conversion reaction process refers to the compound or molecule acted on by the PAL polypeptide. “Product” in the context of an enzymatic conversion process refers to the compound or molecule resulting from the action of the PAL polypeptide on the substrate.

[0091] As used herein the term “culturing” refers to the growing of a population of microbial cells under suitable conditions using any suitable medium (e.g., liquid, gel, or solid).

[0092] Recombinant polypeptides (e.g., PAL enzyme variants) can be produced using any suitable methods known the art. For example, there is a wide variety of different mutagenesis techniques well known to those skilled in the art. In addition, mutagenesis kits are also available from many commercial molecular biology suppliers. Methods are available to make specific substitutions at defined amino acids (site-directed), specific or random mutations in a localized region of the gene (regio-specific), or random mutagenesis over the entire gene (e.g., saturation mutagenesis). Numerous suitable methods are known to those in the art to generate enzyme variants, including but not limited to site-directed mutagenesis of single-stranded DNA or double-stranded DNA using PCR, cassette mutagenesis, gene synthesis, error-prone PCR, shuffling, and chemical saturation mutagenesis, or any other suitable method known in the art. Non-limiting examples of methods used for DNA and protein engineering are provided in the following patents: US Pat. No. 6,117,679; US Pat. No. 6,420,175; US Pat. No. 6,376,246; US Pat. No. 6,586,182; US Pat. No. 7,747,391; US Pat. No. 7,747,393; US Pat. No. 7,783,428; and US Pat. No. 8,383,346. After the variants are produced, they can be screened for any desired property (e.g., high or increased activity, or low or reduced activity, increased thermal activity, increased thermal stability, and/or acidic pH stability, etc.). In some embodiments, “recombinant PAL polypeptides” (also referred to herein as “engineered PAL polypeptides,” “variant PAL enzymes,” and “PAL variants”) find use.

[0093] As used herein, a “vector” is a DNA construct for introducing a DNA sequence into a cell. In some embodiments, the vector is an expression vector that is operably linked to a suitable control sequence capable of effecting the expression in a suitable host of the polypeptide encoded in the DNA sequence. In some embodiments, an “expression vector” has a promoter sequence operably linked to the DNA sequence (e.g., transgene) to drive expression in a host cell, and in some embodiments, also comprises a transcription terminator sequence.

[0094] As used herein, the term “expression” includes any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, and post-translational modification. In some embodiments, the term also encompasses secretion of the polypeptide from a cell.

[0095] As used herein, an amino acid or nucleotide sequence (e.g., a promoter sequence, signal peptide, terminator sequence, etc.) is “heterologous” to another sequence with which it is operably linked if the two sequences are not associated in nature.

[0096] As used herein, the terms “host cell” and “host strain” refer to suitable hosts for expression vectors comprising DNA provided herein (e.g., a polynucleotide sequences encoding at least one AvPAL

variant). In some embodiments, the host cells are prokaryotic or eukaryotic cells that have been transformed or transfected with vectors constructed using recombinant DNA techniques as known in the art.

[0097] The term “analogue” means a polypeptide having more than 70 % sequence identity but less than 100% sequence identity (e.g., more than 75%, 78%, 80%, 83%, 85%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity) with a reference polypeptide. In some embodiments, analogues include non-naturally occurring amino acid residues including, but not limited, to homoarginine, ornithine and norvaline, as well as naturally occurring amino acids. In some embodiments, analogues also include one or more D-amino acid residues and non-peptide linkages between two or more amino acid residues.

[0098] The term “therapeutic” refers to a compound administered to a subject who shows signs or symptoms of pathology having beneficial or desirable medical effects.

[0099] The term “pharmaceutical composition” refers to a composition suitable for pharmaceutical use in a mammalian subject (e.g., human) comprising a pharmaceutically effective amount of an engineered PAL polypeptide encompassed by the invention and an acceptable carrier.

[0100] The term “gene therapy” is used in reference to the use of genes (i.e., genetic material) to treat and/or prevent disease in a mammalian subject (e.g., human). In some embodiments, the genetic material is introduced directly into at least some cells of the mammalian subject. It is not intended that the present invention be limited to any specific method(s) or composition(s) useful for gene therapy.

[0101] The term “mRNA therapy” is used in reference to the use of messenger RNA (mRNA) to treat and/or prevent disease in a mammalian subject (e.g., human). In some embodiments, the genetic material is introduced directly into at least some cells of the mammalian subject. It is not intended that the present invention be limited to any specific method(s) or composition(s) useful for mRNA therapy.

[0102] The term “effective amount” means an amount sufficient to produce the desired result. One of general skill in the art may determine what the effective amount by using routine experimentation.

[0103] The terms “isolated” and “purified” are used to refer to a molecule (e.g., an isolated nucleic acid, polypeptide, etc.) or other component that is removed from at least one other component with which it is naturally associated. The term “purified” does not require absolute purity, rather it is intended as a relative definition.

[0104] The term “subject” encompasses mammals such as humans, non-human primates, livestock, companion animals, and laboratory animals (e.g., rodents and lagamorphs). It is intended that the term encompass females as well as males.

[0105] As used herein, the term “patient” means any subject that is being assessed for, treated for, or is experiencing disease.

[0106] The term “infant” refers to a child in the period of the first month after birth to approximately one (1) year of age. As used herein, the term “newborn” refers to child in the period from birth to the 28th

day of life. The term “premature infant” refers to an infant born after the twentieth completed week of gestation, yet before full term, generally weighing ~500 to ~2499 grams at birth. A “very low birth weight infant” is an infant weighing less than 1500 g at birth.

[0107] As used herein, the term “child” refers to a person who has not attained the legal age for consent to treatment or research procedures. In some embodiments, the term refers to a person between the time of birth and adolescence.

[0108] As used herein, the term “adult” refers to a person who has attained legal age for the relevant jurisdiction (e.g., 18 years of age in the United States). In some embodiments, the term refers to any fully grown, mature organism. In some embodiments, the term “young adult” refers to a person less than 18 years of age, but who has reached sexual maturity.

[0109] As used herein, “composition” and “formulation” encompass products comprising at least one engineered PAL of the present invention, intended for any suitable use (e.g., pharmaceutical compositions, dietary/nutritional supplements, feed, etc.).

[0110] The terms “administration” and “administering” a composition mean providing a composition of the present invention to a subject (e.g., to a person suffering from the effects of PKU).

[0111] The term “carrier” when used in reference to a pharmaceutical composition means any of the standard pharmaceutical carrier, buffers, and excipients, such as stabilizers, preservatives, and adjuvants.

[0112] The term “pharmaceutically acceptable” means a material that can be administered to a subject without causing any undesirable biological effects or interacting in a deleterious manner with any of the components in which it is contained and that possesses the desired biological activity.

[0113] As used herein, the term “excipient” refers to any pharmaceutically acceptable additive, carrier, diluent, adjuvant, or other ingredient, other than the active pharmaceutical ingredient (API; e.g., the engineered PAL polypeptides of the present invention). Excipients are typically included for formulation and/or administration purposes.

[0114] The term “therapeutically effective amount” when used in reference to symptoms of disease/condition refers to the amount and/or concentration of a compound (e.g., engineered PAL polypeptides) that ameliorates, attenuates, or eliminates one or more symptom of a disease/condition or prevents or delays the onset of symptom(s) (e.g., PKU). In some embodiments, the term is used in reference to the amount of a composition that elicits the biological (e.g., medical) response by a tissue, system, or animal subject that is sought by the researcher, physician, veterinarian, or other clinician.

[0115] The term “therapeutically effective amount” when used in reference to a disease/condition refers to the amount and/or concentration of a composition that ameliorates, attenuates, or eliminates the disease/condition.

[0116] It is intended that the terms “treating,” “treat” and “treatment” encompass preventative (e.g., prophylactic), as well as palliative treatment.

Engineered PAL Polypeptides:

[0117] The parent PAL polypeptides from which the engineered PAL polypeptides of the invention are derived from include bacterial strains such as *Anabaena* (e.g., *A. variabilis*), *Nostoc* (e.g., *N. punctiforme*), *Rhodospiridium* (e.g., *R. toruloides*), *Streptomyces* (e.g., *S. maritimus* or *S. verticillatus*), *Oscillatoria* sp., *Gloeocapsa* sp., and *Rivularia* sp. PAL enzymes from these strains have been identified and are well known. Homologous enzyme sequences from *Anabaena* (*A. variabilis*) ATCC 29413 and NCBI YP_324488.1; *Nostoc* (*N. punctiforme*) ATCC 29133 and NCBI YP_00186563.1; *Oscillatoria* sp. PCC 6506 and NCBI ZP_07108482.1 and *Gloeocapsa* sp. PCC7428 and NCBI YP_007127054.1 are provided in Figure 1 of US Pat. Appln. Ser. No. 2014/0314843, incorporated herein by reference.

[0118] Furthermore, when a particular PAL variant (i.e., an engineered PAL polypeptide) is referred to by reference to modification of particular amino acids residues in the sequence of a wild-type PAL or reference PAL it is to be understood that variants of another PAL modified in the equivalent position(s) (as determined from the optional amino acid sequence alignment between the respective amino acid sequences) are encompassed herein. In some embodiments, the engineered PAL polypeptide is derived from any one of the polypeptides listed from the bacterial strains above (i.e., *Nostoc* [*N. punctiforme*], *Rhodospiridium* [*R. toruloides*], *Streptomyces* [*S. maritimus* or *S. verticillatus*], *Oscillatoria* sp., *Gloeocapsa* sp and *Rivularia* sp.). In some additional embodiments, the engineered PAL polypeptide of the present invention comprises the conserved active site Ala167- Ser168-Gly169 and comprises at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO:4. In some embodiments, the engineered PAL polypeptides comprise not only PAL activity but are also active on tyrosine and/or histidine substrates.

[0119] In some embodiments, engineered PAL polypeptides are produced by cultivating a microorganism comprising at least one polynucleotide sequence encoding at least one engineered PAL polypeptide under conditions which are conducive for producing the engineered PAL polypeptide. In some embodiments, the engineered PAL polypeptide is subsequently recovered from the resulting culture medium and/or cells.

[0120] The present invention provides exemplary engineered PAL polypeptides having PAL activity. The Examples provide Tables showing sequence structural information correlating specific amino acid sequence features with the functional activity of the engineered PAL polypeptides. This structure-function correlation information is provided in the form of specific amino acid residue differences relative to the reference engineered polypeptide of SEQ ID NO:2, as well as associated experimentally determined activity data for the exemplary engineered PAL polypeptides.

[0121] In some embodiments, the engineered PAL polypeptides of the present invention having PAL activity comprise a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:2; b) an amino acid residue difference as compared to SEQ ID NO:2 at one or more amino

acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced proteolytic sensitivity, iii) reduced aggregation, iv) increased stability as a lyophilized preparation to elevated temperatures, v) a reduced number of phenylalanine residues in its primary structure, or a combination of any of i), ii), iii), iv), or v) as compared to the reference sequence.

[0122] In some embodiments the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:2, and an amino acid residue difference as compared to SEQ ID NO:2, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 20 or more amino acid positions compared to SEQ ID NO:2 or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:2). In some embodiments, the residue difference as compared to SEQ ID NO:2, at one or more positions includes at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more conservative amino acid substitutions. In some embodiments, the engineered PAL polypeptide is a polypeptide listed in the Tables provided in the Examples.

[0123] In some embodiments, the engineered PAL polypeptides of the present invention having PAL activity comprise a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:4; b) an amino acid residue difference as compared to SEQ ID NO:4 at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced proteolytic sensitivity, iii) reduced aggregation, iv) increased stability as a lyophilized preparation to elevated temperatures, v) a reduced number of phenylalanine residues in its primary structure, or a combination of any of i), ii), iii), iv), or v) as compared to the reference sequence.

[0124] In some embodiments the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:4, and an amino acid residue difference as compared to SEQ ID NO:4, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 20 or more amino acid positions compared to SEQ ID NO:4 or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:4). In some embodiments, the residue difference as compared to SEQ ID NO:4, at one or more positions includes at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more conservative amino acid substitutions. In some embodiments, the engineered PAL polypeptide is a polypeptide listed in the Tables provided in the Examples.

[0125] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence

identity with SEQ ID NO:6. In some embodiments the amino acid difference is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 or greater amino acid positions. In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:6 and at least one amino acid residue difference as compared to SEQ ID NO:6, at one or more amino acid positions selected from 16, 16/150, 44/239/495, 44/56, 44/56/102/239/285/469/470/495, 44/239, 44/239/285/470, 44/239/285/469/495, 44/239/285/470, 44/239/469/470, 44/239/470/546, 44/239/495/546, 44/469/470, 102, 102/470, 162, 162, 165, 188, 239/285/469, 239/285, 239/469/470/495, 264, 267, 267, 285/470, 285/469/470/495, 285/470/495, 364, 455, 469/470, 472, and 482, or any combination thereof, wherein the amino acid positions are numbered relative SEQ ID NO:6. In some embodiments, the amino acid substitutions are selected from F16A/E/K/N/R/S/T/V/W, F16K/F150A, N44C/T239A/T495A, N44H/I56V, N44H/I56V/T102Q/T239A/I285L/V469I/D470E/T495A, N44H/T239A, N44H/T239A/I285L/D470E, N44H/T239A/I285R/V469I/T495A, N44H/T239A/I285R/D470E, N44H/T239A/V469I/D470E, N44H/T239A/D470E/S546A, N44H/T239A/T495A/S546A, N44H/V469I/D470E, T102Q, T102Q/D470E, F162Q/W, I165L, F188I, T239A/I285L/V469I, T239A/I285R, T239A/V469I/D470E/T495A, F264H, F267G/V, I285L/D470E, I285L/V469I/D470E/T495A, I285R/D470E/T495A, L364E/H/S/T, N455S, V469I/D470E, F472A, and F482C/N, wherein the amino acid positions are numbered relative to SEQ ID NO:6.

[0126] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:6. In some embodiments the amino acid difference is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 or greater amino acid positions. In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:6 and at least one amino acid residue difference as compared to SEQ ID NO:6, at one or more amino acid positions selected from 16, 264, 364, and 472, wherein the amino acid positions are numbered relative to SEQ ID NO:8. In some embodiments, the amino acid substitutions are selected from F16S, F264H, L364H, F472A, and F482N, wherein the amino acid positions are numbered relative to SEQ ID NO:6.

[0127] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:6. In some embodiments the amino acid difference is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 or greater amino acid positions. In some embodiments, the engineered PAL polypeptides

exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:6, and at least one amino acid residue difference as compared to SEQ ID NO:8, at one or more amino acid positions selected from 16, 16/150, 162, 188, 264, 267, 398, 434, 472A/L/V, and F482C/N/S, wherein the amino acid positions are numbered relative to SEQ ID NO:6. In some embodiments, the amino acid substitutions are selected from F16A/E/K/L/M/N/P/R/S/T/V/W, F16K/F150A, F162M/Q/W, F188A/I/N, F264H, F267G/L/Q/S/V, F398H, F434V, F472A/L/V, and F482C/N/S, wherein the amino acid positions are numbered relative to SEQ ID NO:6.

[0128] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:8. In some embodiments the amino acid difference is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 or greater amino acid positions. In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:8, and at least one amino acid residue difference as compared to SEQ ID NO:8, at one or more amino acid position selected from 44/102/285/364, 44/285/364, 44/47/204/209/285, 44/47/364/470/495, 44/54/285/470, 44/54/56/204/239/285/364/495, 44/54/56/204/239/470/495, 44/204/285/364/470/495, 44/204/209/285, 44/209/285/460/495, 47/54/209, 47/204/209/239/285/495, 47/204/285/364/495, 47/209/239/364, 47/239/285/364, 47/470, 54, 54/285/470, 54/56/204/209/470, 54/56/204/209/495, 54/56/285/364/470, 54/56, 54/56/285/470, 54/56/204/495, 54/56/209/562, 54/165/204/209/239/285/470/495, 54/239/495, 54/470, 56, 165, 204, 204/209/239/285/470/495, 204/209/364, 204/209/364/495, 204/239, 204/239/285, 204/364, 204/470, wherein the amino acid positions are numbered relative to SEQ ID NO:8. In some embodiments, the amino acid substitutions are selected from N44C/T102Q/I285R/L364H, N44C/I285R/L364E, N44H/A47K/R204K/S209P/I285R, N44H/A47K/L364H/D470E/T495A, N44H/K54P/I285R/D470E, N44H/K54P/I56V/R204K/T239A/I285R/L364S/T495A, N44H/K54P/I56V/R204K/T239A/D470E/T495A, N44H/R204K/I285R/L364H/D470E/T495A, N44H/R204K/S209P/I285R, N44H/S209P/I285R/T460G/T495A, A47K/K54P/S209P, A47K/R204K/S209P/T239A/I285R/T495A, A47K/R204K/I285R/L364H/T495A, A47K/S209P/T239A/L364H, A47K/T239A/I285R/L364H, A47K/T239A/I285R/L364H, A47K/D470E, K54E, K54P/I285L/D470E, K54E/I285R/D470E, K54E/I56V/R204K/S209P/D470E, K54E/I56V/R204K/S209P/T495A, K54E/I56V/I285R/L364S/D470E, K54P/I56V, K54P/I56V/I285L/D470E, K54P/I56V/R204K/T495A, K54P/I56V/S209P/I562N, K54P/I165L/R204K/S209P/T239A/I285R/D470E/T495A, K54P/T239A/T495A, K54P/D470E, I56V,

I165L, R204K, R204K/S209P/T239A/I285R/D470E/T495A, R204K/S209P/L364E/T495A, R204K/S209P/L364H, R204K/S209P/L364H/T495A, R204K/T239A, R204K/T239A/I285R, R204K/L364H, R204K/D470E, S209P/I285L/L364H/D470E, S209P/I285R/L364E/D470E, S209P/I285R/L364E/D470E/T495A, S209P/I285R/L364H/D470E/T495A, S209P/L364H, S209P/L364H/T495A, S209P/D470E, T239A/L364H, I285R/L364H, I285R/L364H/T495A, L364H, L364H/D470E, D470E, and T495A, wherein the amino acid positions are numbered relative to SEQ ID NO:8.

[0129] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:12. In some embodiments the amino acid difference is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 or greater amino acid positions. In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:12 and at least one amino acid residue difference as compared to SEQ ID NO:12, at one or more amino acid position selected from 54/56/162/204/285, 54/56/398/472, 54/162/204/398, 54/162/398, 54/204/398, 54/56/162/204/398, 54/56/204/285, 54/56/204/398, 54/56/204/398/472, 54/56/285, 54/56/398, 54/162/398, 54/204/285/398/472, 54/285/398, 54/285/398/472, 56/162/398, 56/204/285, 56/204/398, 56/204/398/460, 56/204/398/472, 56/285, 56/285/398/472, 56/398, 56/398/472, and 201/204/398, wherein the amino acid positions are numbered relative to SEQ ID NO:12. In some embodiments, the amino acid substitutions are selected from K54E/I56V/F162W/R204K/L285I, K54E/I56V/F398H/F472L, K54E/F162W/R204K/F398H, K54E/F162W/F398H, K54E/R204K/F398H, K54P/I56V/F162W/R204K/F398H, K54P/I56V/R204K/L285I, K54P/I56V/R204K/F398H, K54P/I56V/R204K/F398H/F472L, K54P/I56V/L285I, K54P/I56V/F398H, K54P/F162W/F398H, K54P/R204K/L285I/F398H/F472L, K54P/L285I/F398H, K54P/L285I/F398H/F472L, I56V/F162W/F398H, I56V/R204K/L285I, I56V/R204K/F398H, I56V/R204K/F398H/T460G, I56V/R204K/F398H/F472L, I56V/L285H, I56V/L285I, I56V/L285I/F398H/F472L, I56V/F398H, I56V/F398H, I56V/F398H/F472L, and T201A/R204K/F398H, wherein the amino acid positions are numbered relative to SEQ ID NO:12.

[0130] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 86%, at least 87%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or 99% sequence identity to SEQ ID NO:2, 4, 8, 10, and/or 12. In some embodiments, the engineered PAL polypeptide exhibiting at least one improved property is selected from SEQ ID NOS:4, 6, 8, 10, and 12.

[0131] In some embodiments, the present invention provides functional fragments of engineered PAL polypeptides. In some embodiments, functional fragments comprise at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% of the activity of the engineered PAL polypeptide from which it was derived (i.e., the parent engineered PAL). In some embodiments, functional fragments comprise at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% of the parent sequence of the engineered PAL. In some embodiments the functional fragment is truncated by less than 5, less than 10, less than 15, less than 10, less than 25, less than 30, less than 35, less than 40, less than 45, and less than 50 amino acids.

[0132] In some embodiments, the present invention provides functional fragments of engineered PAL polypeptides. In some embodiments, functional fragments comprise at least about 95%, 96%, 97%, 98%, or 99% of the activity of the engineered PAL polypeptide from which it was derived (i.e., the parent engineered PAL). In some embodiments, functional fragments comprise at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the parent sequence of the engineered PAL. In some embodiments the functional fragment is truncated by less than 5, less than 10, less than 15, less than 10, less than 25, less than 30, less than 35, less than 40, less than 45, less than 50, less than 55, less than 60, less than 65, or less than 70 amino acids.

[0133] In some embodiments, the engineered PAL polypeptide exhibiting at least one improved property has at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:6, 8, 10, 12, and/or 14, and an amino acid residue difference as compared to SEQ ID NO:4, 6, 8, 10, and/or 12, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:4, 6, 8, 10, and/or 12, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:4, 6, 8, 10, and/or 12. In some embodiments, the engineered PALs comprise at least 90% sequence identity to SEQ ID NO:6 and comprise an amino acid difference as compared to SEQ ID NO:4, 6, 8, 10, and/or 12, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:4, 6, 8, 10, and/or 12.

