CLAIMS

1. An engineered carboxyesterase comprising a polypeptide 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 SEQ ID NO: 2~~ or a functional fragment thereof~~, wherein said engineered carboxyesterase comprises ~~at least one~~ a substitution ~~or substitution set~~ in said polypeptide sequence at position 282, ~~and~~ wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 2, and wherein said engineered carboxyesterase exhibits greater enzymatic activity than wild-type *T. fusca* carboxyesterase of SEQ ID NO: 2.

2. The engineered carboxyesterase of Claim 1, wherein ~~said at least one substitution or substitution set in~~ said polypeptide sequence further comprises substitutions at positions selected from: 39, 39/323, 62, 62/117, 63, 64, 65, 66, 68, 69, 70, 71, 71/263, 77, 77/184, 103, 103/147, 104, 104/429, 105, 107, 107/185, 108, 109, 109/117, 110, 111, 113, 114, 115, 117, 118, 118/269, 118/349, 119, 126, 147, 153, 153/215, 164, 164/271, 174, ~~174/282,~~ 183, 184, 184/249, 185, 186, 187, 188, 190, 209, 210, 211, 212, 213, 213/271, 213/345, 214, 215, 215/271, 216, 217, 217/231, 224, 224/268/372, 231, 249, 249/284, 263, 268, 269, 270, 270/470, 271, 271/416, 276, 277, 278, 279, ~~279/280/282,~~ 280, 281, 281/374, ~~282,~~ 283, 283/429, 284, 284/438, 285, 286, 311, 317, 320, 320/323, 320/323/372, 320/372/376, 320/376/377, 321, 323, 324, 345, 349, 372, 372/376, 373, 374, 376, 377, 405, 416, 420, 427, 428, 429, 438, and 470, wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 2.

~~3. An engineered carboxyesterase comprising a polypeptide 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 SEQ ID NO: 8 or a functional fragment thereof, wherein said engineered carboxyesterase comprises at least one substitution or substitution set in said polypeptide sequence, wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 8.~~

~~4. The engineered carboxyesterase of Claim 3, wherein said at least one substitution or substitution set in said polypeptide sequence comprises substitutions at positions selected from: 63, 63/65/108, 63/65/108/189, 63/65/108/377, 63/65/282/285/320/323, 63/65/320/323, 63/108, 63/108/282/285/377, 63/108/285/377, 63/108/320/323, 63/189, 63/212/215, 63/212/215/268/269/343, 63/215, 63/215/269, 63/215/270/271, 63/215/343, 63/268/269/270/429, 63/377, 65/69/70/281/372, 65/69/70/372, 65/69/372, 65/70/372, 65/320, 65/320/323, 68, 68/69/189/214, 68/69/189/214/215, 68/69/189/214/215/271, 68/69/189/214/215/271/281/282/343/381, 68/69/189/214/271/280, 68/69/189/214/372, 68/69/189/214/377/381, 68/69/189/271, 68/69/189/271/280/372/381,~~