[0134] In some embodiments, the engineered PAL polypeptide exhibiting at least one improved property has at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:4, or a functional fragment thereof, and an amino acid residue difference as compared to SEQ ID NO:4, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:4, or a sequence having at least

85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:4. In some embodiments, the engineered PALs comprise at least 90% sequence identity to SEQ ID NO:4 and comprise an amino acid difference as compared to SEQ ID NO:4, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:4.

[0135] In some embodiments, the engineered PAL polypeptide exhibiting at least one improved property has at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:6, or a functional fragment thereof, and an amino acid residue difference as compared to SEQ ID NO:6, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:6, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:6. In some embodiments, the engineered PALs comprise at least 90% sequence identity to SEQ ID NO:6 and comprise an amino acid difference as compared to SEQ ID NO:6, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:6.

[0136] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:8, or a functional fragment thereof and an amino acid residue difference as compared to SEQ ID NO:8, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:8, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:8. In some embodiments, the engineered PALs comprise at least 90% sequence identity to SEQ ID NO:8, and comprise an amino acid difference as compared to SEQ ID NO:8, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:8.

[0137] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:10, or a functional fragment thereof and an amino acid residue difference as compared to SEQ ID NO:10, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:10, or a sequence having at least

85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:10. In some embodiments, the engineered PALs comprise at least 90% sequence identity to SEQ ID NO:10, and comprise an amino acid difference as compared to SEQ ID NO:10, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:10.

[0138] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:12 or a functional fragment thereof and an amino acid residue difference as compared to SEQ ID NO:12 at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:12, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:12. In some embodiments, the engineered PALs comprise at least 90% sequence identity to SEQ ID NO:12, and comprise an amino acid difference as compared to SEQ ID NO:12, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:12.

Variants with Reduced Sensitivity to Proteolysis:

[0139] In some embodiments, the engineered PAL polypeptides of the present invention have PAL activity, exhibit reduced sensitivity to proteolysis, and comprise: a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:4, 6, 8, 10, and/or 12; and b) an amino acid residue difference as compared to SEQ ID NO:4, 6, 8, 10, and/or 12, at one or more amino acid positions.

[0140] In some embodiments, the engineered PAL polypeptides that exhibit reduced sensitivity to proteolysis have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:4, 6, 8, 10, and/or 12, and an amino acid residue difference as compared to SEQ ID NO:4, 6, 8, 10, and/or 12, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 20 or more amino acid positions compared to SEQ ID NO:4, 6, 8, 10, and/or 12, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:4, 6, 8, 10, and/or 12).

[0141] In some embodiments, the proteolytic sensitivity of the engineered PAL polypeptides is reduced by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least

50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, or at least 95%, of that of the wild-type PAL (e.g., AvPAL having SEQ ID NO:2) or as compared to a reference PAL polypeptide under essentially the same conditions. The proteolytic activity can be measured using any suitable methods known in the art, including but not limited to those described in the Examples.

[0142] In some embodiments, the engineered PAL polypeptides having reduced sensitivity to proteolysis have reduced sensitivity to a composition comprising one or more proteases, including, but not limited to pepsin, trypsin, chymotrypsin, carboxypeptidase A and B, peptidases (e.g., amino peptidase, dipeptidase and enteropeptidase) when both the reference PAL and the engineered PAL having reduced sensitivity are compared and exposed to essentially the same amount and kind of protease under essentially the same conditions.

[0143] In some embodiments, the engineered PAL polypeptide having reduced sensitivity to proteolysis have enzyme activity levels that are about 1.0 fold, 2-fold, 5-fold, 10-fold, 20-fold, 25-fold, 50-fold, 75-fold, 100-fold, 150-fold, 200-fold or more of the enzymatic activity of the reference PAL (e.g., AvPAL). In some embodiments, the engineered polypeptides have more enzyme activity, as compared to a reference PAL, when activity is measured at a pH range of 4.5 to 7.5; when activity is measured at a pH range of 4.5 to 6.5; when activity is measured at a pH range of 5.0 to 7.5; when activity is measured at a pH range of 5.0 to 6.5; when activity is measured at a pH range of 5.5 to 7.5; and/or also when activity is measured at a pH range of 5.5 to 6.5. In some other embodiments, the engineered PAL polypeptides have K_M values in the range of 1 μ M to 5mM.

Variants with Increased Tolerance to Storage at Elevated Temperatures:

[0144] In some embodiments, the engineered PAL polypeptides of the invention have PAL activity, are more tolerant to storage at elevated temperatures and comprise: a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:4, 6, 8, 10, and/or 12, or a fragment thereof; and b) an amino acid residue difference as compared to SEQ ID NO:4, 6, 8, 10, and/or 12, at one or more amino acid positions.

[0145] In some embodiments, the engineered PAL polypeptides that exhibit increased tolerance to storage at elevated temperatures as compared to wild-type AvPAL and/or another reference polypeptide have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:4, 6, 8, 10, and/or 12, and an amino acid residue difference as compared to SEQ ID NO:4, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 20 or more amino acid positions compared to SEQ ID NO:4, 6, 8, 10, and/or 12, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:4, 6, 8, 10, and/or 12.

[0146] In some embodiments, when all other assay conditions are essentially the same, the engineered PAL polypeptides having increased tolerance to storage at elevated temperatures as compared to a reference PAL polypeptide have an increased tolerance at a temperature of about 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, etc.

[0147] In some embodiments, the engineered PAL polypeptides that have increased tolerance to storage at elevated temperatures also exhibit greater PAL activity as compared to a reference PAL when measure by a standard assay. Any suitable assay finds use in the present invention, including, but not limited to those provided herein.

Variants that Contain Fewer Phenylalanine Residues:

[0148] In some embodiments, the engineered PAL polypeptides of the present invention have PAL activity, and a reduced number of phenylalanine residues in its primary sequence, and comprise: a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:4, 6, 8, 10, and/or 12; and/or b) an amino acid residue difference as compared to SEQ ID NO:4, 6, 8, 10, and/or 12, at one or more amino acid positions.

[0149] In some embodiments, the engineered PAL polypeptides that contain a reduced number of phenylalanine residues in its primary sequence have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:4, 6, 8, 10, and/or 12, and an amino acid residue difference as compared to SEQ ID NO:4, 6, 8, 10, and/or 12, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 20 or more amino acid positions compared to SEQ ID NO:4, 6, 8, 10, and/or 12, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:4, 6, 8, 10, and/or 12).

[0150] In some embodiments, the engineered PAL polypeptides that contain a reduced number of phenylalanine residues in its primary sequence have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:6 and an amino acid residue difference as compared to SEQ ID NO:6, at one or more amino acid positions are selected from 16, 16/150, 162, 188, 264, 267, 398, 434, 472A/L/V, and F482C/N/S, or any combination thereof, when optimally aligned with the amino acid sequence of SEQ ID NO:6. In some embodiments the amino acid difference is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, or 25, or greater amino acid positions.

[0151] In some embodiments, the engineered PAL polypeptides that contain a reduced number of phenylalanine residues in its primary sequence have at least 85%, at least 88%, 90%, 91%, 92%, 93%,

94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:6, and comprise an amino acid residue difference at position F16, F150, F162, F188, F264, F267, F398, F434, F472, F482 and optionally an amino acid residue difference at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the amino acid residue difference is selected from F16A/E/K/L/M/N/P/R/S/T/V/W, F16K/F150A, F162M/Q/W, F188A/I/N, F264H, F267G/L/Q/S/V, F398H, F434V, F472A/L/V, and F482C/N/S, wherein the amino acid residues are numbered with reference to SEQ ID NO:6.

[0152] In some embodiments, the engineered PAL polypeptides containing fewer phenylalanine residues than wild-type PAL also have proteolytic sensitivity that is reduced by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, or at least 95%, of that of the wild-type PAL (e.g., AvPAL having SEQ ID NO:2) or as compared to a reference PAL polypeptide under essentially the same conditions. The activity can be measured using any suitable methods known in the art, including but not limited to those described in the Examples.

[0153] In some embodiments, the engineered PAL polypeptides containing fewer phenylalanine residues than wild-type PAL in their primary sequences, have reduced sensitivity to compositions comprising one or more proteases, including, but not limited to pepsin, trypsin, chymotrypsin, carboxypeptidase A and B, peptidases (e.g., amino peptidase, dipeptidase and enteropeptidase) when both the reference PAL and the engineered PAL having the reduced phenylalanine content are compared and exposed to essentially the same amount and kind of protease under essentially the same conditions.

[0154] In some embodiments, the engineered PAL polypeptides containing a reduced number of phenylalanine residues in their primary sequences have enzyme activity levels that are about 1.0 fold, 2-fold, 5-fold, 10-fold, 20-fold, 25-fold, 50-fold, 75-fold, 100-fold, 150-fold, 200-fold or more of the enzymatic activity of the reference PAL (e.g., AvPAL). In some embodiments, the engineered polypeptides have more enzyme activity, as compared to a reference PAL, when activity is measured at a pH range of 4.5 to 7.5; when activity is measured at a pH range of 4.5 to 6.5; when activity is measured at a pH range of 5.0 to 7.5; when activity is measured at a pH range of 5.0 to 6.5; when activity is measured at a pH range of 5.5 to 7.5; and/or also when activity is measured at a pH range of 5.5 to 6.5. In some other embodiments, the engineered PAL polypeptides have K_M values in the range of 1 μ M to 5mM.

[0155] It is further contemplated that any of the exemplary engineered polypeptides (i.e., all of the variants provided in the tables and described herein) find use as the starting amino acid sequence for synthesizing other engineered PAL polypeptides, for example by subsequent rounds of evolution by adding new combinations of various amino acid differences from other polypeptides and other residue positions described herein. In some embodiments, additional improvements are generated by including amino acid differences at residue positions that were maintained as unchanged throughout earlier rounds of evolution. It is not intended that the present invention be limited to any particular method for

producing engineered PAL polypeptides, as any suitable method finds use, including but not limited to the methods provided herein.

Polynucleotides Encoding Engineered Polypeptides, Expression Vectors and Host Cells:

[0156] The present invention provides polynucleotides encoding the engineered PAL polypeptides described herein. In some embodiments, the polynucleotides are operatively linked to one or more heterologous regulatory sequences that control gene expression to create a recombinant polynucleotide capable of expressing the polypeptide. In some embodiments, expression constructs containing at least one heterologous polynucleotide encoding the engineered PAL polypeptide(s) is introduced into appropriate host cells to express the corresponding PAL polypeptide(s).

[0157] As will be apparent to the skilled artisan, availability of a protein sequence and the knowledge of the codons corresponding to the various amino acids provide a description of all the polynucleotides capable of encoding the subject polypeptides. 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 an engineered PAL polypeptide. Thus, the present invention provides methods and compositions for the production of each and every possible variation of PAL polynucleotides that could be made that encode the PAL polypeptides described herein by selecting combinations based on the possible codon choices, and all such variations are to be considered specifically disclosed for any polypeptide described herein, including the amino acid sequences presented in the Examples (e.g., in the various Tables).

[0158] In some embodiments, the codons are preferably optimized for utilization by the chosen host cell for protein production. For example, preferred codons used in bacteria are typically used for expression in bacteria. Consequently, codon optimized polynucleotides encoding the engineered PAL polypeptides contain preferred codons at about 40%, 50%, 60%, 70%, 80%, 90%, or greater than 90% of the codon positions in the full length coding region.

[0159] In some embodiments, the PAL polynucleotide encodes an engineered polypeptide having PAL activity with the properties disclosed herein, wherein the polypeptide comprises an amino acid sequence having at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identity to a reference sequence selected from SEQ ID NOS:3, 5, 7, 9, and/or 11, or the amino acid sequence of any variant (e.g., those provided in the Examples), and one or more residue differences as compared to the reference polynucleotide of SEQ ID NOS:3, 5, 7, 9, and/or 11, or the amino acid sequence of any variant as disclosed in the Examples (for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid residue positions). In some embodiments, the reference sequence is selected from SEQ ID NOS:3, 5, 7, 9, and/or 11.

[0160] In some embodiments, the PAL polynucleotide encodes an engineered polypeptide having PAL activity with the properties disclosed herein, wherein the polypeptide comprises an amino acid sequence

having at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to reference sequence SEQ ID NO:4, 6, 8, 10, and/or 12 and one or more residue differences as compared to SEQ ID NO:4, 6, 8, 10, and/or 12.

[0161] In some embodiments, the polynucleotide encoding the engineered PAL polypeptides comprises a polynucleotide sequence selected from a polynucleotide sequence selected from SEQ ID NOS:3, 5, 7, 9, and/or 11. In some embodiments, the polynucleotide encoding an engineered PAL polypeptide has at least 80%, 85%, 90%, 93%, 95%, 96%, 97%, 98%, 99% nucleotide residue identity to SEQ ID NOS:3, 5, 7, 9, and/or 11.

[0162] In some embodiments, the polynucleotides are capable of hybridizing under highly stringent conditions to a reference polynucleotide sequence selected from SEQ ID NOS:3, 5, 7, 9, and/or 11, or a complement thereof, or a polynucleotide sequence encoding any of the variant PAL polypeptides provided herein. In some embodiments, the polynucleotide capable of hybridizing under highly stringent conditions encodes a PAL polypeptide comprising an amino acid sequence that has one or more residue differences as compared to SEQ ID NO:4, 6, 8, 10, and/or 12.

[0163] In some embodiments, an isolated polynucleotide encoding any of the engineered PAL polypeptides herein is manipulated in a variety of ways to facilitate expression of the PAL polypeptide. In some embodiments, the polynucleotides encoding the PAL polypeptides comprise expression vectors where one or more control sequences is present to regulate the expression of the PAL polynucleotides and/or polypeptides. Manipulation of the isolated polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector utilized. Techniques for modifying polynucleotides and nucleic acid sequences utilizing recombinant DNA methods are well known in the art. In some embodiments, the control sequences include among others, promoters, leader sequences, polyadenylation sequences, propeptide sequences, signal peptide sequences, and transcription terminators. In some embodiments, suitable promoters are selected based on the host cells selection. For bacterial host cells, suitable promoters for directing transcription of the nucleic acid constructs of the present disclosure, include, but are not limited to 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 (See e.g., Villa-Kamaroff et al., Proc. Natl Acad. Sci. USA 75:3727-3731 [1978]), as well as the *tac* promoter (See e.g., DeBoer et al., Proc. Natl Acad. Sci. USA 80: 21-25 [1983]). Exemplary promoters for filamentous fungal host cells, include, but are not limited to 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 (See e.g., 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. Exemplary yeast cell promoters can be from the genes 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 known in the art (See e.g., Romanos et al., Yeast 8:423-488 [1992]).

[0164] In some embodiments, the control sequence is also a suitable transcription terminator sequence (i.e., a sequence recognized by a host cell to terminate transcription). In some embodiments, the terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the PAL polypeptide. Any suitable terminator which is functional in the host cell of choice finds use in the present invention. 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. 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 known in the art (See e.g., Romanos et al., supra).

[0165] In some embodiments, the control sequence is also a suitable leader sequence (i.e., a non-translated region of an mRNA that is important for translation by the host cell). In some embodiments, the leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the PAL polypeptide. Any suitable leader sequence that is functional in the host cell of choice find use in the present invention. 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).

[0166] In some embodiments, the control sequence is also a polyadenylation sequence (i.e., 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 suitable polyadenylation sequence which is functional in the host cell of choice finds use in the present invention. Exemplary polyadenylation sequences for filamentous fungal host cells include, but are not limited to 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 known (See e.g., Guo and Sherman, *Mol. Cell. Bio.*, 15:5983-5990 [1995]).

[0167] In some embodiments, the control sequence is also a signal peptide (i.e., a 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). In some embodiments, the 5' end of the coding sequence of the nucleic acid sequence inherently contains a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region that encodes the secreted polypeptide. Alternatively, in some embodiments, the 5' end of the coding sequence contains a signal peptide coding region that is foreign to the coding sequence. Any suitable signal peptide coding region which directs the expressed polypeptide into the secretory pathway of a host cell of choice finds use for expression of the engineered polypeptide(s). Effective signal peptide coding regions for bacterial host cells are the signal peptide coding regions include, but are not limited to those obtained from the genes for *Bacillus* NCIB 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 known in the art (See e.g., Simonen and Palva, *Microbiol. Rev.*, 57:109-137 [1993]). In some embodiments, effective signal peptide coding regions for filamentous fungal host cells include, but are not limited to 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. Useful signal peptides for yeast host cells include, but are not limited to those from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase.

[0168] In some embodiments, the control sequence is also a propeptide coding region that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is referred to as a "proenzyme," "propeptide," or "zymogen." A propeptide can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propeptide. The propeptide coding region may be obtained from any suitable source, including, but not limited to 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 (See e.g., WO 95/33836). 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.

[0169] In some embodiments, regulatory sequences are also utilized. These sequences facilitate the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those that 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, but are not limited to the *lac*, *tac*, and *trp* operator systems. In yeast host cells, suitable regulatory systems include, but are not limited to the ADH2 system or GAL1 system. In filamentous fungi, suitable regulatory sequences include, but are not limited to the TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter.

[0170] In another aspect, the present invention is directed to a recombinant expression vector comprising a polynucleotide encoding an engineered PAL polypeptide, 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. In some embodiments, the various nucleic acid and control sequences described herein are joined together to produce recombinant expression vectors which include one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the PAL polypeptide at such sites. Alternatively, in some embodiments, the nucleic acid sequence of the present invention is expressed by inserting the nucleic acid sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In some embodiments involving the creation of 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.

[0171] The recombinant expression vector may be any suitable vector (e.g., a plasmid or virus), that can be conveniently subjected to recombinant DNA procedures and bring about the expression of the PAL polynucleotide sequence. The choice of the vector typically depends 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.

[0172] In some embodiments, the expression vector is an autonomously replicating vector (i.e., a vector that exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, such as a plasmid, an extra-chromosomal element, a minichromosome, or an artificial chromosome). The vector may contain any means for assuring self-replication. In some alternative embodiments, the vector is one in which, when introduced into the host cell, it is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, in some embodiments, 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, and/or a transposon is utilized.

[0173] In some embodiments, the expression vector 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 include, but are not limited to the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers, which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Suitable markers for yeast host cells include, but are not limited to ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in

filamentous fungal host cells include, but are not limited to, amdS (acetamidase; e.g., from *A. nidulans* or *A. oryzae*), argB (ornithine carbamoyltransferases), bar (phosphinothricin acetyltransferase; e.g., from *S. hygrosopicus*), hph (hygromycin phosphotransferase), niaD (nitrate reductase), pyrG (orotidine-5'-phosphate decarboxylase; e.g., from *A. nidulans* or *A. oryzae*), sC (sulfate adenylyltransferase), and trpC (anthranilate synthase), as well as equivalents thereof. In another aspect, the present invention provides a host cell comprising at least one polynucleotide encoding at least one engineered PAL polypeptide of the present invention, the polynucleotide(s) being operatively linked to one or more control sequences for expression of the engineered PAL enzyme(s) in the host cell. Host cells suitable for use in expressing the 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*, *Vibrio fluvialis*, *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. Exemplary host cells also include various *Escherichia coli* strains (e.g., W3110 (Δ fhuA) and BL21).

[0174] Accordingly, in another aspect, the present invention provides methods of producing the engineered PAL polypeptides, where the methods comprise culturing a host cell capable of expressing a polynucleotide encoding the engineered PAL polypeptide under conditions suitable for expression of the polypeptide. In some embodiments, the methods further comprise the steps of isolating and/or purifying the PAL polypeptides, as described herein.

[0175] Appropriate culture media and growth conditions for host cells are well known in the art. It is contemplated that any suitable method for introducing polynucleotides for expression of the PAL polypeptides into cells will find use in the present invention. Suitable techniques include, but are not limited to electroporation, biolistic particle bombardment, liposome mediated transfection, calcium chloride transfection, and protoplast fusion.

[0176] Engineered PAL polypeptides with the properties disclosed herein can be obtained by subjecting the polynucleotide encoding the naturally occurring or engineered PAL polypeptide to any suitable mutagenesis and/or directed evolution methods known in the art, and/or as described herein. An exemplary directed evolution technique is mutagenesis and/or DNA shuffling (See e.g., Stemmer, Proc. Natl. Acad. Sci. USA 91:10747-10751 [1994]; 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 (See e.g., Zhao et al., Nat. Biotechnol., 16:258-261 [1998]), mutagenic PCR (See e.g., Caldwell et al., PCR Methods Appl., 3:S136-S140 [1994]), and cassette mutagenesis (See e.g., Black et al., Proc. Natl. Acad. Sci. USA 93:3525-3529 [1996]).

[0177] Mutagenesis and directed evolution methods can be readily applied to PAL-encoding polynucleotides to generate variant libraries that can be expressed, screened, and assayed. Any suitable

mutagenesis and directed evolution methods find use in the present invention and are well known in the art (*See e.g.*, US Patent Nos. , 5,605,793, 5,811,238, 5,830,721, 5,834,252, 5,837,458, 5,928,905, 6,096,548, 6,117,679, 6,132,970, 6,165,793, 6,180,406, 6,251,674, 6,265,201, 6,277,638, 6,287,861, 6,287,862, 6,291,242, 6,297,053, 6,303,344, 6,309,883, 6,319,713, 6,319,714, 6,323,030, 6,326,204, 6,335,160, 6,335,198, 6,344,356, 6,352,859, 6,355,484, 6,358,740, 6,358,742, 6,365,377, 6,365,408, 6,368,861, 6,372,497, 6,337,186, 6,376,246, 6,379,964, 6,387,702, 6,391,552, 6,391,640, 6,395,547, 6,406,855, 6,406,910, 6,413,745, 6,413,774, 6,420,175, 6,423,542, 6,426,224, 6,436,675, 6,444,468, 6,455,253, 6,479,652, 6,482,647, 6,483,011, 6,484,105, 6,489,146, 6,500,617, 6,500,639, 6,506,602, 6,506,603, 6,518,065, 6,519,065, 6,521,453, 6,528,311, 6,537,746, 6,573,098, 6,576,467, 6,579,678, 6,586,182, 6,602,986, 6,605,430, 6,613,514, 6,653,072, 6,686,515, 6,703,240, 6,716,631, 6,825,001, 6,902,922, 6,917,882, 6,946,296, 6,961,664, 6,995,017, 7,024,312, 7,058,515, 7,105,297, 7,148,054, 7,220,566, 7,288,375, 7,384,387, 7,421,347, 7,430,477, 7,462,469, 7,534,564, 7,620,500, 7,620,502, 7,629,170, 7,702,464, 7,747,391, 7,747,393, 7,751,986, 7,776,598, 7,783,428, 7,795,030, 7,853,410, 7,868,138, 7,783,428, 7,873,477, 7,873,499, 7,904,249, 7,957,912, 7,981,614, 8,014,961, 8,029,988, 8,048,674, 8,058,001, 8,076,138, 8,108,150, 8,170,806, 8,224,580, 8,377,681, 8,383,346, 8,457,903, 8,504,498, 8,589,085, 8,762,066, 8,768,871, 9,593,326, and all related US and non-US counterparts; Ling et al., *Anal. Biochem.*, 254(2):157-78 [1997]; Dale et al., *Meth. Mol. Biol.*, 57:369-74 [1996]; Smith, *Ann. Rev. Genet.*, 19:423-462 [1985]; Botstein et al., *Science*, 229:1193-1201 [1985]; Carter, *Biochem. J.*, 237:1-7 [1986]; Kramer et al., *Cell*, 38:879-887 [1984]; Wells et al., *Gene*, 34:315-323 [1985]; Minshull et al., *Curr. Op. Chem. Biol.*, 3:284-290 [1999]; Christians et al., *Nat. Biotechnol.*, 17:259-264 [1999]; Cramer et al., *Nature*, 391:288-291 [1998]; Cramer et al., *Nat. Biotechnol.*, 15:436-438 [1997]; Zhang et al., *Proc. Nat. Acad. Sci. U.S.A.*, 94:4504-4509 [1997]; Cramer et al., *Nat. Biotechnol.*, 14:315-319 [1996]; Stemmer, *Nature*, 370:389-391 [1994]; Stemmer, *Proc. Nat. Acad. Sci. USA*, 91:10747-10751 [1994]; WO 95/22625; WO 97/0078; WO 97/35966; WO 98/27230; WO 00/42651; WO 01/75767; WO 2009/152336; and U.S. Pat. Appln. Publ. Nos. 2011/0082055, 2014/0005057, 2014/0214391, 2014/0221216, 2015/0133307, 2015/0134315, and 2015/0050658; all of which are incorporated herein by reference).

[0178] In some embodiments, the enzyme clones obtained following mutagenesis treatment are screened by subjecting the enzyme preparations to a defined temperature (or other assay conditions) and measuring the amount of enzyme activity remaining after heat treatments or other suitable assay conditions. Clones containing a polynucleotide encoding a PAL polypeptide are then isolated from the gene, sequenced to identify the nucleotide sequence changes (if any), and used to express the enzyme in a host cell. Measuring enzyme activity from the expression libraries can be performed using any suitable method known in the art (e.g., standard biochemistry techniques, such as HPLC analysis).

[0179] For engineered polypeptides of known sequence, 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 ligation methods, or polymerase mediated methods) to form any desired continuous sequence. For example, polynucleotides and oligonucleotides disclosed herein can be prepared by chemical synthesis using the classical phosphoramidite method (See e.g., Beaucage et al., Tet. Lett., 22:1859-69 [1981]; and Matthes et al., EMBO J., 3:801-05 [1984]), 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).

[0180] Accordingly, in some embodiments, a method for preparing the engineered PAL polypeptide can comprise: (a) synthesizing a polynucleotide encoding a polypeptide comprising an amino acid sequence selected from the amino acid sequence of any variant as described herein, and (b) expressing the PAL polypeptide encoded by the polynucleotide. In some embodiments of the method, the amino acid sequence encoded by the polynucleotide can optionally have one or several (e.g., up to 3, 4, 5, or up to 10) amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-15, 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-30, 1-35, 1-40, 1-45, or 1-50 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, or 50 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 21, 22, 23, 24, or 25 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the substitutions are conservative or non-conservative substitutions.

[0181] The expressed engineered PAL polypeptide can be evaluated for any desired improved property or combination of properties (e.g., activity, selectivity, stability, acid tolerance, protease sensitivity, etc.) using any suitable assay known in the art, including but not limited to the assays and conditions described herein.

[0182] In some embodiments, any of the engineered PAL polypeptides expressed in a host cell are 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.

[0183] Chromatographic techniques for isolation of the PAL polypeptides include, among others, reverse phase chromatography, high-performance liquid chromatography, ion-exchange chromatography, hydrophobic-interaction chromatography, size-exclusion chromatography, gel electrophoresis, and affinity chromatography. Conditions for purifying a particular enzyme depends, 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. In some embodiments, affinity techniques may be used to isolate the improved PAL enzymes. For affinity chromatography purification, any antibody

that specifically binds a PAL polypeptide of interest may find use. For the production of antibodies, various host animals, including but not limited to rabbits, mice, rats, etc., are immunized by injection with a PAL polypeptide, or a fragment thereof. In some embodiments, the PAL polypeptide or fragment is 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.

[0184] In some embodiments, the engineered PAL polypeptide is produced in a host cell by a method comprising culturing a host cell (e.g., an *E. coli* strain) comprising a polynucleotide sequence encoding an engineered PAL polypeptide as described herein under conditions conducive to the production of the engineered PAL polypeptide and recovering the engineered PAL polypeptide from the cells and/or culture medium. In some embodiments, the host cell produces more than one engineered PAL polypeptide.

[0185] In some embodiments, the present invention provides a method of producing an engineered PAL polypeptide comprising culturing a recombinant bacterial cell comprising a polynucleotide sequence encoding an engineered PAL polypeptide having at least 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to reference sequences SEQ ID NO:4, 6, 8, 10, and/or 12, and one or more amino acid residue differences as compared to SEQ ID NO:4, 6, 8, 10, and/or 12, as provided herein, under suitable culture conditions to allow the production of the engineered PAL polypeptide and optionally recovering the engineered PAL polypeptide from the culture and/or cultured bacterial cells. In some embodiments, the host cell produces more than one engineered PAL polypeptide.

[0186] In some embodiments, once the engineered PAL polypeptides are recovered from the recombinant host cells and/or culture medium, they are further purified by any suitable method(s) known in the art. In some additional embodiments, the purified PAL polypeptides are combined with other ingredients and compounds to provide compositions and formulations comprising the engineered PAL polypeptide as appropriate for different applications and uses (e.g., pharmaceutical compositions).

Compositions:

[0187] The present invention provides engineered PAL polypeptides suitable for use in numerous compositions. These compositions find use in many fields, including but not limited to pharmaceuticals, dietary/nutritional supplements, food, feed, and fine chemical production. For example, in some embodiments, the present invention provides food and/or feeds comprising at least one engineered PAL variant and/or at least one polynucleotide sequence encoding at least one PAL variant. In some embodiments, the present invention provides beverages comprising at least one engineered PAL variant.

[0188] In some embodiments, the engineered PAL variant in food, feed, and/or nutritional/dietary supplement is glycosylated. Furthermore, the engineered PAL variants find use in any suitable edible enzyme delivery matrix. In some embodiments, the engineered PAL variants are present in an edible

enzyme delivery matrix designed for rapid dispersal of the PAL variant within the digestive tract of an animal upon ingestion of the variant.

[0189] The present invention also provides engineered PAL polypeptides suitable for use in production of fine chemicals and other industrially important compounds (See e.g., US Pat. Appln. Nos. 2013/0340119, 2013/0005012, and 2005/0260724, and WO 2012/122333).

Pharmaceutical and Other Compositions:

[0190] The present invention provides engineered PAL polypeptides suitable for use in pharmaceutical and other compositions, such as dietary/nutritional supplements.

[0191] Depending on the mode of administration, these compositions comprising a therapeutically effective amount of an engineered PAL according to the invention are in the form of a solid, semi-solid, or liquid. In some embodiments, the compositions include other pharmaceutically acceptable components such as diluents, buffers, excipients, salts, emulsifiers, preservatives, stabilizers, fillers, and other ingredients. Details on techniques for formulation and administration are well known in the art and described in the literature.

[0192] In some embodiments, the engineered PAL polypeptides are formulated for use in oral pharmaceutical compositions. Any suitable format for use in delivering the engineered PAL polypeptides find use in the present invention, including but not limited to pills, tablets, gel tabs, capsules, lozenges, dragees, powders, soft gels, sol-gels, gels, emulsions, implants, patches, sprays, ointments, liniments, creams, pastes, jellies, paints, aerosols, chewing gums, demulcents, sticks, suspensions (including but not limited to oil-based suspensions, oil-in water emulsions, etc.), slurries, syrups, controlled release formulations, suppositories, etc. In some embodiments, the engineered PAL polypeptides are provided in a format suitable for injection (i.e., in an injectable formulation). In some embodiments, the engineered PAL polypeptides are provided in biocompatible matrices such as sol-gels, including silica-based (e.g., oxysilane) sol-gels. In some embodiments, the engineered PAL polypeptides are encapsulated. In some alternative embodiments, the engineered PAL polypeptides are encapsulated in nanostructures (e.g., nanotubes, nanotubules, nanocapsules, or microcapsules, microspheres, liposomes, etc.). Indeed, it is not intended that the present invention be limited to any particular delivery formulation and/or means of delivery. It is intended that the engineered PAL polypeptides be administered by any suitable means known in the art, including but not limited to parenteral, oral, topical, transdermal, intranasal, intraocular, intrathecal, via implants, etc.

[0193] In some embodiments, the engineered PAL polypeptides are chemically modified by glycosylation, pegylation (i.e., modified with polyethylene glycol [PEG] or activated PEG, etc.) or other compounds (See e.g., Ikeda, Amino Acids 29:283-287 [2005]; US Pat. Nos. 7,531,341, 7,534,595, 7,560,263, and 7,53,653; US Pat. Appln. Publ. Nos. 2013/0039898, 2012/0177722, etc.). Indeed, it is not intended that the present invention be limited to any particular delivery method and/or mechanism.

[0194] In some additional embodiments, the engineered PAL polypeptides are provided in formulations comprising matrix-stabilized enzyme crystals. In some embodiments, the formulation comprises a cross-linked crystalline engineered PAL enzyme and a polymer with a reactive moiety that adheres to the enzyme crystals. The present invention also provides engineered PAL polypeptides in polymers.

[0195] In some embodiments, compositions comprising the engineered PAL polypeptides of the present invention include one or more commonly used carrier compounds, including but not limited to sugars (e.g., lactose, sucrose, mannitol, and/or sorbitol), starches (e.g., corn, wheat, rice, potato, or other plant starch), cellulose (e.g., methyl cellulose, hydroxypropylmethyl cellulose, sodium carboxymethylcellulose), gums (e.g., arabic, tragacanth, guar, etc.), and/or proteins (e.g., gelatin, collagen, etc.). Additional components in oral formulations may include coloring and or sweetening agents (e.g., glucose, sucrose, and mannitol) and lubricating agents (e.g., magnesium stearate), as well as enteric coatings (e.g., methacrylate polymers, hydroxyl propyl methyl cellulose phthalate, and/or any other suitable enteric coating known in the art). In some embodiments, disintegrating or solubilizing agents are included (e.g., cross-linked polyvinyl pyrrolidone, agar, alginic acid or salts thereof, such as sodium alginate). In some embodiments, the engineered PAL polypeptide are combined with various additional components, including but not limited to preservatives, suspending agents, thickening agents, wetting agents, alcohols, fatty acids, and/or emulsifiers, particularly in liquid formulations.

[0196] In some embodiments, the engineered PAL polypeptide are be combined with various additional components, including but not limited to preservatives, suspending agents, thickening agents, wetting agents, alcohols, fatty acids, and/or emulsifiers, particularly in liquid formulations. In some embodiments, the engineered PAL polypeptides are administered to subjects in combination with other compounds used in the treatment of PKU, including but not limited to KUVAN[®] tetrahydrobiopterin (BioMarin Pharmaceutical, Inc., Novato, CA), antacids (e.g., omeprazole, esomeprazole and other prazoles), as well as any other suitable compounds.

[0197] In some embodiments, the present invention provides engineered PAL polypeptides suitable for use in decreasing the concentration of phenylalanine in fluids such as blood, cerebrospinal fluid, etc. The dosage of engineered PAL polypeptide(s) administered to a patient with elevated blood phenylalanine levels depends upon the genotype of the patient, the general condition of the patient, and other factors known to those in the art. In some embodiments, PKU patients with elevated blood phenylalanine levels have greater than 360 uM blood phenylalanine concentrations. However, it is not intended that the present invention be limited to administration to patients with blood phenylalanine concentrations that are greater than 360 uM, as the present invention finds use with patients with lower phenylalanine blood concentrations. In some embodiments, the compositions are intended for single or repeat administration to a patient. In some embodiments, it is contemplated that the concentration of engineered PAL polypeptide(s) in the composition(s) administered to a patient is sufficient to effectively treat, ameliorate and/or prevent the symptoms of the disease (e.g., PKU and/or PKU-related conditions, diseases and/or

symptoms). In some embodiments, the engineered PAL polypeptides are administered in combination with other pharmaceutical and/or dietary compositions.

[0198] In some embodiments, PAL enzymes find use as a therapeutic protein for the treatment of disorders of tyrosine metabolism, such as tyrosinemia Type I, tyrosinemia Type II, tyrosinemia Type III, and alkaptonuria. These disorders of tyrosine metabolism are autosomal metabolic genetic disorders in which one of the enzymes involved in the degradation of tyrosine is partially functional or non-functional, due to a mutation in the corresponding gene. This lack of functionality results in elevated levels of tyrosine and other tyrosine-metabolites in the bloodstream. Because tyrosine is derived from phenylalanine, it is beneficial for patients with a disorder of tyrosine metabolism to limit phenylalanine and tyrosine intake. PAL enzymes are thus a potential treatment of disorders of tyrosine metabolism.

[0199] If patients with a disorder of tyrosine metabolism are not treated early, high levels of tyrosine and some of its breakdown products can cause significant medical problems including liver and kidney failure, liver cancer, intellectual disability, and even death.

[0200] In some embodiments, the present invention provides engineered PAL polypeptides suitable for use in decreasing the concentration of phenylalanine in fluids such as blood, cerebrospinal fluid, etc. of tyrosinemia and alkaptonuria patients. The dosage of engineered PAL polypeptide(s) administered to a patient with elevated blood tyrosine levels depends upon the genotype of the patient, the general condition of the patient, and other factors known to those in the art. In some embodiments, the compositions are intended for single or repeat administration to a tyrosinemia or alkaptonuria patient. In some embodiments, it is contemplated that the concentration of engineered PAL polypeptide(s) in the composition(s) administered to a patient is sufficient to effectively treat, ameliorate and/or prevent the symptoms of the disease (e.g., tyrosinemia Type I, Type II, or Type III, or alkaptonuria, diseases and/or symptoms). In some embodiments, the engineered PAL polypeptides are administered to tyrosinemia and alkaptonuria patients in combination with nitisinone or other pharmaceutical and/or dietary compositions.

Industrial Compositions:

[0201] It is contemplated that the engineered PAL polypeptides of the present invention will find use in industrial compositions. In some embodiments, the engineered PAL polypeptides are formulated for use in the food and/or feed industries. In some embodiments, the engineered PAL polypeptides are formulated in granulated or pelleted products which are mixed with animal feed components such as additional enzymes (for example, cellulases, laccases, and amylases). In some alternative embodiments, the engineered PAL polypeptides are used in liquid animal feed compositions (e.g., aqueous or oil based slurries). Thus, in some embodiments, the engineered PAL variants of the present invention are sufficiently thermotolerant and thermostable to withstand the treatment used to produce pellets and other processed feed/foods.

[0202] The engineered PAL variants of the present invention also find use in the production of phenylalanine and/or phenylalanine derivatives.

[0203] The foregoing and other aspects of the invention may be better understood in connection with the following non-limiting examples. The examples are provided for illustrative purposes only and are not intended to limit the scope of the present invention in any way.

EXPERIMENTAL

[0204] The following Examples, including experiments and results achieved, are provided for illustrative purposes only and are not to be construed as limiting the present invention.

[0205] In the experimental disclosure below, the following abbreviations apply: ppm (parts per million); M (molar); mM (millimolar), μ M and μ M (micromolar); nM (nanomolar); mol (moles); gm and g (gram); mg (milligrams); μ g and μ g (micrograms); L and l (liter); ml and mL (milliliter); cm (centimeters); mm (millimeters); μ m and μ m (micrometers); sec. (seconds); min(s) (minute(s)); h(s) and hr(s) (hour(s)); U (units); MW (molecular weight); rpm (rotations per minute); psi and PSI (pounds per square inch); °C (degrees Centigrade); RT and rt (room temperature); CDS (coding sequence); DNA (deoxyribonucleic acid); RNA (ribonucleic acid); AUC (area under the curve); *E. coli* W3110 (commonly used laboratory *E. coli* strain, available from the Coli Genetic Stock Center [CGSC], New Haven, CT); HTP (high throughput); HPLC (high pressure liquid chromatography); CFSE (carboxyfluorescein succinimidyl ester); IPTG (isopropyl β -D-1-thiogalactopyranoside); PES (polyethersulfone); PHE and phe (phenylalanine); BSA (bovine serum albumin); PBMC (peripheral blood mononuclear cells); PKU (phenylketonuria); MHC (major histocompatibility complex); HLA (human leukocyte antigen); HLA-DR (an MHC Class II cell surface receptor encoded by the HLA complex on chromosome #6); FIOPC (fold improvements over positive control); LB (Luria broth); Athens Research (Athens Research Technology, Athens, GA); ProSpec (ProSpec Tany Technogene, East Brunswick, NJ); Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO); Ram Scientific (Ram Scientific, Inc., Yonkers, NY); Pall Corp. (Pall, Corp., Pt. Washington, NY); Millipore (Millipore, Corp., Billerica MA); Difco (Difco Laboratories, BD Diagnostic Systems, Detroit, MI); Molecular Devices (Molecular Devices, LLC, Sunnyvale, CA); Kuhner (Adolf Kuhner, AG, Basel, Switzerland); Biospringer (Biospringer North America, Milwaukee, WI); Cambridge Isotope Laboratories, (Cambridge Isotope Laboratories, Inc., Tewksbury, MA); Applied Biosystems (Applied Biosystems, part of Life Technologies, Corp., Grand Island, NY); Agilent (Agilent Technologies, Inc., Santa Clara, CA); Thermo Scientific (part of Thermo Fisher Scientific, Waltham, MA); Corning (Corning, Inc., Palo Alto, CA); Constant Systems (Constant Systems Ltd., Daventry, United Kingdom); Megazyme (Megazyme International, Wicklow, Ireland); Enzo (Enzo Life Sciences, Inc., Farmingdale, NY); GE Healthcare (GE Healthcare Bio-Sciences, Piscataway, NJ); Harlan (Harlan Laboratories, Indianapolis, IN); AB Sciex

(AB Sciex, Framingham, MA); PetAg Inc. (PetAg, Hampshire, IL); and Bio-Rad (Bio-Rad Laboratories, Hercules, CA).

[0206] The following polynucleotide and polypeptide sequences find use in the present invention. In some cases (as shown below), the polynucleotide sequence is followed by the encoded polypeptide.