68/69/189/280/281/282/372/377, 68/69/189/281/282/372/377/381, 68/69/189/343/381, 68/69/189/372, 68/69/189/381, 68/69/214/215/271, 68/69/214/343, 68/69/215, 68/69/271, 68/69/282/287, 68/69/343/372, 68/108/377, 68/184, 68/184/189, 68/189/271/372, 68/189/343, 68/214/215/271/281/282/372, 68/215/271/343/372/381, 68/215/377, 68/271/372, 68/377, 69, 69/70, 69/70/331/372, 69/70/372, 69/70/459, 69/108, 69/108/270/372/377, 69/108/281/285, 69/110/215/281, 69/189, 69/189/214/271/281/282/343, 69/189/214/343/372, 69/189/215/343, 69/189/271, 69/189/271/281/282, 69/189/271/343, 69/189/271/343/381, 69/189/280/282/343/372/381, 69/189/281/373, 69/189/282, 69/189/343/381, 69/189/372, 69/189/372/377, 69/189/377, 69/212/213/215/280/281, 69/214/215/271/372/377/381, 69/214/271/282, 69/214/271/343, 69/215, 69/215/269/270/377, 69/215/271/280/281/282, 69/215/271/282, 69/215/271/372, 69/215/285/317, 69/215/323, 69/215/343, 69/215/343/372/381, 69/271, 69/271/372, 69/282, 69/282/343/372, 69/285/373, 69/372, 70, 70/212, 108, 108/189, 108/189/282/285/320, 108/189/320, 108/189/377, 108/215, 108/215/377, 108/269/270, 108/270, 108/282/285/377, 108/285, 108/320/323, 108/377, 126, 126/184/213/280/281/285/320, 126/184/213/372, 126/189/285/372, 126/215, 126/372, 181/215, 189, 189/214, 189/214/215/271/282, 189/215, 189/215/249/277, 189/215/271/281/282/377, 189/215/343/372, 189/270/285, 189/270/372, 189/280/282, 189/320/377, 189/343, 189/343/377, 189/372/377, 189/377, 189/381, 213/215/320, 214/215/271, 214/215/271/377, 214/271, 214/280/282/343/377/381, 215, 215/249/280/281/285/372, 215/271/372, 215/280/281/285/372, 215/281/285/372, 215/281/285/373, 215/281/373, 215/285, 215/285/317, 215/285/346, 215/285/445, 215/320, 215/320/372, 215/323, 215/372, 215/372/377, 215/373, 215/377, 215/381, 249/377, 269/270/281/372/377, 270/377, 271, 271/343, 271/343/372, 271/343/372/381, 280/285/372, 281/372, 282/285/320/323, 285/323, 320, 343/372, 372, 372/377, 372/381, 373, and 377, wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 8.

5. An engineered carboxyesterase comprising a polypeptide 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 SEQ ID NO: 138 or a functional fragment thereof, wherein said engineered carboxyesterase comprises at least one substitution or substitution set in said polypeptide sequence, and wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 138.

6. An engineered carboxyesterase comprising a polypeptide 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 SEQ ID NO: 140 or a functional fragment thereof, wherein said engineered carboxyesterase comprises at least one substitution or substitution set in said polypeptide sequence,

~~and wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 140.~~

~~7. The engineered carboxyesterase of Claim 1, wherein said engineered carboxyesterase comprises an amino acid substitution at position 282, wherein said position is numbered with reference to SEQ ID NO: 2.~~

~~8~~3. The engineered carboxyesterase of Claim ~~7~~1, wherein said amino acid at position 282 is ~~selected from: aliphatic, non-polar, basic,~~ a polar~~, and aromatic~~ amino acid~~s~~.

~~9~~4. The engineered carboxyesterase of Claim ~~7~~1, wherein said engineered carboxyesterase comprises an amino acid substitution selected from: ~~X282T, X282G, X282A, X282V, X282M, X282C, X282W,~~ X282Q, X282S, and X282T, ~~and X282R~~ and wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 2.

~~10~~5. The engineered carboxyesterase of Claim 1, wherein said polypeptide sequence is selected from: SEQ ID NO~~s~~: 4, 6, 8, ~~10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66,~~ 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132~~,~~ and 134~~, and 136~~.

~~11~~6. The engineered carboxyesterase of ~~any of~~ Claim~~s~~ 1~~-4 and 7-10~~, wherein said engineered carboxyesterase comprises ~~at least one improved property~~ improved amidation activity as compared to the wild-type *T. fusca* carboxyesterase of SEQ ID NO:2.