Polynucleotide Sequence of WT AvPAL (SEQ ID NO:1):

ATGAAAACCCTGAGCCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGG
CAATAGCAGCGCAAATGTGATTATTGGTAATCAGAACTGACCATCAATGATGTTGCAC
GTGTTGCCCGTAATGGCACCCCTGGTTAGCCTGACCAATAATACCGATATTCTGCAGGGTA
TTCAGGCCAGCTGTGATTATATCAATAATGCAGTTGAAAGCGGTGAACCGATTTATGGTG
TTACCAGCGGTTTTGGTGGTATGGCAAATGTTGCAATTAGCCGTGAACAGGCAAGCGAA
CTGCAGACCAATCTGGTTTGGTTTCTGAAAACCGGTGCAGGTAATAAACTGCCGCTGGCA
GATGTTTCGTGCAGCAATGCTGCTGCGTGCAAATAGCCACATGCGTGGTGCAAGCGGTATT
CGTCTGGAAGTGAATTAACGCATGGAAATCTTTCTGAATGCCGGTGTTACCCCGTATGTT
TATGAATTTGGTAGCATTGGTGCCAGCGGTGATCTGGTTCCGCTGAGCTATATTACCGGT
AGCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATGGCAAAGAAATGGACGC
ACCGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCTGCCGAAAGAAGGTCT
GGCAATGATGAATGGCACCAGCGTTATGACCGGTATTGCAGCAAATTGTGTTTATGATAC
CCAGATTCTGACCGCAATTGCAATGGGTGTTTCATGCACTGGATATTCAGGCACTGAATGG
TACAAATCAGAGCTTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTGAGCTGTG
GGCAGCAGATCAGATGATTAGCCTGCTGGCCAATAGCCAGCTGGTTCGTGATGAACTGG
ATGGTAAACATGATTATCGTGATCATGAACTGATCCAGGATCGTTATAGCCTGCGTTGTC
TGCCGCACTATCTGGGTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAA
TTGAGATTAACAGCGTTACCGATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATC
ATGGTGGTAATTTTCTGGGTCAGTATGTTGGTATGGGTATGGATCATCTGCGCTATTATAT
CGGTCTGCTGGCAAAACATCTGGATGTTTCAGATTGCACTGCTGGCATCACCGGAATTTAG
CAATGGTCTGCCTCCGAGTCTGCTGGGTAATCGTGAACGTAAAGTTAATATGGGTCTGAA
AGGTCTGCAGATTTGCGGTAATAGCATTATGCCGCTGCTGACCTTTTATGGTAATAGTAT
TGCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGAATATTAACAGCCAGGGTTA
TACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTTTCAGAATTATGTTGCCATTGC
CCTGATGTTTGGTGTTCAGGCAGTTGATCTGCGTACCTACAAAAAACCGGTCATTATGA
TGCACGTGCCTGTCTGTACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTTCATGTTGT
TGGTCAGAAACCGACCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGG
ATGAACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTATTGTTTCAGGCCG
TTCAGGACATTCTGCCGTGTCTGCAT (SEQ ID NO:1)

Polypeptide Sequence of WT AvPAL (SEQ ID NO:2):

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVARVARNGTLVSLTNNTDILQGIQAS
 CDYINNAVESGEPIYGVTSFGFGGMANVAISREQASELQTNLVWFLKTGAGNKLPLADVRAA
 MLLRANSHMRGASGIRLELIKRMEIFLNAGVTPYVYEFSGSIGASGDLVPLSYTTGSLIGLDPSF
 KVDENGKEMDAPTALRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAMGV
 HALDIQALNGTNQSFHPFIHNSKPHPGQLWAADQMISLLANSQLVRDELDGKHHDYRDHELIQ
 DRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGMDH
 LRYIYIGLLAKHLDVQIALLASPEFSNGLPPSLLGNRERKVNMGMLKGLQICGNSIMPLLTIFYGN
 SIADRFPTHAEQFNQINSQGYTSATLARRSVDIFQNYVAIALMFGVQAVDLRTYKKTGHYD
 ARACLSPATERLYSAVRHVVGQKPTSDRPYIWNQNEQGLDEHIARISADIAAGGVIVQAVQDI
 LPCLH (SEQ ID NO:2)

Polynucleotide Sequence of Variant #1 (SEQ ID NO:3)

ATGAAAACCTGAGTCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCCATACCGG
 CAATAGCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGTACG
 TGTTGCCCGTAATGGCACCGCGGTTAGCCTGACCAATAATAAAGATATTCTGCAGCGTATT
 CAGGCCAGCTGTGATTATATCAATAATGCAGTTGAAAAAGGTGAACCGATTTATGGTGTT
 ACCAGCGGTTTTGGTGGTATGGCAAATGTTGTAATTAGCCGTGAACAGGCAAGCGAACTG
 CAGACCAATCTGGTTTTGGTTTTCTGAAAACCGGTGCAGGTAATAAACTGCCGCTGGCAGAT
 GTTCGTGCAGCAATGCTGCTGCGTGCAAATAGCCACATGCGTGGTGCAAGCGGTATTCTGT
 CTGGAACCTGATTAAACGCATGGAAATCTTTCTGAATGCCGGTGTTACCCCGTATGTTTATG
 AATTTGGTAGCATTGGTGCCAGCGGTGATCTGGTTCCGCTGAGCTATATTACCGGTAGCCT
 GATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATGGCAAAGAAATGGACGCACCGAC
 CGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCAGCCGAAAGAAGGTCTGGCAAT
 GATGAATGGCACCGCGTTATGACCGGTATTGCAGCAAATTGTGTTTATGATACCCAGAT
 TCTGACCGCAATTGCAATGGGTGTTTCATGCACTGGATATTAGGCACTGAATGGTACAAA
 TCAGAGCTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTGAGCTGTGGGCAGC
 AGATCAGATGATTAGCCTGCTGGCCGGTAGCCAGCTGGTTCGTGATGAACTGGATGGTAA
 ACATGATTATATGGATGGTGAACCTGATCCAGGATCGTTATAGCCTGCGTTGTCTGCCGCAG
 TATCTGGGTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAATTGAGATT
 AACAGCGTTACCGATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATCATGGTGGT
 AATTTTCTGGGTCAGTATGTTGGTATGGGTATGGATCATCTGCGCTATTATATCGGTCTGC
 TGGCAAAACATCTGGATGTTTCAGATTGCACTGCTGGCATCACCGGAATTTAGCAATGGTC
 TGCCTCCGAGTCTGGTGGGTAATCGTGAACGTAAAGTTAATATGGGTCTGAAAGGTCTGC

AGATTTGCGGTAATAGCATTATGCCGCTGCTGACCTTTTATGGTAATAGTATTGCAGATCG
 TTTTCCGACCCATGCCGAACAGTTTAACCAGAATATTAACAGCCAGGGTTATACCAGCGC
 AACCCTGGCACGTCGTAGCGTTGATATTTTTCAGAATTATGTTGCCATTGCCCTGATGTTT
 GGTGTTTCAGGCAGTTGATCTGCGTACCTACAAAAAACC GGTCATTATGATGCACGTGCC
 CAGCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATGTTGTTGGTAAAAAA
 CCGAGCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGGATGAACATATT
 GCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTTCAGGCCGTTTCAGGACATTC
 TGCCGCCGCTGCAT (SEQ ID NO:3)

Polypeptide Sequence of Variant #1 (SEQ ID NO:4)

MKTL SQAQSKTSSQQFSHTGNSSANVIIGNQKL TINDVVRVARNGTAVSLTNNKDILQRI
 QASCDYINNAVEKGEPIYGVTS GF GGMANVVISREQASELQTNL VWFLKTGAGNKLPLADV
 RAAMLLRANSHMRGASGIRLELIKRM EIFLNAGVTPYVYEFSGISGDLVPLSYITGSLIGLD
 PSFKVDFNGKEMDAPTALRQLNLSPLTLQPK EGLAMMNGTSVMTGIAANCVYDTQILTAIA
 MGVHALDIQALNGTNQSFHPFIHNSKPHPGQLWAADQMISLLAGSQLVRDEL DGKHDYMD
 GELIQDRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVT DNPLIDVDNQASYHGGNFLGQYVGM
 GMDHLRYIYIGLLAKHLDVQIALLASPEFSNGLP PSLVGNRERKVN MGLKGLQICGNSIMPLL
 TFYGN SIADRFPTHAEQFNQ NINSQGYTSATLARRSVDIFQNYVAIALMFGVQAVDLRTYKK
 TGHYDARAQLSPATERLYSAVRHVVGKKPSSDRPYIWN DNEQGLDE
 HIARISADIAAGGVIVQAVQDILPPLH (SEQ ID NO:4)

EXAMPLE 1

PAL Gene Acquisition and Construction of Expression Vectors

[0207] The *Anabaena variabilis* phenylalanine ammonia lyase (AvPAL) gene sequence was designed with codons for optimized expression in *E. coli* and cloned into the *E. coli* expression vector pET16b to provide pET16b-AvPAL and subcloned as described in Example 1, of US Pat. No. 9,611,468. The plasmid construct was transformed into an *E. coli* strain derived from W3110. Directed evolution techniques generally known by those skilled in the art were used to generate libraries of gene variants from this plasmid construct (See e.g., US Pat. No. 8,383,346, and WO2010/144103) as well as AvPAL derivatives. In some embodiments, expression vectors lacking antimicrobial resistance markers find use.

EXAMPLE 2

High-Throughput (HTP) Growth and Assays

High-Throughput (HTP) Growth of PAL and PAL Variants

[0208] Transformed *E. coli* cells were selected by plating onto LB agar plates containing 1% glucose and 30 µg/ml chloramphenicol. After overnight incubation at 37°C, colonies were placed into the wells of 96-well shallow flat bottom plates (NUNC™, Thermo-Scientific) filled with 180 µl/well LB supplemented with 1% glucose and 30 µg/ml chloramphenicol. The cultures were allowed to grow overnight for 18-20 hours in a shaker (200 rpm, 30 °C, and 85% relative humidity; Kuhner). Overnight growth samples (20 µL) were transferred into Costar 96-well deep plates filled with 380 µL of Terrific Broth supplemented with 30 µg/ml chloramphenicol. The plates were incubated for 135 minutes in a shaker (250 rpm, 30 °C, and 85% relative humidity; Kuhner). The cells were then induced with 40 µL of 10 mM IPTG in sterile water and incubated overnight for 20-24 hours in a shaker (250 rpm, 30°C, and 85% relative humidity; Kuhner). The cells were pelleted (4000 rpm x 20 min), the supernatants were discarded, and the cells were frozen at -80°C prior to analysis.

Lysis of HTP Pellets

[0209] First, 500 µL of lysis buffer (20 mM sodium phosphate pH 8, 150 mM NaCl, 1 mg/ml lysozyme, and 0.5 mg/ml polymyxin B sulfate) were added to the cell pellets. The mixture was agitated for 1.5-2 h at room temperature, and centrifuged (4000 rpm x 5 min) prior to use of the clarified lysates in the various HTP assays described herein. Analysis of these lysates by SDS-PAGE revealed the presence of an overexpressed protein at an apparent MW of ~60 kDa, consistent with the expected MW of PAL.

Analysis of Clarified Lysates

[0210] PAL variant activity was determined by measuring the formation of cinnamic acid by the change in absorbance at 290 nm over time. For this assay, 100 µL of either 200 mM Tris/50 mM phenylalanine, pH 7.5, or 200 mM sodium phosphate/50 mM phenylalanine pH 7.0, 80 µL of water, and 20 µL of clarified lysate were added to the wells of a poly-acrylate 96-well microtiter plate (Costar #3635, Corning). The reactions were mixed briefly and the activity was determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus 384 or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader.

HTP-Analysis of Clarified Lysates Pretreated with Protease

[0211] The activity of PAL variants was determined after incubation with chymotrypsin and trypsin to simulate the environment of the intestinal tract (e.g., the upper intestine). First, 30 µL of protease mix (0.01 - 100 mg/ml chymotrypsin (C4129 Sigma Aldrich), 0.01 -100 mg/ml trypsin (T7409 Sigma Aldrich), 1 mM CaCl₂, and 1 mM HCl), 0- 30 µL of 20 mM sodium taurocholate in 500 mM sodium phosphate pH 7.0, and 90 -120 µL of clarified lysate were added to the wells of a 96-well round bottom

microtiter plate (Costar #3798, Corning). The plates were sealed and incubated at 37 °C with shaking (Thermotron® shaker HT Infors AJ185, 400 rpm, 1" throw) for 1h prior to analysis. For the assay, 100 µL of either 200 mM Tris/50 mM phenylalanine pH 7.5 or 200 mM sodium phosphate/50 mM phenylalanine pH 7.0 and 100 µL of the protease treated lysate were added to the wells of a polyacrylate 96-well microtiter plate (Costar #3635, Corning). The reactions were mixed briefly and the activity was determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus 384 or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader. The results are provided in the Tables below.

HTP-Analysis of PAL Variants after Storage at Elevated Temperatures

[0212] Lysates of PAL variants grown in high throughput were incubated at 65°C for 1hr and insoluble material was pelleted (4000 rpm x 10 min). The supernatant was transferred to a new 96-well microtiter plate (NUNC™, Thermo-Scientific), frozen, and lyophilized to a dry powder. Lyophilized enzymes were incubated at 45°C in a Thermotron® HT shaker (Infors AJ185) for up to 10 days. Subsequently the lyophilized enzymes were resuspended in 400 µL of ddH₂O and mixed by agitation for 10 min. For the assay, 100 µL of 200 mM sodium phosphate/50 mM phenylalanine pH 7.0, 80 µL of ddH₂O and 20 µL of the enzyme suspension were added to the wells of a poly-acrylate 96-well microtiter plate (Costar #3635, Corning). The reactions were mixed briefly and the activity was determined by following the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus 384 or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader.

EXAMPLE 3

Lyophilized Lysates from Shake Flask (SF) Cultures

[0213] Selected HTP cultures grown as described in Example 2 above, were plated onto LB-agar plates with 1% glucose and 30 µg/ml chloramphenicol and grown overnight at 37°C. A single colony from each culture was transferred to 50 ml Luria Bertani medium with 1% glucose and 30 µg/ml chloramphenicol. The cultures were grown for 18 h at 30°C, 250 rpm, and subcultured at a dilution of approximately 1:10 into 250 ml of Terrific Broth with 30 µg/ml of chloramphenicol, to a final OD₆₀₀ of 0.2. The cultures were incubated for 135 minutes at 30°C, 250 rpm, to an OD₆₀₀ of 0.6 and induced with 1 mM of IPTG. The induced cultures were incubated for 20 h at 30°C, 250 rpm. Following this incubation period, the cultures were centrifuged 4000 rpm x 10 min. The supernatant was discarded, and the pellets were resuspended in 30 ml of 20 mM sodium phosphate pH 8, 150 mM NaCl. The cells were pelleted 2860*g for 10 min), resuspended in 12 ml of 50 mM sodium phosphate pH 7.5, and lysed using a One Shot Cell

Disruption system (Constant Systems) at 17,000 psi. Lysate was pelleted (10,000 rpm x 30 min) and the supernatant was frozen and lyophilized to generate an enzyme powder.

Purification of PAL From Shake Flask Cultures

[0214] Select variants were grown in shake flask cultures to saturation, as described above. Saturated cultures were pelleted by centrifugation (4000 rpm x 20 min) and the cell pellets were stored at -80 °C prior to purification. The cell pellets were thawed at room temperature and resuspended in 20 mM sodium Phosphate pH 8, 150 mM NaCl at 5 mL of buffer per g of cells. The sample slurry was lysed using a microfluidizer with a pressure setting of 110 psi. The resulting lysate was clarified by centrifugation at 10,000 rpm for 1 hour, followed by filtration through a 0.2 µm PES filter (Millipore).

[0215] After filtration, the resulting lysate was heated at 55-65 °C for 1.5-2 hours in the presence or absence of 10 mM phenylalanine. The lysate was removed from the heat and clarified by centrifugation at 10,000 rpm at 4 °C for 1 hour. The supernatant containing soluble PAL was then filtered through a 0.2 µm PES filter.

[0216] Bulk crystallization was done with a particulate free solution. The filtered concentrate and anti-solvent (0.1M HEPES, 0.2M NaCl pH 7.5 25% (w/v) PEG 3350) were heated separately to 40°C. Anti-solvent was added slowly in small increments until a cloud point was sustained. Contact seeding was achieved by adding a small amount of existing crystals to the solution after it reached its cloud point (this step is not essential). The solution was cooled linearly from 40°C to 4°C over 6 hours. Periodically the suspension was gently mixed to resuspend solids. The suspension was held for 12 to 24 hours at 4°C. The liquid was decanted retaining the settled crystals. The crystalline solids were washed with water to remove residual PEG. Washed crystals were dried by lyophilization overnight.

EXAMPLE 4

Characterization of Purified PAL and PAL Variants

[0217] In this Example, assays conducted to characterize wild-type and variant PALs are described.

Tolerance to Storage Conditions

[0218] Lyophilized enzyme variants were incubated in a Thermotron® HT shaker (Infors AJ185) at 45°C for 1, 5, 7, or 10 days. The lyophilized enzyme was resuspended in 400µL of ddH₂O and solubilized by agitation for 10 min. For the assay, 100 µL of 200 mM sodium phosphate/50 mM phenylalanine pH 7.0, 80µL of ddH₂O and 20 µL of the resuspension were added to the wells of a polyacrylate 96-well microtiter plate (Costar #3635, Corning). The reactions were mixed briefly and the activity was determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min)

using a SpectraMax[®] Plus 384 or a SpectraMax[®] 190 (Molecular Devices) absorbance microplate reader.

Resistance to Intestinal Fluids

[0219] PAL variant samples prepared as described in Example 3, were dissolved at 2 g/L in simulated intestinal fluids. PAL variants were dissolved into each of four simulated intestinal fluids: fasted state simulated intestinal fluid (sodium taurocholate 3 mM, lecithin 0.2 mM, maleic Acid 19.12 mM, sodium hydroxide 34.8 mM, sodium chloride 68.62 mM, pH 6.5), early fed state simulated intestinal fluid (sodium taurocholate 10 mM, lecithin 3 mM, maleic acid 28.6 mM, sodium hydroxide 52.5 mM, sodium chloride 145.2 mM, glyceryl monocholate 6.5 mM, sodium oleate 40 mM, pH, 6.5), middle fed state simulated intestinal fluid (sodium taurocholate 7.5 mM, lecithin 2 mM, maleic acid 44 mM, sodium hydroxide 65.3 mM, sodium chloride 122.8 mM, glyceryl monocholate 5 mM, sodium oleate 30 mM, pH 5.8), late fed state simulated intestinal fluid (sodium taurocholate 4.5 mM, lecithin 0.5 mM, maleic acid 58.09 mM, sodium hydroxide 72 mM, sodium chloride 51 mM, glyceryl monocholate 1 mM, sodium oleate 0.8 mM, pH 5.4). Porcine trypsin and bovine chymotrypsin (100 mg each) were dissolved in 2 ml of 100 mM sodium phosphate pH 7.0, and diluted to 1.5 mg/mL in each simulated fluid. Enzyme activity was measured in each simulated fluid over time, with and without trypsin and chymotrypsin. The reaction mixtures were incubated at 37 °C for 1-24 h in a Thermotron[®] HT shaker (Infors AJ185) at 400 rpm (1" throw). Then, 20 µL of the reaction was mixed with 80 µL of water and 100 µL of 100 mM sodium phosphate, 50 mM phenylalanine pH 7.0. Each solution was mixed briefly, and the activity was determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax[®] Plus 384 or a SpectraMax[®] 190 (Molecular Devices) absorbance microplate reader.

EXAMPLE 5

Production of PAL Variants by Fermentation and Crystallization

[0220] Fermentation batch media ((NH₄)₂SO₄ 0.52 g/L, sodium citrate dihydrate 0.59 g/L, K₂HPO₄·3H₂O 7.5 g/L, KH₂PO₄ 3.7 g/L, yeast extract (Biospringer) 2.0 g/L, polypropylene glycol (PPG 2000) 0.3 ml/L, ammonium iron (III) citrate 0.05 g/L, Trace element stock solution 5.0 ml/L (CaCl₂·2H₂O 2.0 g/L, ZnSO₄·7H₂O 2.2 g/L, MnSO₄·H₂O 0.5 g/L, CuSO₄·5H₂O 1.0 g/L, (NH₄)₆Mo₇O₂₄·4H₂O 0.1 g/L, Na₂B₄O₇·10H₂O 0.02 g/L, Adjust to pH 2-3 with H₂SO₄)) was supplemented with glucose at an initial concentration of approximately 10 g/L. After inoculation, the culture was controlled at 37°C and a dissolved oxygen level not less than 45%. Fed-batch feeding (glucose monohydrate 500 g/L and magnesium sulfate, anhydrous 5.1 g/L) was initialized after depletion of the batch glucose as indicated by the spike in pH from set point of 7.00 to 7.05. The glucose feed was started at a rate of

0.41ml/min and was increased to a final feed rate of 2.42ml/min using an exponential profile over 12 hours. The feed was then kept at a constant rate of 2.42ml/min for the remainder of the fermentation.

[0221] When a cumulative volume of 0.94 L of feed solution had been fed into the fermenter, the temperature was reduced to 30°C, and expression of the PAL variant was induced by addition of IPTG to a concentration of 1mM. The expression phase ended when a total feed volume of 3.84 L was delivered to the fermenter. The total induction time was approximately 20 hours.

[0222] The cells were harvested by centrifugation (3752 xg) for 10 min and the pellet used directly or stored frozen. Cold buffer (4°C) consisting of 20 mM sodium phosphate, 150 mM NaCl pH 8.0 was used to resuspend the pellet to the original harvest volume. During the resuspension process, the pH was adjusted to 8.0 ± 0.2 with NaOH. The suspension was cooled to 6°C prior to homogenization. The intracellular enzyme was released from the cell using a high-pressure homogenizer (APV 1000) fitted with the appropriate cell disruption valve at $11,500 \pm 500$ psig. Immediately after cell disruption, the homogenate was cooled to $15 \pm 2^\circ\text{C}$ and the pH of the homogenate was adjusted to 8.0 ± 0.2 using NaOH. 10% w/v SuperFloc C581 was added to the cell homogenate. During the addition, the homogenate and C581 mixture was agitated to ensure complete blending. The suspension was incubated with agitation at $10 \pm 2^\circ\text{C}$ for 30 minutes, then heated to $55^\circ\text{C} \pm 2^\circ\text{C}$ and held at 55°C for 1 hour. The heat-treated suspension was cooled to $10 \pm 2^\circ\text{C}$. Separation of the solids from the liquid fraction was achieved by centrifugation. The homogenate was centrifuged at 5050xg for 35 min. A concentration step was performed in order to bring the protein concentration between 30 g/L and 40 g/L. The concentrated clarified lysate was dia-filtered with two volumes of 20 mM sodium phosphate/150 mM NaCl pH 8.0. The concentration and buffer exchange of the solution was achieved with TangenX 30kD PES NovaSet™-LS cassettes. The concentrate was filter-sterilized through a 0.2µm Nalgene™ Rapid-Flow™ disposable filter unit with PES membrane.

[0223] Filtered concentrate and anti-solvent (0.1 M HEPES, 0.2 M NaCl pH 7.5 25% (w/v) PEG 3350) were heated separately to 40°C. Anti-solvent was added slowly to the PAL variant concentrate until a cloud point was sustained. Contact seeding was achieved by adding a small amount of crystals to the solution after it reached the cloud point. The solution was cooled linearly from 40°C to 4°C over 6 hours with periodical gentle mixing. The suspension was held for 12 to 24 hours at 4°C. The liquid was decanted and the crystalline solids washed with water to remove residual PEG. The washed crystalline solids were then lyophilized overnight to dryness.