~~12. The engineered carboxyesterase of Claim 11, wherein said improved property is selected from: improved amidation activity, solvent tolerance, thermostability, pH stability, regioselectivity, stereoselectivity, substrate scope, and/or reduced substrate or product inhibition, and reduced toxicity to bacterial host cells producing said engineered carboxyesterase.~~

~~13. The engineered carboxyesterase of Claim 12, wherein said bacterial host cells comprise *E. coli*.~~

~~14. The engineered carboxyesterase of Claim 12, wherein said solvent is selected from: acetone, acetonitrile, toluene, tetrahydrofuran, isopropanol, isopropyl acetate, dimethyl sulfoxide, and methyl ethyl ketone.~~

~~15~~7. The engineered carboxyesterase of ~~any of~~ Claims ~~1-14~~, wherein said engineered carboxyesterase exhibits greater enzymatic activity than wild-type *T. fusca* carboxyesterase on ~~at least one~~ a substrate ~~selected from: aniline;~~ comprising isobutylamine~~; n-butylamine; t-butylamine; *N*'-t-butoxycarbonyl-benzhydrazide; 4-methylpiperidine; *O*-t-butylhdroxylamine; benzylamine; 2,6-dimethylaniline; (*S*)-(-)-α-methylbenzylamine; (*R*)-(+)-α-methylbenzylamine; methyl phenylacetate; ethyl acetate; ethyl benzoate; 2-pyrazinyl ethyl ester; 4-ethyl-*1H*-indole-ester; and *N*,*N*-diethylglycyl methyl ester~~.

~~16~~8. The engineered carboxyesterase of ~~any of~~ Claims ~~1-15~~, wherein said engineered carboxyesterase exhibits greater enzymatic activity than wild-type *T. fusca* carboxyesterase on ~~at least one substrate or~~ the substrate set ~~selected from: aniline;~~ comprising isobutylamine~~; n-butylamine; t-butylamine; *N*'-t-butoxycarbonyl-benzhydrazide; 4-methylpiperidine; *O*-t-butylhdroxylamine; benzylamine; 2,6-dimethylaniline; (*S*)-(-)-α-methylbenzylamine; (*R*)-(+)-α-methylbenzylamine; methyl phenylacetate; ethyl acetate;~~ and ethyl benzoate~~; 2-pyrazinyl ethyl ester; 4-ethyl-*1H*-indole-ester; and *N*,*N*-diethylglycyl methyl ester~~.

~~17~~9. The engineered carboxyesterase of ~~any of~~ Claims ~~1-16~~, wherein said engineered carboxyesterase exhibits greater enzymatic activity than wild-type *T. fusca* carboxyesterase in producing ~~at least one~~ a product ~~selected from: acetanilide; *N*-n-butyl-benzylacetamide; *N*-[(*S*)-1-phenylethyl]-pyrazinylamide; *N*-[(*S*)-1-phenylethyl]-benzamide; *N*-[(*R*)-1-phenylethyl]-benzamide; *N'*-t-butoxycarbonyl-benzhydrazide; 1-benzoyl-4-methylpiperidine; 2-pyrazinyl-4-methylpiperidine;~~ comprising *N*-isobutyl-benzamide~~; *N*-t-butyl-benzamide; *N*-t-butylhydroxyl-benzamide; *N*-isobutyl-*1H*-indol-4-amide; *N'*,*N'*-(diethylamino)-*N*-phenylacetamide; *N'*,*N'*-(diethylamino)-*N*-benzylacetamide; and *N'*,*N'*-(diethylamino)-*N*-2,6-dimethylphenylacetamide~~.

~~18~~10. The engineered carboxyesterase of Claim 1, further comprising at least one substitution selected from: ~~X~~L343V, ~~X~~L372L, ~~X~~L320W/G, ~~X~~L214R, ~~X282C, X~~L271Y~~,~~ and ~~X~~L65G, wherein said engineered carboxyesterase exhibits greater enzymatic activity than wild-type *T. fusca* carboxyesterase~~, on a hindered amine for formation of *N'*,*N'*-(diethylamino)-*N*-2,6-dimethylphenylacetamide from ethyl benzoate; and 2,6-dimethylaniline~~and wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 2.

~~19~~11. The engineered carboxyesterase of Claim 1, further comprising at least one substitution selected from: ~~X~~L268A, ~~X~~L63A/R, ~~X~~L189Q/I/E, ~~X~~L214R, ~~X282G/C, X~~L381L~~,~~ and ~~X~~L69W, wherein said engineered carboxyesterase exhibits greater enzymatic activity than wild-type

T. fusca carboxyesterase, ~~on a secondary amine for formation of 1-benzoyl-4-methyl-piperidine from ethyl benzoate and 4-methyl-piperidine~~and wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 2.