EXAMPLE 6

Screening Results

[0224] Variants of SEQ ID NO:4 ("Variant #1") were produced, including Variant #2 (SEQ ID NO:6), which has an additional F450A mutation incorporated into the sequence of Variant #1. Variants were

also produced using Variant #2 as a starting backbone. These variants were tested for PAL activity after incubation in lyophilized form at 45°C for 0 or 10 days as described in Example 2. In addition, variants with mutations of phenylalanine residues were tested at day 0 only. Retest data of active variants with corresponding fold- improvement over positive control (FIOP) are shown in Table 6.1, below. In this data set the positive control is Variant #2.

Table 6.1 Screening of Variants Derived From SEQ ID NO:6 (Variant #2) for PAL Activity			
Variant #	Amino Acid Changes Relative to Variant #2 (SEQ ID NO:6)	FIOP (T=0)	FIOP (T=10 days)
3	T102Q/D470E	+	++
4	I285R/D470E/T495A	++	++
5	T102Q	+	++
6	N44H/T239A/I285R/V469I/T495A	+	+
7	T239A/I285R	++	+
8	N44H/I56V	++	++
9	I285L/D470E	++	+++
10	N44H/T239A/V469I/D470E	++	+++
11	N44H/I56V/T102Q/T239A/I285L/V469I/D470E/T495A	+	++
12	N44H/T239A/I285L/D470E	++	++
13	I285L/V469I/D470E/T495A	+	+
14	N44C/T239A/T495A	+	++
15	N44H/T239A/T495A/S546A	+	+
16	N44H/V469I/D470E	++	+
17	N44H/T239A/D470E/S546A	++	+
18	T239A/I285L/V469I	+	++
19	T239A/V469I/D470E/T495A	++	+
20	V469I/D470E	++	+
21	N44H/T239A/I285R/D470E	++	+
22	N44H/T239A	+	+
23	F16S	++++	
24	F472A	+++	
25	F16W	++	
26	F16A	++	
27	F16E	+++	

Table 6.1 Screening of Variants Derived From SEQ ID NO:6 (Variant #2) for PAL Activity			
Variant #	Amino Acid Changes Relative to Variant #2 (SEQ ID NO:6)	FIOP (T=0)	FIOP (T=10 days)
28	F162W	++	
29	F482N	++	
30	F16V	+++	
31	F16T	+++	
32	F162Q	+	
33	F16W	++	
34	F16R	++	
35	F267V	++	
36	F267G	++	
37	F188I	++	
38	F16T	++	
39	F482C	++	
40	F264H	+++	
41	F16N	+++	
42	F16K/F150A	++	
43	L364E	+	+++
44	N455S	++	+
45	L364S	+	++++
46	L364T	+	+
47	L364H	+++	++++
48	I165L	++	++

[0225] In the above Table, the activity fold improvement (FIOP) is compared to the backbone SEQ ID NO:6 (Variant #2), and defined as follows: “-“ = less than 0.2-fold activity; “+” = greater than 0.2-fold, but less than 1-fold activity; “++” = greater than 1-fold, but less than 1.25-fold increased activity; “+++” = greater than 1.25-fold, but less than 1.5-fold increased activity; and “++++” = greater than 1.5-fold activity.

EXAMPLE 7**Confirmation of Hits**

[0226] Based on the results shown in Table 6.1 above, the activities of the variants listed in Table 7.1 (below) were confirmed for enzyme samples prepared per the description in Example 3.

Table 7.1 Confirmation of Activity of Selected Variants Derived From Variant #2 (SEQ ID NO:6)			
Variant #	Amino Acid Changes Relative to Variant #2 (SEQ ID NO:6)	FIOP (T=0)	FIOP (T=10 days)
2		+	+
24	F472A	++	+++
29	F482N	++	+
47	L364H	++	+
23	F16S	+++	+++
40	F264H	+	+

[0227] In the above Table, the activity fold improvement (FIOP) is compared to the backbone SEQ ID NO:6 (Variant #2), and defined as follows: “+” = greater than 0.95-fold, but less than 1-fold increased activity; “++” = greater than 1 fold, but less than 1.25-fold increased activity; and “+++” = greater than 1.25-fold increased activity.

EXAMPLE 8**Screening Results for Additional Variants**

[0228] Beneficial mutations identified as described in Example 6 were recombined into SEQ ID NO:8 (Variant No.23) (shown in Tables 6.1 and 7.1), and the new variants were tested for PAL activity after incubation in lyophilized form at 45°C for 0 and 10 days. Retest data of active variants with corresponding fold improvement over positive control (FIOP) are shown in Table 8.1, with Variant #23 as the positive control. In the following Table, some of the variants contain the same substitutions (i.e., are duplicates, such as variants 52 and 61), as these assay test results were obtained before sequencing data were obtained for the variants.

Table 8.1 Screening Results for Variants Derived from SEQ ID NO:8 (Variant #23)			
Variant #	Amino Acid Changes Relative to Variant #23 (SEQ ID NO:8)	FIOP (Primary T=0)	FIOP (Primary T=10 days)
49	S209P/I285R/L364H/D470E/T495A	++++	+++
50	R204K/S209P/L364H	+++	+++
51	S209P/I285L/L364H/D470E	+++	++
52	A47K/T239A/I285R/L364H	+++	++
53	L364H	++	+++
54	R204K/L364H	+++	+
55	N44H/R204K/I285R/L364H/D470E/T495A	+++	++++
56	A47K/R204K/I285R/L364H/T495A	+++	+++
57	L364H/D470E	+++	++
58	R204K/S209P/L364H/T495A	+++	++
59	A47K/S209P/T239A/L364H	++	++
60	S209P/L364H/T495A	+++	++
61	A47K/T239A/I285R/L364H	+++	++
62	N44H/A47K/L364H/D470E/T495A	+++	++
63	A47K/D470E	+++	++
64	I56V	+++	++
65	T239A/L364H	+++	+++
66	K54E/I56V/R204K/S209P/D470E	+++	+++
67	R204K	+++	++
68	N44H/K54P/I285R/D470E	+++	+++
69	R204K/D470E	+++	+++
70	S209P/L364H	+++	++
71	R204K/S209P/T239A/I285R/D470E/T495A	++	+++
72	A47K/K54P/S209P	+++	+++
73	K54E/I285R/D470E	+++	+++
74	D470E	++	++
75	R204K/T239A	+++	++
76	K54P/D470E	+++	+++
77	S209P/D470E	++	++
78	N44H/S209P/I285R/T460G/T495A	++++	++

Table 8.1 Screening Results for Variants Derived from SEQ ID NO:8 (Variant #23)			
Variant #	Amino Acid Changes Relative to Variant #23 (SEQ ID NO:8)	FIOP (Primary T=0)	FIOP (Primary T=10 days)
79	R204K/T239A/I285R	++++	+++
80	K54E/I56V/R204K/S209P/T495A	+++	+++
81	R204K/T239A/I285R	+++	++
82	R204K/T239A/I285R	++	++
83	N44H/K54P/I56V/R204K/T239A/D470E/T495A	+++	++
84	A47K/R204K/S209P/T239A/I285R/T495A	++	++
85	N44H/R204K/S209P/I285R	++	++
86	K54E	++	++
87	K54P/I285L/D470E	++	++
88	K54P/I56V/R204K/T495A	+++	++
89	K54P/I56V/I285L/D470E	+++	+++
90	N44H/A47K/R204K/S209P/I285R	++	++
91	K54P/I56V	++++	++
92	K54E/I56V/I285R/L364S/D470E	++	+++
93	K54P/I56V/S209P/I562N	++	++
94	K54P/T239A/T495A	++	++
95	I165L	++	++
96	N44H/K54P/I56V/R204K/T239A/I285R/L364S/T495A	++++	++
97	R204K/T239A/I285R	++++	+++
98	S209P/I285R/L364E/D470E	++	++
99	K54P/I165L/R204K/S209P/T239A/I285R/D470E/T495A	++	++
100	S209P/I285R/L364E/D470E/T495A	+++	++
101	R204K/S209P/L364E/T495A	++	++
102	N44C/I285R/L364E	++++	+
103	I285R/L364H	++++	+++
104	I285R/L364H/T495A	+++	+++
105	L364H	+++	++
106	N44C/T102Q/I285R/L364H	+++	+++
107	T495A	+++	+

[0229] In the above Table, the activity fold improvement (FIOP) is compared to the backbone (SEQ ID NO:8 (Variant #23); and defined as follows: “-“ = smaller than 0.2-fold activity; “+” = greater than 0.2-fold, but less than 1-fold activity; “++” = greater than 1-fold, but less than 1.25-fold increased activity; “+++” = greater than 1.25-fold activity, but less than 1.5-fold increased activity; and “++++” = greater than 1.5-fold activity.

EXAMPLE 9

Removal of Phenylalanine Residues

[0230] Activity results for variants of SEQ ID NO:6 (Variant #2), in which phenylalanine residues were changed without resulting in a loss of activity (i.e., phenylalanine mutations that are either neutral or beneficial for activity) are shown in Table 9.1. As in Table 8.1, in the following Table, some of the variants contain the same substitutions, as these assay test results were obtained before sequencing data were obtained for the variants.

Table 9.1 Mutations of Phenylalanine Residues in SEQ ID NO:6 (Variant #2) That Can be Replaced Without Loss of Function		
Variant #	Amino Acid Changes Relative to SEQ ID NO:6 (Variant #2)	FIOP Primary (T=0)
108	F162M	+
32	F162Q	+
28	F162W	+
26	F16A	+
109	F16A	+
27	F16E	+
110	F16K	+
42	F16K/F150A	+
111	F16L	+
112	F16M	+
41	F16N	+
113	F16P	+
34	F16R	+
23	F16S	+
31	F16T	+
38	F16T	+

Table 9.1 Mutations of Phenylalanine Residues in SEQ ID NO:6 (Variant #2) That Can be Replaced Without Loss of Function		
Variant #	Amino Acid Changes Relative to SEQ ID NO:6 (Variant #2)	FIOP Primary (T=0)
30	F16V	+
114	F16V	+
25	F16W	+
33	F16W	+
115	F188A	+
37	F188I	+
116	F188N	+
40	F264H	+
36	F267G	+
117	F267L	+
118	F267Q	+
119	F267S	+
35	F267V	+
120	F398H	+
121	F434V	+
24	F472A	+
122	F472A	+
123	F472L	+
124	F472V	+
39	F482C	+
29	F482N	+
125	F482S	+

[0231] In the above Table, the activity fold improvement (FIOP) is compared to the backbone SEQ ID NO:6 (Variant #2), and defined as follows: “+” = similar activity to SEQ ID NO:6 (variant #2) or SEQ ID NO:8 (variant #23)

EXAMPLE 10

Round 9 Screening Results

[0232] A combinatorial library was designed based on analysis of the previous results, using SEQ ID NO:12 (Variant #126) as the backbone, as Variant #126 showed improved storage stability as well as protease resistance in high throughput screening. The polynucleotide sequence encoding Variant #126 (SEQ ID NO:11) contains an a21t mutation compared to Variant #51 (SEQ ID NO:9). This variant was compared to wild-type AvPAL in various simulated intestinal fluids as described in Example 4. Active mutations with corresponding fold improvement in activity at T=0 and at T=10 days after incubation of lyophilized enzyme at 45°C over Variant #126 are shown in Table 10.1, below. As in other Tables in the Examples, in the following Table, some of the variants contain the same substitutions, as these assay test results were obtained before sequencing data were obtained for the variants.

Table 10.1 Screening of Variants Derived From Variant #126 for PAL Activity			
Variant #	Amino Acid Changes Relative to SEQ ID NO:12	FIOP (T=0)	FIOP (T=10)
127	I56V/F162W/F398H	++	++
128	K54E/I56V/F162W/R204K/L285I	+++	+++
129	I56V/R204K/F398H/F472L	++	+
130	K54P/I56V/R204K/F398H/F472L	++	++
131	I56V/L285I	+++	++
132	I56V/R204K/F398H	++	+
133	K54P/I56V/R204K/F398H	++	++
134	K54P/R204K/L285I/F398H/F472L	++	+
135	I56V/F398H	++	+
136	K54E/I56V/F398H/F472L	++	+
137	I56V/L285I/F398H/F472L	++	+
138	K54P/I56V/F398H	++	+
139	I56V/F398H	++	++
140	K54P/L285I/F398H	++	+
141	K54P/I56V/F162W/R204K/F398H	++	++
142	K54P/I56V/L285I	++	++
143	K54P/I56V/R204K/L285I	++	+
144	I56V/F398H/F472L	++	++
145	K54E/R204K/F398H	++	+
146	I56V/R204K/L285I	++	++
147	T201A/R204K/F398H	++	++

Table 10.1 Screening of Variants Derived From Variant #126 for PAL Activity			
Variant #	Amino Acid Changes Relative to SEQ ID NO:12	FIOP (T=0)	FIOP (T=10)
148	I56V/L285H	++	+
149	K54E/F162W/F398H	++	+
150	K54P/F162W/F398H	++	++
151	K54E/F162W/R204K/F398H	++	++
152	K54P/L285I/F398H/F472L	++	+
153	I56V/R204K/F398H/T460G	++	+

[0233] In this Table, the activity fold improvement (FIOP) is compared to the backbone SEQ ID NO:12 (Variant #126), and are defined as follows: “-” = less than 0.2-fold activity; “+” = greater than 0.2-fold, but less than 1-fold activity; “++” = greater than 1-fold, but less than 1.25-fold increased activity; and “+++” = greater than 1.25-fold activity.

EXAMPLE 11

Plasma Phenylalanine Levels

[0234] Plasma samples were analyzed for phenylalanine and cinnamic acid. Internal standards were generated by dissolving phenylalanine or cinnamic acid in 50/50 acetonitrile:DMSO, and then diluted to working concentrations of 10-40,000 ng/ml in 50% acetonitrile. These samples were diluted into blank dog plasma (10 µL:90 µL) and then diluted with 300 µL of internal standard (d₈-Phe or d₇-CA at 250 ng/ml in acetonitrile). Animal plasma samples (20 µL) were diluted with acetonitrile (80 µL), and then diluted with 300 µL of internal standard. For analysis of phenylalanine concentrations, 30 µL of samples were injected using a Shimadzu HPLC/CTC Autosampler with Luna C18 column (2.1x100mm, 5µm). Samples were eluted using a gradient of 0.5% Formic acid, 5mM ammonium acetate in water and 90% Acetonitrile and 10% water with 0.5% formic acid over 2.5 minutes, with phenylalanine eluting at 1.3 min.

[0235] For analysis of cinnamic acid, 30 µL of sample was injected using a Shimadzu HPLC/CTC Autosampler with Thermo Silica column (2.1x100mm, 5µm). Samples were eluted using a gradient of 5mM ammonium acetate in water and 90% Acetonitrile/10% water over 3.5 minutes, with cinnamic acid eluting at 2.3 min. Analytes were detected using an API-4000Q-trap Mass Spectrometer, performing MRM scans in ESI positive mode (phenylalanine) or negative mode (cinnamic acid). Transitions for isotopically labeled phenylalanine derivatives were 166.2/120.0, 171.2/125.1, and 174.2/128.2. Transitions for isotopically labeled cinnamic acid were 146.8/102.9, 151.9/107.9, and 153.9/110.1.

EXAMPLE 12

Intestinal Activity of Variant #126 in a Dog Model

[0236] To assess the stability and activity of PAL variants as they transit through the gastrointestinal tract, beagle dogs were gavaged with purified enzyme variants. The effect of these variants on concentrations of phenylalanine and cinnamic acid in the dogs' blood was measured. Dogs were fasted overnight (12-16 hours) and through the study until 8 hours after enzyme dosing. All dogs were administered an oral dose of famotidine (40 mg/dog) 2 hours before study start and again at +3 hours to minimize gastric acidification. Pre-study plasma samples (~2mL) were taken at -2 hours (just prior to first famotidine dose), -30 min, and -10 min (the average of these 3 measurements was used to normalize post-dose values). Exactly 5 minutes after the dose of vehicle or Variant #126, at t=0, all dogs received a 900 mg/kg PO dose of powdered Goat Milk Esbilac (PetAg) protein in suspension (4 mL/kg). Additional plasma samples were taken at +15 min, +30 min, and +1, +2, +4, +6, +8, and +24 h post-treatment dose. Plasma samples were analyzed for Phe and CA as described in Example 11.

[0237] Results are shown in Figures 3, 4, and 5. In Figure 3, the relative blood phenylalanine levels for the different groups are shown. Upon administration of vehicle a 1.35-fold increase in phenylalanine level was observed. Administration of all doses of Variant #126 suppress this immediate phenylalanine spike and led to a reduction in blood phenylalanine at t= 1 hr, except for the 200 mg/kg group. At the 2 hr time point, the 120 mg/kg group showed a 34% reduction in blood phenylalanine.

[0238] Figure 5 shows the cinnamic acid levels for the different groups. All groups except for vehicle showed rapid appearance of cinnamic acid upon administration of Variant #126 administration. The rapid onset of cinnamic acid formation leads to a C_{max} at 15 minutes for the 120 mg/kg dose, at 30 min for PAL-the 60 mg/kg dose, and 1hr for the 200 mg/kg dose. Cinnamic acid is detected in plasma for 10 hrs in all dose groups. In the 200 mg/kg dose group, cinnamic acid continues to be above baseline for 24 hrs.

[0239] In Figure 5 the amount of cinnamic acid formed during the first 4 hrs after Variant #126 administration was quantified as the AUC. The AUC for the 60, 120, and 200 mg/kg groups is statistically significantly different from the vehicle response.

EXAMPLE 13

Intestinal Activity of Variant #126 in the Cynomolgus Monkey

[0240] Cynomolgus monkeys were used to assess the efficacy of Variant #126 to convert phenylalanine to cinnamic acid, by measuring plasma phenylalanine and cinnamic acid levels. Some of the animals received a dose of famotidine (20 mg per monkey) two hours prior to administration of Variant #126.

Enzyme was administered by gavage and followed 5 minutes later with 900 mg/kg Goat Milk Esbilac (PetAg) protein to start the study ($t=0$). Plasma samples were analyzed for Phe and CA as described in Example 11.

[0241] In Figure 6, the relative plasma phenylalanine levels for the different groups are plotted over the first 8 hrs. after administration of Variant #126. Administration of vehicle led to a 1.7-fold increase in phenylalanine levels at 1 hr. Administration of all doses of Variant #126 suppressed this phenylalanine spike and led to a reduction in blood phenylalanine after 1 to 2 hrs. All groups treated with Variant #126, showed reduction of plasma phenylalanine between 20% and 38% at the 1 hour compared to vehicle.

[0242] As shown in Figure 7, the relative reduction of phenylalanine was quantified as the AUC for the 0 to 4 hrs time period. All groups dosed with Variant #126 had on average, a 22% lower AUC for phenylalanine compared to vehicle control.

[0243] As shown in Figure 8, the time dependent cinnamic acid concentrations in plasma for the different dose groups are shown. Figure 8 (top) shows cinnamic acid formation for groups that were pretreated with famotidine; Figure 8 (bottom) shows such data for groups that were not treated with famotidine prior to dosing. Cinnamic acid levels rose rapidly to reach C_{max} at 1 hr, and then receded to close to baseline by 4 hrs. No cinnamic acid was detected in the vehicle control group.

[0244] As shown in Figure 9, the formation of cinnamic acid during the initial 0 to 4 hrs after administration was quantified as the AUC. Figure 9 (top) shows the AUC of groups pretreated with famotidine. A 10-fold increase in dose resulted in a less than 2-fold increase in cinnamic acid production. Figure 9 (middle) shows the AUC of groups not pre-treated with famotidine and all groups showed a similar level of cinnamic acid formation. Figure 9 (bottom) shows a direct comparison for groups that were dosed with the same amount of Variant #126 but either had been pretreated with famotidine or not. Famotidine treatment showed no statistically significant effect.

[0245] The results provided in Figures 6 through 9, show that oral administration of Variant #126 to healthy cynomolgus monkeys has a statistically significant effect on phenylalanine levels in plasma. In addition, upon oral administration of Variant #126 to cynomolgus monkeys, an amount of cinnamic acid is formed that corresponds to removal of approximately 9 mg/kg of phenylalanine. This amount is relevant in the context of phenylalanine intake by healthy individuals and PKU patients.

EXAMPLE 14

Screening Results for Individual Mutation Variants

[0246] All amino acid mutations were individually made into Variant No.126 (SEQ ID NO:12) and tested for PAL activity after incubation in lyophilized form at 45°C for 0 ($T=0$) or 10 ($T=10$) days, and

for protease stability, each was tested in triplicate. In the following Table, the mutations and assay results are provided.