~~20~~12. The engineered carboxyesterase of ~~any of~~ Claim~~s~~ 1~~—19~~, wherein said engineered carboxyesterase is purified.

~~21~~13. The engineered carboxyesterase of ~~any of~~ Claim~~s~~ 1~~—20~~, wherein said engineered carboxyesterase is immobilized on a solid support.

~~22~~14. A composition comprising at least one engineered carboxyesterase of ~~any of~~ Claim~~s~~ 1~~-21~~.

~~23. A polynucleotide sequence encoding at least one engineered carboxyesterase of any of Claims 1-19.~~

~~24. A polynucleotide sequence encoding at least one engineered carboxyesterase comprising a polypeptide 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 SEQ ID NO: 2 or a functional fragment thereof, wherein said engineered carboxyesterase comprises at least one substitution or substitution set in said polypeptide sequence, wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 2.~~

~~25. The polynucleotide sequence of Claim 24, wherein said encoded at least one engineered carboxyesterase comprises at least one substitution or substitution set at positions selected from: 39, 39/323, 62, 62/117, 63, 64, 65, 66, 68, 69, 70, 71, 71/263, 77, 77/184, 103, 103/147, 104, 104/429, 105, 107, 107/185, 108, 109, 109/117, 110, 111, 113, 114, 115, 117, 118, 118/269, 118/349, 119, 126, 147, 153, 153/215, 164, 164/271, 174, 174/282, 183, 184, 184/249, 185, 186, 187, 188, 190, 209, 210, 211, 212, 213, 213/271, 213/345, 214, 215, 215/271, 216, 217, 217/231, 224, 224/268/372, 231, 249, 249/284, 263, 268, 269, 270, 270/470, 271, 271/416, 276, 277, 278, 279, 279/280/282, 280, 281, 281/374, 282, 283, 283/429, 284, 284/438, 285, 286, 311, 317, 320, 320/323, 320/323/372, 320/372/376, 320/376/377, 321, 323, 324, 345, 349, 372, 372/376, 373, 374, 376, 377, 405, 416, 420, 427, 428, 429, 438, and 470, wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 2.~~

~~26. A polynucleotide sequence encoding at least one engineered carboxyesterase comprising a polypeptide 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 SEQ ID NO: 8 or a functional fragment thereof, wherein said engineered carboxyesterase comprises at least one~~

substitution or substitution set in said polypeptide sequence, wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 8.