Table 14.1 Screening of Variants Derived From Variant #126 (SEQ ID NO:12) for PAL Activity				
Variant #	Amino Acid Changes (Relative to SEQ ID NO:12)	FIOP (T=0)	FIOP Protease	FIOP (T=10)
154	S16E	+++	+++	+
155	S16I	+++	+++	+
156	S16L	++	++	+
157	S16D	++	++	+
158	S16K	++	++	+
159	S16H	++	++	+
160	S16M	++	++	+
161	Q214N	++	++	+++
162	S16C	++	++	+
163	S16T	++	++	+
164	S16A	++	++	+
165	H364M	++	++	+
166	G307I	++	++	+
167	S16Q	++	++	+
168	V39Y	++	+	++
169	S16F	++	++	+
170	Q214C	++	++	++
171	S16N	++	+	+
172	S16R	++	++	+
173	S16P	++	+	+
174	R59F	++	+	++
175	Q503E	++	++	++
176	H364Y	++	++	+
177	S16V	++	+	+
178	L285N	++	+	+
179	K521Y	++	++	++
180	Q214D	+	++	+
181	S16W	+	++	+
182	H364E	+	+	+
183	H364L	+	+	+
184	L285C	+	+	+
185	G307Q	+	+	+
186	L285A	+	+	+
187	V39H	+	+	+
188	G307A	+	+	+
189	H364K	+	+	+
190	L285H	+	+	+
191	G307L	+	+	+
192	Q214W	+	+	+
193	G307H	+	+	+

Table 14.1 Screening of Variants Derived From Variant #126 (SEQ ID NO:12) for PAL Activity				
Variant #	Amino Acid Changes (Relative to SEQ ID NO:12)	FIOP (T=0)	FIOP Protease	FIOP (T=10)
194	R59N	+	+	+
195	A47D	+	+	+
196	V39N	+	+	+
197	G307E	+	+	+
198	V39Q	+	+	+
199	L285E	+	+	+
200	G307C	+	+	+
201	L285G	+	+	+
202	K54Q	+	+	+
203	H364N	+	+	+
204	K521G	+	+	+
205	H364T	+	+	+
206	V39R	+	+	+
207	Q214E	+	+	+
208	L285V	+	+	+
209	K54I	+	+	+
210	Q214G	+	+	+
211	H18V	+	+	+
212	L285M	+	+	+
213	K521P	+	+	+
214	A47E	+	+	+
215	H18P	+	+	+
216	Q503G	+	+	+
217	H364Q	+	+	+
218	K73I	+	+	+
219	G290D	+	++	+
220	Q503A	+	+	+
221	Q503V	+	+	+
222	Q503I	+	+	+
223	K521M	+	+	+
224	V407I	+	+	+
225	H364I	+	+	+
226	K54D	+	+	+
227	V39E	+	+	+
228	V39K	+	+	+
229	G307F	+	+	+
230	G307V	+	+	+
231	V39T	+	+	+
232	G307S	+	+	+
233	K73R	+	+	+
234	K521V	+	+	+
235	V39G	+	+	+
236	V407R	+	+	+
237	Q503Y	+	+	+

**Table 14.1 Screening of Variants
Derived From Variant #126 (SEQ ID NO:12) for PAL Activity**

Variant #	Amino Acid Changes (Relative to SEQ ID NO:12)	FIOP (T=0)	FIOP Protease	FIOP (T=10)
238	G307T	+	+	+
239	K521C	+	+	+
240	M305A	+	++	+
241	V407K	+	+	+
242	P565E	+	+	+
243	V39F	+	+	+
244	L285Q	+	+	+
245	M305N	+	+	+
246	K521L	+	+	+
247	H18N	+	+	+
248	V91T	+	+	+
249	K54C	+	+	+
250	P209S	+	+	+
251	K521A	+	+	+
252	L285T	+	+	+
253	A47S	+	+	+
254	K54A	+	+	+
255	P209N	+	+	+
256	G307M	+	+	+
257	L285K	+	+	+
258	V39W	+	+	+
259	K521H	+	+	+
260	K73V	+	+	+
261	K73Y	+	+	+
262	L285F	+	+	+
263	K521W	+	+	+
264	K54L	+	+	+
265	P565V	+	+	+
266	H18Q	+	+	+
267	Q503D	+	+	+
268	M305L	+	+	+
269	M305S	+	+	+
270	M305K	+	+	+
271	G290P	+	-	+
272	S524L	+	+	+
273	H18Y	+	+	+
274	G307R	+	+	+
275	K73L	+	+	+
276	V407C	+	+	+
277	K54M	+	+	+
278	G290S	+	++	+
279	H364V	+	+	+
280	P565D	+	+	+
281	V407M	+	+	+

Table 14.1 Screening of Variants Derived From Variant #126 (SEQ ID NO:12) for PAL Activity				
Variant #	Amino Acid Changes (Relative to SEQ ID NO:12)	FIOP (T=0)	FIOP Protease	FIOP (T=10)
282	G290Q	+	+	+
283	E470M	+	+	+
284	V91S	+	+	+
285	Q503S	+	+	+
286	H18D	+	+	+
287	K54T	+	+	+
288	K73E	+	+	+
289	E470T	+	+	+
290	P565G	+	+	+
291	K521E	+	+	+
292	G307Y	+	+	+
293	V407A	+	+	+
294	K54S	+	+	+
295	L285R	+	+	+
296	S524H	+	+	+
297	A47G	+	+	+
298	P565K	+	+	+
299	K54V	+	+	+
300	P209A	+	+	+
301	V39L	+	+	+
302	A47I	+	+	+
303	Q503T	+	+	+
304	S524W	+	+	+
305	Q214S	+	+	+
306	K73T	+	+	+
307	K521S	+	+	+
308	P565R	+	+	+
309	E470Q	+	+	+
310	Q503C	+	+	+
311	E470W	+	+	+
312	V39S	+	+	+
313	R59L	+	+	+
314	V39D	+	+	+
315	S524E	+	+	+
316	Q214T	+	+	+
317	A47K	+	+	+
318	K54E	+	+	+
319	V39M	+	+	+
320	L285S	+	+	+
321	K73A	+	+	+
322	K521D	+	+	+
323	S524R	+	+	+
324	Q503F	+	+	+
325	K521H	+	+	+

Table 14.1 Screening of Variants Derived From Variant #126 (SEQ ID NO:12) for PAL Activity				
Variant #	Amino Acid Changes (Relative to SEQ ID NO:12)	FIOP (T=0)	FIOP Protease	FIOP (T=10)
326	A47T	+	+	+
327	Q214A	+	+	+
328	Q214F	+	+	+
329	P565F	+	+	+
330	V39I	+	+	+
331	G290N	+	++	+
332	K73M	+	+	+
333	V407L	+	+	+
334	M305D	+	+	+
335	K73S	+	+	+
336	M305C	+	+	+
337	E470D	+	+	+
338	K54R	+	+	+
339	S524K	+	+	+
340	A47H	+	+	+
341	R59C	+	+	+
342	H364A	+	+	+
343	R59S	+	+	+
344	V91A	+	+	+
345	V407T	+	+	+
346	E470K	+	+	+
347	S524C	+	+	+
348	E470A	+	+	+
349	K54P	+	+	+
350	P565H	+	+	+
351	Q214H	+	+	+
352	A47Q	+	+	+
353	A47V	+	+	+
354	A47M	+	+	+
355	K54H	+	+	+
356	P209D	+	+	+
357	G307D	+	+	+
358	V407H	+	+	+
359	Q503M	+	+	+
360	K73G	+	+	+
361	M305E	+	+	+
362	E470I	+	+	+
363	K521F	+	+	+
364	Q503L	+	+	+
365	P565Q	+	+	+
366	G290K	+	+	+
367	P209Y	+	+	+
368	P565Y	+	+	+
369	P209G	+	+	+

Table 14.1 Screening of Variants Derived From Variant #126 (SEQ ID NO:12) for PAL Activity				
Variant #	Amino Acid Changes (Relative to SEQ ID NO:12)	FIOP (T=0)	FIOP Protease	FIOP (T=10)
370	A47R	+	+	+
371	Q214Y	+	+	+
372	A47W	+	+	+
373	Q503K	+	+	+
374	V407N	+	+	+
375	G307W	+	+	+
376	P209C	+	+	+
377	K73Q	+	+	+
378	E470H	+	+	+
379	E470R	+	+	+
380	R59G	+	+	+
381	K521N	+	+	+
382	P565S	+	+	+
383	P209E	+	+	+
384	G290T	+	++	+
385	V39C	+	+	+
386	E470V	+	+	+
387	K73C	+	+	+
388	S524G	+	+	+
389	L285W	+	+	+
390	K54N	+	+	+
391	E470G	+	+	+
392	V91C	+	+	+
393	L285D	+	+	+
394	K521R	+	+	+
395	K521T	+	+	+
396	P565N	+	+	+
397	R59A	+	+	+
398	K54Y	+	+	+
399	E470F	+	+	+
400	P565I	+	+	+
401	P209K	+	+	+
402	H18W	+	+	+
403	E470C	+	+	+
404	M305T	+	+	+
405	S524T	+	+	+
406	P209R	+	+	+
407	R59D	+	+	+
408	K521Q	+	+	+
409	Q503R	+	+	+
410	A47P	+	+	+
411	A47Y	+	+	+
412	S524V	+	+	+
413	R59H	+	+	+

Table 14.1 Screening of Variants Derived From Variant #126 (SEQ ID NO:12) for PAL Activity				
Variant #	Amino Acid Changes (Relative to SEQ ID NO:12)	FIOP (T=0)	FIOP Protease	FIOP (T=10)
414	V407Q	+	+	+
415	P565L	+	+	+
416	S524D	+	+	+
417	L285I	+	+	+
418	V39P	+	+	+
419	P209W	+	+	+
420	K54F	+	+	+
421	P209Q	+	+	+
422	Q214M	+	+	+
423	K73W	+	+	+
424	K73N	+	+	+
425	A47C	+	+	+
426	P209V	+	+	+
427	K73D	+	+	+
428	A47F	+	+	+
429	Q503N	+	+	+
430	P209I	+	+	+
431	R59K	+	+	+
432	Q214L	+	+	+
433	Q503H	+	+	+
434	M305Q	+	+	+
435	E470Y	+	+	+
436	S524M	+	+	+
437	V91P	+	+	+
438	K73H	+	+	+
439	S524A	+	+	+
440	R59E	+	+	+
441	P565M	+	+	+
442	S16G	+	+	+
443	P209H	+	+	+
444	S524I	+	+	+
445	P209F	+	+	+
446	P565T	+	+	+
447	R59P	+	+	+
448	K54W	+	+	+
449	H18R	+	+	+
450	K73F	+	+	+
451	G290H	+	+	+
452	M305H	+	+	+
453	R59Y	+	++	+
454	P209L	+	+	+
455	A47L	+	+	+
456	P565C	+	+	+
457	M305I	+	+	+

**Table 14.1 Screening of Variants
Derived From Variant #126 (SEQ ID NO:12) for PAL Activity**

Variant #	Amino Acid Changes (Relative to SEQ ID NO:12)	FIOP (T=0)	FIOP Protease	FIOP (T=10)
458	H18K	+	+	+
459	E470L	+	+	+
460	E470N	+	+	+
461	E470S	+	+	+
462	P209M	+	+	+
463	S524Y	+	+	+
464	S524Q	+	+	+
465	E470P	+	+	+
466	S524P	+	+	+
467	S524N	+	+	+
468	G290R	+	+	+
469	R59M	+	+	+
470	M305V	+	+	+
471	S524F	+	+	+
472	G290A	+	+	+
473	M305W	+	+	+
474	V39A	+	+	+
475	M305G	+	+	+
476	R59Q	+	+	+
477	G290C	+	+	+
478	R59W	+	+	+
479	A450R	+	+	+
480	V91G	+	+	+
481	V407Y	+	+	+
482	H18A	+	+	+
483	R59V	+	+	+
484	R59I	+	+	+
485	M305F	+	+	+
486	H18F	+	+	+
487	V407F	+	+	+
488	V91I	+	+	+
489	A450G	+	+	+
490	H364C	+	+	+
491	P565W	+	+	+
492	A450S	+	+	+
493	P209T	+	+	+
494	H18S	+	+	+
495	G290E	+	+	+
496	R59T	+	-	+
497	H18I	+	+	+
498	A450T	+	+	+
499	P565A	+	+	+
500	M305Y	+	+	+
501	H18E	+	+	+

Table 14.1 Screening of Variants Derived From Variant #126 (SEQ ID NO:12) for PAL Activity				
Variant #	Amino Acid Changes (Relative to SEQ ID NO:12)	FIOP (T=0)	FIOP Protease	FIOP (T=10)
502	G307N	+	+	+
503	A450V	+	+	+
504	Q214I	+	+	+
505	V91N	+	+	+
506	H18G	+	+	+
507	M305P	+	+	+
508	A450K	+	+	+
509	K73P	+	-	+
510	G290M	+	+	+
511	V407S	+	+	+
512	G290Y	+	+	+
513	Q214V	+	+	+
514	H18T	+	+	+
515	Q214P	+	-	+
516	H18C	+	+	+
517	Q214K	+	+	+
518	G290F	+	+	+
519	A450M	+	+	+
520	A450N	+	+	+
521	A450Q	+	+	+
522	Q503P	+	+	+
523	V91F	+	-	+
524	V91L	+	+	+
525	V91D	+	+	+
526	H18M	+	+	+
527	Q214R	+	+	+
528	A450C	+	+	+
529	L285Y	+	+	+
530	L285P	+	+	-
531	A450H	+	+	+
532	H364S	+	+	+
533	A450F	+	+	+
534	G290L	+	+	+
535	G290V	+	+	+
536	A450I	+	+	+
537	V407G	+	+	-
538	V91M	+	+	+
539	A450E	+	+	+
540	V91H	+	+	+
541	V91Y	+	-	+
542	G307K	+	+	+
543	A450Y	+	+	+
544	G290I	+	+	+
545	H18L	+	+	+

Table 14.1 Screening of Variants Derived From Variant #126 (SEQ ID NO:12) for PAL Activity				
Variant #	Amino Acid Changes (Relative to SEQ ID NO:12)	FIOP (T=0)	FIOP Protease	FIOP (T=10)
546	V91Q	+	+	+
547	V407P	+	+	+
548	G290W	+	+	+
549	H364F	+	+	-
550	V91E	+	+	+
551	V91K	+	+	-
552	A450W	+	+	+
553	M305R	+	+	+
554	V91R	+	-	+
555	A450D	-	+	-
556	K54G	-	+	+
557	V91W	-	-	-
558	V407E	-	-	-
559	G307P	-	-	-
560	H364G	-	-	-
561	V407D	-	-	-
562	V407W	-	-	-
563	H364D	-	-	-
564	S16Y	-	-	-
565	H364P	-	-	-
566	A450P	-	-	-
567	Q503W	-	-	-
568	A47N	-	-	-
569	H364R	-	-	-
570	H364W	-	-	-
571	A450L	-	-	-

[0247] In the above Table, the activity fold improvement (FIOP) is compared to SEQ ID NO:12 (Variant #126), and defined as follows: “-” = less than 0.2-fold activity; “+” = greater than 0.2-fold, but less than 1-fold activity; “++” = greater than 1-fold, but less than 1.25-fold increased activity; and “+++” = greater than 1.25- fold activity.

EXAMPLE 15

Combinatorial Screening Results for Additional Variants

[0248] Mutations identified in Variant No. 126 (SEQ ID NO:12) were added combinatorially in SEQ ID NO:2. The new variants were tested for PAL activity after incubation in lyophilized form at 45°C for 0 (T=0) or 7 days (T=7), as well as stability in the presence of protease. Data of variants with corresponding fold improvement over positive control (FIOP) are shown in Table 15.1, with SEQ ID NO:12 (Variant #126) as the positive control.

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	FIOP (T=0)	FIOP Protease	FIOP (T=7)
572	A39V/G59R/A202T/L407V/T524S/C565P	-	-	-
573	A39V/G59R/L364H/F450A/D470E/C565P	-	-	-
574	A39V/G59R/S73K/S209P/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
575	A39V/L214Q/H307G/C503Q/T524S	-	+	-
576	A39V/L214Q/I285L/N290G/R305M/H307G/L364H/C503Q/Q521K/T524S	+	-	+
577	A39V/L47A/A91V/L214Q	-	-	-
578	A39V/L47A/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
579	A39V/L47A/G59R/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S	+	-	+
580	A39V/L47A/G59R/A91V/S98G/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/P403T/L407V/F450A/D470E/C503Q/T524S/C565P	+	+	+
581	A39V/L47A/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	+
582	A39V/L47A/I285L/C503Q	-	-	-
583	A39V/L47A/S73K/A91V/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	++
584	A39V/L47A/S73K/A91V/L214Q/I285L/N290G/R305M/L364H/L407V/F450A/C503Q/Q521K/T524S	+	+	+
585	A39V/L47A/T54K/A91V/L214Q/I285L/N290G/H307G/L364H/D470E/C503Q/Q521K/T524S	+	+	+
586	A39V/L47A/T54K/A91V/S209P/I285L/N290G/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
587	A39V/L47A/T54K/A91V/S209P/L214Q/I285L/R305M/H307G/L407V/C503Q/Q521K/C565P	+	+	+
588	A39V/L47A/T54K/G59R/A91V/S98G/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/T524S	+	-	+
589	A39V/L47A/T54K/G59R/S73K/A91V/I285L/N290G/R305M/H307G/F450A/D470E/C503Q/Q521K/T524S	+	-	+
590	A39V/L47A/T54K/G59R/S73K/A91V/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/C565P	+	+	+
591	A39V/L47A/T54K/G59R/S73K/A91V/I285L/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S/C565P	+	+	+
592	A39V/L47A/T54K/G59R/S73K/A91V/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
593	A39V/L47A/T54K/G59R/S73K/A91V/I285L/N290G/R305M/H307G/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	F10P (T=0)	F10P Protease	F10P (T=7)
594	A39V/L47A/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
595	A39V/L47A/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/R305M/H307G/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
596	A39V/L47A/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/R305M/H307G/L407V/R412H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
597	A39V/L47A/T54K/G59R/S73K/A91V/S209P/I285L/N290G/H307G/L407V/F450A/D470E/C503Q/Q521K/C565P	+	+	+
598	A39V/L47A/T54K/G59R/S73K/A91V/S209P/I285L/N290G/L364H/F450A/D470E/C503Q/Q521K/T524S	+	-	+
599	A39V/L47A/T54K/G59R/S73K/A91V/S209P/I285L/N290G/R305M/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
600	A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/L364H/L407V/C503Q/Q521K/T524S/C565P	+	+	+
601	A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S/C565P	+	+	+
602	A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q	+	-	+
603	A39V/L47A/T54K/G59R/S73K/S209P/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S	+	+	+
604	A39V/L47A/T54K/G59R/S73K/S209P/L214Q/I285L/N290G/H307G/L407V/F450A/C503Q/Q521K/T524S	+	+	+
605	A39V/L47A/T54K/S73K/A91V/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
606	A39V/L47A/T54K/S73K/A91V/L214Q/I285L/N290G/R305M/L364H/L407V/F450A/C503Q/T524S	+	+	+
607	A39V/L47A/T54K/S73K/A91V/S209P/I285L/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S	+	+	+
608	A39V/L47A/T54K/S73K/A91V/S209P/I285L/N290G/R305M/L364H/F450A/D470E/C503Q/Q521K/T524S	+	+	+
609	A39V/L47A/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L407V/D470E/C503Q/Q521K/T524S/C565P	+	+	+
610	A39V/S209P/F450A/D470E/C503Q	-	-	-
611	A39V/S209P/L214Q/L407V/F450A/D470E/C503Q/Q521K	-	-	-
612	A39V/S73K/N290G/H307G/L364H/L407V/F450A/T524S/C565P	-	+	-
613	A39V/T54K/G59R/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/T524S/C565P	+	-	+
614	A39V/T54K/G59R/A91V/S98G/I285L/N290G/C503Q/Q521K/T524S/C565P	-	-	-
615	A39V/T54K/G59R/A91V/S98G/S209P/L287Q/N290V/S291G/H307G/L364H/L407V/F450A/D470E/Q521K/T524S	-	++	-

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	FPOP (T=0)	FPOP Protease	FPOP (T=7)
616	A39V/T54K/G59R/S73K/A91V/I285L/N290G/H307G/L364H/L407V/F450A/C503Q/T524S	+	+	+
617	A39V/T54K/G59R/S73K/A91V/S209P/I285L/N290G/R305M/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
618	A39V/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/L364H/L407V/D470E/C503Q/T524S/C565P	+	+	+
619	A39V/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/D470E/C503Q	+	+	+
620	A39V/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
621	A39V/T54K/G59R/S73K/A91V/S209P/L214Q/N290G/R305M/H307G/L407V/C503Q/Q521K/T524S/C565P	+	+	+
622	A39V/T54K/G59R/S73K/A91V/S209P/L214Q/N290G/R305M/L407V/F450A/C503Q/T524S/C565P	+	+	+
623	A39V/T54K/G59R/S73K/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S/C565P	+	+	+
624	A39V/T54K/G59R/S73K/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/C503Q/Q521K/C565P	+	+	+
625	A39V/T54K/G59R/S73K/S98G/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q	+	+	+
626	A39V/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/L364H/C503Q/T524S/C565P	+	+	+
627	A91V/A97T/S209P/I285L	-	-	-
628	A91V/S209P/L214Q/D470E/C565P	-	-	-
629	A91V/S209P/L214Q/F450A/Q521K/T524S/C565P	-	-	-
630	A91V/S209P/L214Q/L364H/L407V/F450A/D470E/C565P	-	-	-
631	A91V/S209P/L214Q/L407V/C503Q/C565P	-	-	-
632	A91V/S209P/L214Q/Q255L/I285L/N290G/H307G/F450A	-	-	-
633	A91V/S209P/L407V	-	-	-
634	C503Q	-	-	+
635	D55H/N290G/R305M/L364H/F450A/C503Q	-	-	-
636	F16S/A39V/D55E/G59R/C503Q/C565P	-	-	-
637	F16S/A39V/L214Q/L407V/C503Q/C565P	-	-	-
638	F16S/A39V/L214Q/Q255R/L407V/C565P	-	-	-
639	F16S/A39V/L214Q/T524S	-	-	-
640	F16S/A39V/L407V/F450A/D470E/Y475F	-	-	-
641	F16S/A39V/L47A/G59R/S73K/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/T524S/C565P	++	+	++
642	F16S/A39V/L47A/S73K/A91V/I285L/N290G/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
643	F16S/A39V/L47A/S73K/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	++	-	++

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	F1OP (T=0)	F1OP Protease	F1OP (T=7)
644	F16S/A39V/L47A/T54K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	++	+	++
645	F16S/A39V/L47A/T54K/G59R/L214Q/L364H/F450A/D470E/C503Q/Q521K/T524S	+	-	+
646	F16S/A39V/L47A/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/H307G/L364H/L407V/F450A/C503Q/Q521K/T524S/C565P	+	-	+
647	F16S/A39V/L47A/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/R305M/H307G/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
648	F16S/A39V/L47A/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/R305M/L364H/L407V/F450A/C503Q/T524S/C565P	+	++	+
649	F16S/A39V/L47A/T54K/G59R/S73K/A91V/L214Q/N290G/L364H/L407V/F450A/D470E/T524S/C565P	+	-	+
650	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S209P/I285L/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S	+	++	+
651	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/H307G/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
652	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S/C565P	+	+	+
653	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/C503Q/Q521K/T524S/C565P	+	++	+
654	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	++	++	+
655	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	++	+	+
656	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/L364H/L407V/C503Q/Q521K/T524S/C565P	+	+	+
657	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/L364H/L407V/F450A/C503Q/Q521K	++	+	++
658	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	++
659	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
660	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/N290G/R305M/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	F1OP (T=0)	F1OP Protease	F1OP (T=7)
661	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S98G/S209P/L214Q/I285L/N290G/H307G/L364H/L407V/F450A/D470E/Q521K/T524S/C565P	+	-	+
662	F16S/A39V/L47A/T54K/G59R/S73K/A91V/S98G/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
663	F16S/A39V/L47A/T54K/G59R/S73K/H307G/L364H/L407V/C503Q/Q521K/T524S	+	+	+
664	F16S/A39V/L47A/T54K/G59R/S73K/I285L/N290G/R305M/H307G/L364H/L407V/T524S	+	+	+
665	F16S/A39V/L47A/T54K/G59R/S73K/S98G/S209P/I285L/R305M/H307G/L407V/F450A/D470E/C503Q/T524S	+	+	+
666	F16S/A39V/L47A/T54K/S209P/H307G/C503Q	-	+	-
667	F16S/A39V/L47A/T54K/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/D470E/C503Q/Q521K/T524S/C565P	+	+	+
668	F16S/A39V/L47A/T54K/S73K/A91V/S209P/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S/C565P	+	+	+
669	F16S/A39V/L47A/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/L407V/F450A/D470E/Q521K/T524S/C565P	+	+	+
670	F16S/A39V/Q521K/C565P	-	-	-
671	F16S/A39V/S73K/L364H	-	-	-
672	F16S/A39V/T54K/G59R/A91V/I285L/N290G/R305M/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
673	F16S/A39V/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K	+	+	+
674	F16S/A39V/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
675	F16S/A39V/T54K/G59R/S73K/A91V/L214Q/N290G/R305M/H307G/L407V/D470E/C503Q/Q521K/T524S/C565P	+	++	+
676	F16S/A39V/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/D470E/C503Q/Q521K/T524S/C565P	+	+	+
677	F16S/A91V/I285L/N290G/L364H/L407V	-	-	-
678	F16S/A91V/L214Q/L407V/C503Q	-	-	-
679	F16S/A91V/S209P/L214Q	+	-	+
680	F16S/A91V/S209P/L214Q/L364H/F450A	-	-	-
681	F16S/C565P	-	-	-
682	F16S/F18H/A39V/A91V/C503Q/Q521K	-	-	-
683	F16S/F18H/A39V/G45D/L47A/T54K/G59R/S73K/S98G/S209P/L214Q/I285L/N290G/R305M/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
684	F16S/F18H/A39V/L47A/A91V/N290G/L407V/	-	-	-
685	F16S/F18H/A39V/L47A/G59R/L214Q/C503Q/C565P	-	-	-
686	F16S/F18H/A39V/L47A/G59R/S73K/A91V/L214Q/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K	+	+	+

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	F1OP (T=0)	F1OP Protease	F1OP (T=7)
687	F16S/F18H/A39V/L47A/S49I/T54K/G59R/S73K/S209P/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
688	F16S/F18H/A39V/L47A/T54K/A91V/L214Q/I285L/N290G/L364H/L407V/F450A/D470E/C503Q/Q521K	+	-	+
689	F16S/F18H/A39V/L47A/T54K/G59R/A91V/I285L/N290G/R305M/H307G/L364H/L407V/F450A/C503Q/Q521K/T524S	+	-	+
690	F16S/F18H/A39V/L47A/T54K/G59R/A91V/L214Q/I285L/N290G/R305M/H307G/L364H/F450A/C503Q/Q521K/T524S	+	+	+
691	F16S/F18H/A39V/L47A/T54K/G59R/A91V/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
692	F16S/F18H/A39V/L47A/T54K/G59R/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
693	F16S/F18H/A39V/L47A/T54K/G59R/A91V/S98G/S209P/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	+
694	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K	+	+	+
695	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
696	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/I285L/N290G/R305M/L364H/L407V/D470E/C503Q/Q521K/T524S	+	+	+
697	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/C503Q	+	-	+
698	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/I285L/N290G/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S/C565P	+	+	+
699	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/I285L/N290G/R305M/H307G/L407V/F450A/C503Q/Q521K/T524S/C565P	+	-	+
700	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/H307G/L364H/L407V/F450A/D470E/C503Q/T524S/C565P	+	+	+
701	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/F450A/D470E/Q521K/T524S/C565P	+	-	+
702	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/C503Q/Q521K/T524S	++	++	+
703	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	++	++	+

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	F1OP (T=0)	F1OP Protease	F1OP (T=7)
704	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	++	+	++
705	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/Q521K/T524S/C565P	+	++	+
706	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/R313H/L364H/F450A/C503Q/Q521K/T524S/C565P	-	-	-
707	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/L364H/L407V/D470E/C503Q/Q521K/T524S	+	+	+
708	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/L364H/L407V/F450A/C503Q/Q521K/T524S/C565P	+	+	+
709	F16S/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S98G/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/T524S/C565P	+	+	+
710	F16S/F18H/A39V/L47A/T54K/G59R/S73K/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
711	F16S/F18H/A39V/L47A/T54K/S209P/L214Q/L407V/C503Q/T524S/C565P	-	-	-
712	F16S/F18H/A39V/L47A/T54K/S73K/A91V/S209P/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	+
713	F16S/F18H/A39V/L47A/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K/C565P	+	+	+
714	F16S/F18H/A39V/L47A/T54K/S73K/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	++	+	++
715	F16S/F18H/A39V/R43G/N44K/G59R/S209P/C503Q/C565P	-	-	-
716	F16S/F18H/A39V/T54K/A91V/L214Q/I285L/N290G/L364H/F450A/D470E/C503Q/Q521K/T524S	+	-	+
717	F16S/F18H/A39V/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	+
718	F16S/F18H/A39V/T54K/G59R/S73K/L214Q/I285L/N290G/R305M/L364H/L407V/F450A/Q521K/T524S/C565P	+	-	+
719	F16S/F18H/A39V/T54K/G59R/S73K/S209P/L214Q/I285L/N290G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
720	F16S/F18H/A39V/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	+

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	F1OP (T=0)	F1OP Protease	F1OP (T=7)
721	F16S/F18H/A39V/T54K/S73K/A91V/S209P/P210S/I285L/N290G/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
722	F16S/F18H/A39V/T54K/S73K/S209P/I285L/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S/C565P	+	+	+
723	F16S/F18H/A39V/T54K/S73K/S98G/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S/C565P	+	+	+
724	F16S/F18H/A91V/L214Q/L407V/Q521K/T524S/C565P	-	-	-
725	F16S/F18H/A91V/S209P/L407V/C503Q	-	-	-
726	F16S/F18H/C503Q	+	-	-
727	F16S/F18H/D55H/S209P/I285L/R305M	-	-	-
728	F16S/F18H/G59R/S209P/F450A/C503Q/T524S	-	-	-
729	F16S/F18H/I285L/N290G/D470E	-	-	-
730	F16S/F18H/L214Q/L407V/F450A/C503Q	-	-	-
731	F16S/F18H/L47A/G59R/I285L/L407V/F450A/C565P	-	-	-
732	F16S/F18H/L47A/I285L/L364H/C503Q/Q521K	-	-	-
733	F16S/F18H/L47A/L364H/L407V	-	-	+
734	F16S/F18H/L47A/S209P	-	-	-
735	F16S/F18H/L47A/S73K/A91V/S209P/I285L/N290G/F450A/D470E/C503Q/Q521K/T524S	+	+	+
736	F16S/F18H/L47A/S73K/L214Q/H307G/C503Q	-	-	-
737	F16S/F18H/L47A/S73K/N290G/L407V/F450A	-	-	-
738	F16S/F18H/L47A/T54K/G59R/S73K/A91V/S98G/I285L/N290G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
739	F16S/F18H/L47A/T54K/G59R/S73K/S209P/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
740	F16S/F18H/L47A/T54K/G59R/S73K/S98G/S209P/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
741	F16S/F18H/L47A/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
742	F16S/F18H/S209P/F450A/C503Q	-	-	-
743	F16S/F18H/S23G/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	++	++	+
744	F16S/F18H/S73K/S209P/L214Q/F450A	+	-	-
745	F16S/F18H/T19I/L214Q/L407V	-	-	-
746	F16S/F18H/T54K/A91V/S209P/L214Q/I285L/N290G/H307G/L364H/L407V/F450A/C503Q/Q521K/T524S/C565P	+	-	+
747	F16S/F18H/T54K/G59R/N290G/L407V/F450A/C503Q/T524S	+	-	-
748	F16S/F18H/T54K/G59R/S73K/A91V/S209P/I285L/N290G/R305M/H307G/L364H/L407V/F450A/C503Q/Q521K	+	+	+

Table 15.1 Screening of Variants for PAL Activity				
Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	F1OP (T=0)	F1OP Protease	F1OP (T=7)
749	F16S/F18H/T54K/L407V	-	-	-
750	F16S/F18H/T54K/S73K/A91V/L214Q/I285L/N290G/L407V/D561G	-	-	-
751	F16S/F450A/D470E/T524S	+	-	-
752	F16S/G59R/L364H/L407V/F450A/T524S	-	-	-
753	F16S/G59R/S73K/A91V/M370Q/C503Q/T524S	-	-	-
754	F16S/G59R/S73K/C503Q/C565P	-	-	-
755	F16S/G59R/S73K/L407V/F450A/C503Q/C565P	-	-	-
756	F16S/L214Q/D470E	-	-	-
757	F16S/L214Q/L364H/D470E	+	-	+
758	F16S/L214Q/L407V/C565P	-	-	-
759	F16S/L364H/C503Q/C565F	-	-	-
760	F16S/L407V/D470E	-	-	-
761	F16S/L407V/T524S/C565P	-	-	-
762	F16S/L47A/A91V/L214Q/H307G/L407V/D470E/C503Q/C565P	-	-	-
763	F16S/L47A/F450A/D470E/C565P	-	-	-
764	F16S/L47A/F450A/Q521K	-	-	-
765	F16S/L47A/G59R/L364H/L407V/P523T	-	-	-
766	F16S/L47A/G59R/T524S	-	-	-
767	F16S/L47A/L407V/F450A/T524S	-	-	-
768	F16S/L47A/S209P/L214Q/L364H/L407V/D470E/C503Q/Q521K/C565P	-	-	-
769	F16S/L47A/S209P/L407V	-	-	-
770	F16S/L47A/S73K/A91V/L214Q/I285L/L407V/C565P	-	-	-
771	F16S/L47A/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
772	F16S/L47A/S73K/L214Q/R305M/L407V/C565P	-	-	-
773	F16S/L47A/T54K/A91V/I285L/N290G/R305M/H307G/L407V/F450A/C503Q/Q521K/T524S/C565P	+	++	+
774	F16S/L47A/T54K/A91V/S209P/D470E/T524S/C565P	-	-	-
775	F16S/L47A/T54K/G59R/A91V/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	-	-	-
776	F16S/L47A/T54K/G59R/S73K/A91V/S209P/I285L/N290G/H307G/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
777	F16S/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/F450A/D470E/C503Q/T524S/C565P	+	+	+
778	F16S/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
779	F16S/L47A/T54K/L214Q/N290G/L364H/L407V/C503Q/Q521K/T524S	+	-	+
780	F16S/L47A/T54K/S73K/A91V/S209P/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
781	F16S/N290G/R305M/L364H/L407V/D470E/A479T	-	-	-
782	F16S/S209P/L214Q/L407V/C503Q/T524S	-	-	+

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	F1OP (T=0)	F1OP Protease	F1OP (T=7)
783	F16S/S22G/L214Q/C503Q/T524S/C565P	-	+	-
784	F16S/S73K/A91V/S209P/D470E	-	-	-
785	F16S/S73K/L214Q/T524S	+	-	-
786	F16S/T54K/G59R/A91V/S209P/L364H/L407V/F450A/Q521K	-	-	-
787	F16S/T54K/G59R/L214Q/C565P	-	-	-
788	F16S/T54K/G59R/S209P/L214Q/I285L/F450A	-	-	-
789	F16S/T54K/G59R/S73K/A91V/N290G/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
790	F16S/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/H307G/F450A/D470E/C503Q/T524S	+	+	+
791	F16S/T54K/S73K/A91V/I285L/N290G/R305M/H307G/L407V/F450A/D470E/T524S	+	+	+
792	F16S/T54K/S73K/L407V/F450A/D470E/C503Q	+	-	-
793	F18H	-	-	-
794	F18H/A39V/D55N/L407V/D470E/C503Q	-	-	-
795	F18H/A39V/L214Q/L364H/F450A/D470E/Q521K/T524S	+	-	-
796	F18H/A39V/L214Q/L364H/L407V/D470E/C503Q	+	-	-
797	F18H/A39V/L47A/G59R/S73K/A91V/L214Q/I285L/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
798	F18H/A39V/L47A/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
799	F18H/A39V/L47A/G59R/S73K/S98G/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/T524S	+	+	+
800	F18H/A39V/L47A/S209P/L214Q/N290G/C565P	-	-	-
801	F18H/A39V/L47A/S73K/A91V/S209P/L214Q/N290G/R305M/H307G/L364H/F450A/D470E/C503Q	+	+	+
802	F18H/A39V/L47A/T54K/A91V/L214Q/I285L/N290G/R305M/H307G/L407V/F450A/C503Q/Q521K/T524S	+	++	+
803	F18H/A39V/L47A/T54K/G59R/A91V/S209P/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	+
804	F18H/A39V/L47A/T54K/G59R/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
805	F18H/A39V/L47A/T54K/G59R/A91V/S209P/L214Q/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S/C565P	+	++	+
806	F18H/A39V/L47A/T54K/G59R/A91V/S98G/S209P/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	+
807	F18H/A39V/L47A/T54K/G59R/L214Q/D470E/C503Q/Q521K/T524S	-	-	-
808	F18H/A39V/L47A/T54K/G59R/S209P/I285L/N290G/R305M/H307G/L407V/F450A/C503Q/Q521K/T524S	+	-	+

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	F1OP (T=0)	F1OP Protease	F1OP (T=7)
809	F18H/A39V/L47A/T54K/G59R/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/C565P	+	+	+
810	F18H/A39V/L47A/T54K/G59R/S73K/A91V/I285L/N290G/H307G/L364H/F450A/D470E/C503Q/T524S	+	-	+
811	F18H/A39V/L47A/T54K/G59R/S73K/A91V/I285L/N290G/R305M/H307G/L364H/L407V/F450A/C503Q/Q521K/T524S	+	+	+
812	F18H/A39V/L47A/T54K/G59R/S73K/A91V/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
813	F18H/A39V/L47A/T54K/G59R/S73K/A91V/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/T524S/C565P	+	++	+
814	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q	+	+	+
815	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
816	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/C503Q/Q521K/T524S	+	++	+
817	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S	+	++	++
818	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/C503Q/Q521K/T524S/C565P	+	-	+
819	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	+
820	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/T524S/C565P	+	+	+
821	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/Q521K/T524S/C565P	+	+	+
822	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	++	-	+
823	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/N290G/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
824	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/N290G/R305M/H307G/L407V/C503Q/T524S/C565P	+	++	+
825	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/N290G/H307G/L364H/L407V/D470E/C503Q/Q521K/C565P	+	-	+

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	FIOP (T=0)	FIOP Protease	FIOP (T=7)
826	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S98G/L214Q/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/T524S	+	-	+
827	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S98G/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
828	F18H/A39V/L47A/T54K/G59R/S73K/A91V/S98G/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/T524S	+	+	+
829	F18H/A39V/L47A/T54K/G59R/S73K/L214Q/I285L/N290G/R305M/L364H/L407V/F450A/D470E/C503Q/T524S	++	-	++
830	F18H/A39V/L47A/T54K/G59R/S73K/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/C503Q/Q521K/C565P	+	+	+
831	F18H/A39V/L47A/T54K/S73K/A91V/G154S/S209P/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
832	F18H/A39V/L47A/T54K/S73K/A91V/I285L/N290G/R305M/H307G/L364H/L407V/F450A/C503Q/Q521K/T524S	+	+	+
833	F18H/A39V/L47A/T54K/S73K/A91V/S209P/I285L/N290G/R305M/H307G/L364H/L407V/D470E/Q521K/T524S/C565P	+	+	+
834	F18H/A39V/L47A/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/H307G/L364H/L407V/F450A/D470E/C503Q/C565P	+	-	+
835	F18H/A39V/L47A/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
836	F18H/A39V/L47A/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/F450A/C503Q/Q521K/T524S/C565P	+	++	+
837	F18H/A39V/L47A/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	++	+	+
838	F18H/A39V/L47A/T54K/S73K/A91V/S209P/R305M/L364H/L407V/F450A/D470E/C503Q/Q521K	+	-	+
839	F18H/A39V/L47A/T54K/S73K/A91V/S98G/I285L/N290G/R305M/F450A/C503Q/Q521K/T524S/C565P	+	+	+
840	F18H/A39V/L47A/T54K/S73K/A91V/S98G/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
841	F18H/A39V/L47A/T54K/S73K/A91V/S98G/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S	+	+	+
842	F18H/A39V/S73K/A91V/L214Q/I285L/N290G/R305M/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	++
843	F18H/A39V/S73K/A91V/S209P/L214Q/I285L/N290G/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	F1OP (T=0)	F1OP Protease	F1OP (T=7)
844	F18H/A39V/T54K/G59R/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
845	F18H/A39V/T54K/G59R/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/T524S	+	-	+
846	F18H/A39V/T54K/G59R/S73K/A91V/I285L/N290G/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
847	F18H/A39V/T54K/G59R/S73K/A91V/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
848	F18H/A39V/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/H307G/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
849	F18H/A39V/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/H307G/L364H/L407V/D470E/C503Q/Q521K	+	+	+
850	F18H/A39V/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
851	F18H/A39V/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/C503Q/Q521K/T524S	+	-	+
852	F18H/A39V/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K	+	+	+
853	F18H/A39V/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	+
854	F18H/A39V/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
855	F18H/A39V/T54K/G59R/S73K/A91V/S98G/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/D470E/C503Q/Q521K/T524S/C565P	+	+	+
856	F18H/A39V/T54K/G59R/S73K/A91V/S98G/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/Q521K/T524S	-	-	-
857	F18H/A39V/T54K/G59R/S73K/I285L/N290G/H307G/L364H/D470E/C503Q/Q521K/T524S/C565P	+	+	+
858	F18H/A39V/T54K/S73K/A91V/S209P/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q	+	+	+
859	F18H/A39V/T54K/S73K/A91V/S98G/I285L/N290G/R305M/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
860	F18H/A39V/T54K/S73K/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/C503Q/Q521K/T524S	+	++	+
861	F18H/A91V/L214Q	-	-	-
862	F18H/A91V/L214Q/L407V/T524S/C565P	-	-	-
863	F18H/A91V/L364H/F450A/C565P	-	-	-
864	F18H/A91V/L364H/L407V	-	-	-

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	F1OP (T=0)	F1OP Protease	F1OP (T=7)
865	F18H/A91V/L364H/L407V/F450A	-	-	-
866	F18H/A91V/L407V/C503Q	-	-	-
867	F18H/A91V/L407V/D470E/Q521K/T524S	-	-	-
868	F18H/A91V/S209P/L364H/L407V	-	-	-
869	F18H/F450A/C503Q	-	-	-
870	F18H/G59R/A91V/C565P	-	-	-
871	F18H/G59R/A91V/S209P/L214Q/L364H	-	-	-
872	F18H/G59R/C503Q/T524S	-	-	-
873	F18H/G59R/L407V/F450A	-	-	-
874	F18H/G59R/R305M/L364H/D470E/C565P	-	-	-
875	F18H/G59R/S209P	-	-	-
876	F18H/L214Q/L364H/L407V/D470E/C503Q/T524S	+	-	-
877	F18H/L214Q/L407V	+	-	+
878	F18H/L364H/C503Q	-	-	-
879	F18H/L364H/F450A/Q521K/T524S/C565P	+	-	-
880	F18H/L407V/F450A/C503Q/C565P	-	-	-
881	F18H/L47A/A97T/S98G/I182F/S209P/L364H/L407V/C503Q	-	-	-
882	F18H/L47A/F450A/D470E	-	-	-
883	F18H/L47A/G59R/A97T/S98G/L407V/C503Q	-	-	-
884	F18H/L47A/G59R/L214Q/F450A/Q521K	+	-	-
885	F18H/L47A/G59R/L214Q/I285L/C565P	-	-	-
886	F18H/L47A/G59R/L214Q/N362Y/L407V	-	-	-
887	F18H/L47A/L214Q/I285L/L287Q/N290G/D470E/C503Q	+	-	+
888	F18H/L47A/L214Q/L407V/T524S/C565P	-	-	-
889	F18H/L47A/L364H/C503Q	-	-	-
890	F18H/L47A/L364H/C503Q/C565P	-	-	-
891	F18H/L47A/L364H/D470E	-	-	-
892	F18H/L47A/Q521K/C565P	-	-	-
893	F18H/L47A/S209P/L407V/F450A/C503Q	-	-	-
894	F18H/L47A/S209P/L407V/F450A/D470E/C503Q/Q521K	+	-	-
895	F18H/L47A/S209P/N290G/L364H/D470E	-	-	-
896	F18H/L47A/S73K/A91V/L364H/L407V/C503Q	-	-	-
897	F18H/L47A/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/H307G/L364H/L407V/Q485H/C503Q/T524S/C565P	+	+	+
898	F18H/L47A/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	+
899	F18H/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/N290G/R305M/L364H/L407V/F450A/C503Q/T524S	+	+	+
900	F18H/L47A/T54K/S73K/A91V/S209P/I285L/N290G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
901	F18H/L47A/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L407V/D470E/C503Q/Q521K/T524S	+	+	+
902	F18H/N290G/C565P	-	-	+
903	F18H/R43S/R305M/L364H/M416I/F450A	-	-	-
904	F18H/S209P	-	-	-
905	F18H/S209P/L364H/L407V/Q521K/C565P	-	-	-