27. The polynucleotide sequence of Claim 26, wherein said encoded engineered carboxyesterase comprises at least one substitution or substitution set in said polypeptide sequence at positions selected from: 63, 63/65/108, 63/65/108/189, 63/65/108/377, 63/65/282/285/320/323, 63/65/320/323, 63/108, 63/108/282/285/377, 63/108/285/377, 63/108/320/323, 63/189, 63/212/215, 63/212/215/268/269/343, 63/215, 63/215/269, 63/215/270/271, 63/215/343, 63/268/269/270/429, 63/377, 65/69/70/281/372, 65/69/70/372, 65/69/372, 65/70/372, 65/320, 65/320/323, 68, 68/69/189/214, 68/69/189/214/215, 68/69/189/214/215/271, 68/69/189/214/215/271/281/282/343/381, 68/69/189/214/271/280, 68/69/189/214/372, 68/69/189/214/377/381, 68/69/189/271, 68/69/189/271/280/372/381, 68/69/189/280/281/282/372/377, 68/69/189/281/282/372/377/381, 68/69/189/343/381, 68/69/189/372, 68/69/189/381, 68/69/214/215/271, 68/69/214/343, 68/69/215, 68/69/271, 68/69/282/287, 68/69/343/372, 68/108/377, 68/184, 68/184/189, 68/189/271/372, 68/189/343, 68/214/215/271/281/282/372, 68/215/271/343/372/381, 68/215/377, 68/271/372, 68/377, 69, 69/70, 69/70/331/372, 69/70/372, 69/70/459, 69/108, 69/108/270/372/377, 69/108/281/285, 69/110/215/281, 69/189, 69/189/214/271/281/282/343, 69/189/214/343/372, 69/189/215/343, 69/189/271, 69/189/271/281/282, 69/189/271/343, 69/189/271/343/381, 69/189/280/282/343/372/381, 69/189/281/373, 69/189/282, 69/189/343/381, 69/189/372, 69/189/372/377, 69/189/377, 69/212/213/215/280/281, 69/214/215/271/372/377/381, 69/214/271/282, 69/214/271/343, 69/215, 69/215/269/270/377, 69/215/271/280/281/282, 69/215/271/282, 69/215/271/372, 69/215/285/317, 69/215/323, 69/215/343, 69/215/343/372/381, 69/271, 69/271/372, 69/282, 69/282/343/372, 69/285/373, 69/372, 70, 70/212, 108, 108/189, 108/189/282/285/320, 108/189/320, 108/189/377, 108/215, 108/215/377, 108/269/270, 108/270, 108/282/285/377, 108/285, 108/320/323, 108/377, 126, 126/184/213/280/281/285/320, 126/184/213/372, 126/189/285/372, 126/215, 126/372, 181/215, 189, 189/214, 189/214/215/271/282, 189/215, 189/215/249/277, 189/215/271/281/282/377, 189/215/343/372, 189/270/285, 189/270/372, 189/280/282, 189/320/377, 189/343, 189/343/377, 189/372/377, 189/377, 189/381, 213/215/320, 214/215/271, 214/215/271/377, 214/271, 214/280/282/343/377/381, 215, 215/249/280/281/285/372, 215/271/372, 215/280/281/285/372, 215/281/285/372, 215/281/285/373, 215/281/373, 215/285, 215/285/317, 215/285/346, 215/285/445, 215/320, 215/320/372, 215/323, 215/372, 215/372/377, 215/373, 215/377, 215/381, 249/377, 269/270/281/372/377, 270/377, 271, 271/343, 271/343/372, 271/343/372/381, 280/285/372, 281/372, 282/285/320/323, 285/323, 320, 343/372, 372, 372/377, 372/381, 373, and 377, wherein the amino acid positions of said polypeptide sequence are numbered with reference to SEQ ID NO: 8.

28. A polynucleotide sequence encoding at least one engineered carboxyesterase or a functional fragment thereof, said polynucleotide sequence comprising at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, sequence identity to SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131,133, 135, 137, and/or 139.

29. The polynucleotide sequence of any of Claims 23-28, wherein said polynucleotide sequence is operably linked to a control sequence.

30. The polynucleotide sequence of any of Claims 23-29, wherein said polynucleotide sequence is codon optimized.

31. An expression vector comprising at least one polynucleotide sequence of any of Claims 23-30.

32. A host cell comprising at least one expression vector of Claim 31.

33. A host cell comprising at least one polynucleotide sequence of any of Claims 23-30.

34. The host cell of Claim 32 and/or 33, wherein said host cell is *E. coli*.

35. A method of producing an engineered carboxyesterase in a host cell, comprising culturing the host cell of any of Claims 32-34, under suitable conditions, such that at least one engineered carboxyesterase is produced.

36. The method of Claim 35, further comprising recovering at least one engineered carboxyesterase from the culture and/or host cell.

37. The method of Claim 35 and/or 36, further comprising the step of purifying said at least one engineered carboxyesterase.

ABSTRACT

The present invention provides engineered carboxyesterase enzymes having improved properties as compared to a naturally occurring wild-type carboxyesterase enzymes, as well as polynucleotides encoding the engineered carboxyesterase enzymes, host cells capable of expressing the engineered carboxyesterase enzymes, and methods of using the engineered carboxyesterase enzymes in amidation reactions.

[NO FIGURE]