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	FIOP (T=0)	FIOP Protease	FIOP (T=7)
906	F18H/S22G/A39V/A91V/L214Q/I285L/F450A/D470E/C503Q	-	-	-
907	F18H/S73K/A91V/I182F/L287P/L407V/F450A/C503Q	-	-	-
908	F18H/S73K/I285L/L364H/L407V/D470E	-	-	-
909	F18H/S73K/I285L/Q521K/T524S	-	-	-
910	F18H/S73K/L364H/F450A/C565P	-	-	-
911	F18H/S73K/L407V	-	-	-
912	F18H/T54K/G59R/S73K/A91V/I285L/N290G/R305M/H307G/L364H/L407V/D470E/C503Q/Q521K/T524S	+	+	+
913	F18H/T54K/S73K/L214Q/H307G	-	+	-
914	F18I	-	-	-
915	F450A/C503Q	-	-	-
916	F450A/T524S	+	-	+
917	G59R/A91V/L407V/F450A	-	-	-
918	G59R/C565P	-	-	-
919	G59R/F450A/C503Q	-	-	-
920	G59R/L214Q	-	-	-
921	G59R/L214Q/F450A/C503Q	+	-	-
922	G59R/L214Q/L364H/L407V/Q452R/C503Q	-	-	-
923	G59R/L407V/C503Q/Q521K/T524S	-	-	-
924	G59R/S209P/L364H/D470E/C503Q	-	-	-
925	G59R/S73K/N290G/L407V/C503Q/V554I	-	-	-
926	G59R/S73K/R305M/H307G/C503Q	-	-	-
927	L214Q/D470E/C503Q	-	-	-
928	L214Q/D470E/Q521K	-	-	-
929	L214Q/I285L/H307G/C503Q/C565P	-	-	-
930	L214Q/L407V/C503Q/C565P	-	-	-
931	L364H	-	-	-
932	L364H/D470E/C503Q/Q521K/T524S	+	-	-
933	L364H/L407V/C565P	-	-	-
934	L364H/L407V/K419N/F450A/D470E/Q521K/C565P	-	-	-
935	L407V	-	-	-
936	L407V/C503Q	-	-	-
937	L407V/F450A/C565P	-	-	-
938	L47A	-	-	-
939	L47A/D55N	-	-	-
940	L47A/G59R/A91V/C503Q/Q521K	-	-	-
941	L47A/G59R/A91V/L407V/D470E/C503Q/C565P	-	-	-
942	L47A/G59R/F450A/D470E/C503Q	-	-	-
943	L47A/G59R/S209P	-	-	-
944	L47A/I285L/N290G/L364H/C503Q/C565P	-	-	-
945	L47A/L214Q/L364H/C503Q	+	-	-
946	L47A/L214Q/L407V	-	-	-
947	L47A/L364H/L407V/D470E/Q521K/T524S	+	-	-
948	L47A/L364H/T524S/C565P	-	-	-
949	L47A/L407V	-	-	-
950	L47A/L407V/C565P	-	-	-
951	L47A/L407V/F450A/Q521K/T524S	-	-	-

Table 15.1 Screening of Variants for PAL Activity

Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	F10P (T=0)	F10P Protease	F10P (T=7)
952	L47A/R305M	-	-	-
953	L47A/S209P/C503Q/T524S/C565P	-	-	-
954	L47A/S209P/L214Q/R305M/H307G/L364H/C503Q/Q521K/T524S	-	+	-
955	L47A/S73K	-	-	-
956	L47A/S73K/A91V/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S	+	+	+
957	L47A/S73K/A91V/L214Q	-	-	-
958	L47A/S73K/A91V/N290G/L407V/M416I/F450A/C503Q/Q521K/T524S	-	-	-
959	L47A/S73K/L214Q/L364H/L407V	+	-	+
960	L47A/S73K/L214Q/R305M/H307G/L364H/C503Q/C565P	-	+	-
961	L47A/S73K/L214Q/R305M/L407V/C503Q/C565P	-	+	-
962	L47A/S73K/L407V/D470E/C503Q	+	-	+
963	L47A/S73K/S209P/L364H/F450A/Q521K	-	-	-
964	L47A/T524S/C565P	-	-	-
965	L47A/T54K/A91V/A97T/S209P/L364H/C503Q/C565P	-	-	-
966	L47A/T54K/G59R/S73K/A91V/I285L/N290G/Q311R/L364H/L407V/C503Q/Q521K/T524S/C565P	-	+	-
967	L47A/T54K/G59R/S73K/A91V/I285L/R305M/H307G/L407V/D470E/Q521K/T524S	+	++	+
968	L47A/T54K/G59R/S73K/A91V/S209P/I285L/N290G/R305M/H307G/L364H/L407V/F450A/C503Q/T524S/C565P	+	-	+
969	L47A/T54K/G59R/S73K/A91V/S209P/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	+	+
970	L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
971	L47A/T54K/G59R/S73K/S209P/I285L/N290G/L364H/L407V/L432P/F450A/D470E/C503Q/Q521K/T524S/C565P	-	+	-
972	L47A/T54K/G59R/S73K/S98G/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S	+	-	+
973	L47A/T54K/S73K/A91V/I285L/N290G/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S/C565P	+	-	+
974	L47A/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
975	N290G/D470E	-	-	-
976	Q15H/F18H/A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
977	R305M/L407V/Q521K	-	-	-
978	S209P	-	-	-
979	S209P/D470E	+	-	-
980	S209P/L214Q/H307G/L407V/F450A/C503Q	-	-	-
981	S209P/L214Q/L407V/F450A/D470E/C503Q	-	-	-

Table 15.1 Screening of Variants for PAL Activity				
Variant #	Amino Acid Sequence Changes (Relative to SEQ ID NO:2)	FIOF (T=0)	FIOF Protease	FIOF (T=7)
982	S209P/L214Q/R305M	-	-	-
983	S73K/A91V/L214Q/T524S/C565P	-	-	-
984	S73K/A91V/Q521K	-	-	-
985	T511/L407V/D470E/C503Q/T524S	-	-	-
986	T524S	-	-	-
987	T54K/C503Q	-	-	-
988	T54K/F450A	-	-	-
989	T54K/G59R/A91V/S209P/L407V	-	-	-
990	T54K/G59R/S73K/A91V/L214Q/I285L/N290G/L364H/L407V/D470E/C503Q/T524S/C565P	+	+	+
991	T54K/S209P/D470E/C503Q/T524S/C565P	-	-	-
992	T54K/S209P/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/C565P	+	+	+
993	T54K/S209P/L407V/F450A/D470E/C565P	-	-	-
994	T54K/S73K/A91V/L214Q/F450A/C503Q/Q521K/T524S	-	-	-
995	T54K/S73K/A91V/R305M/L364H/L407V/F450A/D470E/C503Q/Q521K	-	-	-
996	T54K/S73K/F450A	+	-	-
997	A39V/L47A/T54K/G59R/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	+	+
998	A39V/L47A/T54K/S73K/A91V/S209P/L214Q/I285L/N290G/R305M/H307G/L364H/L407V/F450A/D470E/C503Q/Q521K/T524S/C565P	+	++	+
999	A91V/L214Q	-	-	-
1000	F16S/A39V/L47A/T54K/G59R/S73K/A91V/L214Q/I285L/N290G/R305M/H307G/L364H/F450A/D470E/C503Q/Q521K/T524S	+	+	+

[0249] In the above Table, the activity fold improvement (FIOF) is compared to that of SEQ ID NO:12 (Variant #126), and defined as follows: “-” = less than 0.2-fold; “+” = greater than 0.2-fold, but less than 1-fold; “++” = greater than 1-fold, but less than 1.25-fold increased activity; and “+++” = greater than 1.25-fold activity.

[0250] While the invention has been described with reference to the specific embodiments, various changes can be made and equivalents can be substituted to adapt to a particular situation, material, composition of matter, process, process step or steps, thereby achieving benefits of the invention without departing from the scope of what is claimed.

[0251] For all purposes in the United States of America, each and every publication and patent document cited in this disclosure is incorporated herein by reference as if each such publication or document was specifically and individually indicated to be incorporated herein by reference. Citation of

publications and patent documents is not intended as an indication that any such document is pertinent prior art, nor does it constitute an admission as to its contents or date.

CLAIMS

We claim:

1. An engineered polypeptide comprising an amino acid sequence having at least 85% sequence identity to at least one of SEQ ID NO:4, 6, 8, 10, and/or 12, wherein the amino acid positions of said amino acid sequences are numbered with reference to the amino acid sequence of SEQ ID NO:2.
2. The engineered polypeptide of Claim 1, wherein the engineered polypeptide comprises at least one substitution or substitution set at one or more amino acid positions selected from 16, 16/150, 44/56, 44/56/102/239/285/469/470/495, 44/239, 44/239/285/469/495, 44/239/285/470, 44/239/469/470, 44/239/470/546, 44/239/495, 44/239/495/546, 44/469/470, 102, 102/470, 162, 165, 188, 239/285, 239/285/469, 239/469/470/495, 264, 267, 285/469/470/495, 285/470, 285/470/495, 364, 455, 469/470, 472, and 482, wherein the amino acid positions are numbered with reference to SEQ ID NO:6.
3. The engineered polypeptide of Claim 1, wherein the engineered polypeptide comprises at least one substitution at one or more amino acid positions selected from 16, 264, 364, 472, 482, and/or any combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:6.
4. The engineered polypeptide of Claim 1, wherein the engineered polypeptide comprises at least one substitution or substitution set at one or more amino acid positions selected from 44/47/204/209/285, 44/47/364/470/495, 44/54/56/204/239/285/364/495, 44/54/56/204/239/470/495, 44/54/285/470, 44/102/285/364, 44/204/209/285, 44/204/285/364/470/495, 44/209/285/460/495, 44/285/364, 47/54/209, 47/204/209/239/285/495, 47/204/285/364/495, 47/209/239/364, 47/239/285/364, 47/470, 54, 54/56, 54/56/204/209/470, 54/56/204/209/495, 54/56/204/495, 54/56/209/562, 54/56/285/364/470, 54/56/285/470, 54/165/204/209/239/285/470/495, 54/239/495, 54/285/470, 54/470, 56, 165, 204, 204/209/239/285/470/495, 204/209/364, 204/209/364/495, 204/239, 204/239/285, 204/364, 204/470, 209/285/364/470, 209/285/364/470/495, 209/364, 209/364/495, 209/470, 239/364, 285/364, 285/364/495, 364, 364/470, 470, and 495, and/or any combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:8.
5. The engineered polypeptide of Claim 1, wherein the engineered polypeptide comprises at least one substitution or substitution set at one or more amino acid positions selected from 16,

16/150, 162, 188, 264, 267, 398, 434, 472, and 482, and/or any combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:6.

6. The engineered polypeptide of Claim 1, wherein the engineered polypeptide comprises at least one substitution or substitution set at one or more amino acid positions selected from 54/56/162/204/285, 54/56/162/204/398, 54/56/204/285, 54/56/204/398, 54/56/204/398/472, 54/56/285, 54/56/398, 54/56/398/472, 54/162/204/398, 54/162/398, 54/204/285/398/472, 54/204/398, 54/285/398, 54/285/398/472, 56/162/398, 56/204/285, 56/204/398, 56/204/398/460, 56/204/398/472, 56/285, 56/285/398/472, 56/398, 56/398/472, and 201/204/398, and/or any combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:12.

7. The engineered polypeptide of Claim 1, wherein the engineered polypeptide comprises at least one substitution or substitution set at one or more amino acid positions selected from 16, 18, 39, 47, 54, 59, 73, 91, 209, 214, 285, 290, 305, 307, 364, 407, 450, 470, 503, 521, 524, and 565, and/or any combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:12.

8. The engineered polypeptide of Claim 1, wherein the engineered polypeptide comprises at least one substitution or substitution set at one or more amino acid positions selected from 15/18/39/47/54/59/73/91/209/214/285/290/307/364/407/450/470/503/521/524/565, 16/18/19/214/407, 16/18/23/47/54/59/73/91/209/214/285/290/305/307/364/407/450/470/503/521/524/565, 16/18/39/43/44/59/209/503/565, 16/18/39/45/47/54/59/73/98/209/214/285/290/305/364/407/450/470/503/521/524/565, 16/18/39/47/49/54/59/73/209/285/290/305/307/364/450/470/503/521/524/565, 16/18/39/47/54/59/73/91/98/285/290/305/307/364/450/470/503/524/565, 16/18/39/47/54/59/73/91/209/214/285/290/305/307/313/364/450/503/521/524/565, 16/18/39/47/54/59/73/91/209/214/285/290/305/307/364/407/450/470/503/521/524, 16/18/39/47/54/59/73/91/209/214/285/290/305/307/364/407/450/470/503/521/524/565, 16/18/39/47/54/59/73/91/209/214/285/290/305/307/364/407/450/470/521/524/565, 16/18/39/47/54/59/73/91/209/214/285/290/305/307/364/407/450/503/521/524, 16/18/39/47/54/59/73/91/209/214/285/290/305/364/407/450/503/521/524/565, 16/18/39/47/54/59/73/91/209/214/285/290/305/364/407/470/503/521/524, 16/18/39/47/54/59/73/91/209/214/285/290/307/364/407/450/470/503/524/565, 16/18/39/47/54/59/73/91/209/285/290/305/307/407/450/503/521/524/565, 16/18/39/47/54/59/73/91/209/285/290/307/364/407/470/503/521/524/565, 16/18/39/47/54/59/73/91/214/285/290/305/307/364/407/503,

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 364/470/503/521/524, 407, 407/450/565, 407/503, 450/503, 450/524, 503, and 524, and/or any
 combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:2.

9. An engineered phenylalanine ammonia lyase polypeptide comprising an amino acid sequence having at least 90% sequence identity to SEQ ID NO:2, 4, 6, 8, 10, and/or 12, or a functional fragment thereof.

10. An engineered phenylalanine ammonia lyase polypeptide, wherein said engineered phenylalanine ammonia lyase polypeptide comprises an engineered polypeptide provided in any of Claims 1-8.

11. The engineered phenylalanine ammonia lyase polypeptide of Claim 9 and/or 10,

wherein said engineered phenylalanine ammonia lyase polypeptide comprises an amino acid sequence having at least about 95% sequence identity to SEQ ID NO:2, 4, 6, 8, 10, and/or 12, or a functional fragment thereof.

12. The engineered phenylalanine ammonia lyase polypeptide of any of Claims 9-11, wherein said engineered phenylalanine ammonia lyase polypeptide is a variant phenylalanine ammonia lyase polypeptide provided in any of Table 6.1, Table 7.1, Table 8.1, Table 9.1, Table 10.1, Table 14.1, and/or Table 15.1.

13. The engineered phenylalanine ammonia lyase polypeptide of any of Claims 9-12, wherein said engineered polypeptide is an *Anabaena variabilis* variant enzyme.

14. The engineered phenylalanine ammonia lyase polypeptide of any of Claims 9-13, wherein said engineered polypeptide is more thermostable than wild-type *Anabaena variabilis* phenylalanine ammonia lyase.

15. The engineered phenylalanine ammonia lyase polypeptide of any of Claims 9-14, wherein said engineered polypeptide more resistant to proteolysis than wild-type *Anabaena variabilis* phenylalanine ammonia lyase.

16. The engineered phenylalanine ammonia lyase polypeptide of Claim 15, wherein said engineered phenylalanine ammonia lyase polypeptide is resistant to proteolysis by at least one digestive tract enzyme, than wild-type *Anabaena variabilis* phenylalanine ammonia lyase.

17. The engineered phenylalanine ammonia lyase polypeptide of Claim 15, wherein said engineered phenylalanine ammonia lyase polypeptide is more resistant to proteolysis by chymotrypsin, trypsin, carboxypeptidases, and/or elastases than wild-type *Anabaena variabilis* phenylalanine ammonia lyase.

18. The engineered phenylalanine ammonia lyase polypeptide of any of Claims 9-17, wherein said engineered polypeptide is more acid stable than wild-type *Anabaena variabilis* phenylalanine ammonia lyase.

19. The engineered polypeptide of any of Claims 1-8 or the engineered phenylalanine ammonia lyase polypeptide of any of Claims 9-18, wherein said polypeptide is purified.

20. An engineered polynucleotide sequence encoding at least one engineered polypeptide

of Claims 1-8, or 19, and/or a phenylalanine ammonia lyase polypeptide of any one of Claims 9-19.

21. The engineered polynucleotide sequence of Claim 20, wherein said sequence is operably linked to a control sequence.

22. The engineered polynucleotide sequence of Claim 20 and/or 21, wherein said polynucleotide is codon-optimized.

23. An expression vector comprising at least one engineered polynucleotide sequence of any of Claims 20-22, and at least one control sequence.

24. The expression vector of Claim 23, wherein said control sequence comprises a promoter.

25. The expression vector of Claim 24, wherein said promoter is a heterologous promoter.

26. A host cell transformed with at least one polynucleotide sequence of any of Claims 20-22, and/or the expression vector of any of Claims 23-25.

27. The host cell of Claim 26, wherein said host cell is *E. coli*.

28. A method of producing an engineered phenylalanine ammonia lyase polypeptide in a host cell comprising culturing a host cell comprising at least one polynucleotide encoding at least one engineered polypeptide and/or phenylalanine ammonia lyase polypeptide of any of Claims 1 to 19, and/ or at least one polynucleotide sequence of Claim 20-22, and/or at least one expression vector of any of Claim 23-25, under suitable culture conditions, such that at least one engineered phenylalanine ammonia lyase polypeptide is produced.

29. The method of Claim 28, further comprising recovering at least one engineered phenylalanine ammonia lyase polypeptide from the culture and/or host cells.

30. The method of Claim 29, further comprising the step of purifying said at least one engineered phenylalanine ammonia lyase polypeptide.

31. A composition comprising at least one engineered polypeptide and/or engineered phenylalanine ammonia lyase polypeptide of any of Claims 1 to 19.

32. A composition comprising at least one engineered polynucleotide of any of Claims 20-22.

33. The composition of Claim 31 and/or 32, wherein said composition is a pharmaceutical composition.

34. The composition of Claim 32 and/or 33, further comprising at least one pharmaceutically acceptable excipient and/or carrier.

35. The composition of any of Claims 31-34, wherein said composition is suitable for the treatment of phenylketonuria.

36. The composition of any of Claims 31-35, wherein said composition is suitable for the treatment of elevated blood phenylalanine.

37. The composition of any of Claims 31-36, wherein said composition is suitable for the treatment of tyrosinemia.

38. The composition of any of Claims 31-37, wherein said composition is suitable for the treatment of elevated blood tyrosine.

39. The composition of Claim 31, wherein said composition is suitable for use in gene therapy.

40. The composition of Claim 39, wherein said composition is suitable for use in gene therapy to treat phenylketonuria, elevated blood phenylalanine, tyrosinemia, and/or elevated blood tyrosinemia.

41. The composition of Claim 31, wherein said composition is suitable for use in mRNA therapy.

42. The composition of Claim 41, wherein said composition is suitable for use in mRNA therapy to treat phenylketonuria, elevated blood phenylalanine, tyrosinemia, and/or elevated blood tyrosinemia.

43. The composition of any of Claims 31-42, wherein said composition is suitable for oral administration to a human.

44. The composition of any of Claims 31-38, and/or 43, wherein said composition is in the form of a pill, tablet, capsule, gelcap, liquid, or emulsion.

45. The composition of Claim 44, wherein said pill, tablet, capsule, or gelcap further comprises an enteric coating.

46. The composition of any of Claims 31-43, wherein said composition is suitable for parenteral injection into a human.

47. The composition of any of Claims 31-46, wherein said composition is coadministered with at least one additional therapeutically effective compound.

48. The composition of Claim 47, wherein said composition comprises at least one additional therapeutically effective compound.

49. A method for treating and/or preventing the symptoms of phenylketonuria in a subject, comprising providing a subject having phenylketonuria, and providing the composition of any of Claims 31-48, to said subject.

50. The method of Claim 49, wherein the phenylalanine concentration in the blood of said subject is reduced upon providing said composition to said subject.

51. The method of Claim 49 and/or 50, wherein the cinnamic acid concentration in the blood of said subject is increased upon providing said composition to said subject.

52. The method of any of Claims 49-51, wherein said symptoms of phenylketonuria are ameliorated.

53. The method of any of Claims 49-52, wherein said subject is able to eat a diet that is less restricted in its phenylalanine content than diets required by subjects who have not been provided at least one pharmaceutical composition comprising at least one engineered polypeptide and/or phenylalanine ammonia lyase, as set forth in Claims 1-19.

54. The method of any of Claims 49-53, wherein said subject is an infant, child, young adult, or adult.

55. Use of the compositions provided in any of Claims 31-48.