

CLAIMS

What is claimed is:

1. An engineered host cell for expressing a heterologous protein, said engineering host cell comprising at least three different expression cassettes integrated into the genome of the engineered host cell wherein;
 - a. a first expression cassette comprising a first promoter operably linked to a heterologous gene sequence encoding the heterologous protein
 - b. a second expression cassette comprising a second promoter operably linked to a heterologous gene sequence encoding the heterologous protein;
 - c. a third expression cassette comprising a third promoter operably linked to a helper factor sequence; and
 - d. a copy number ratio of the helper factor encoding sequence to the heterologous protein encoding sequence is at least 1:10.
2. An engineered host cell for expressing a heterologous protein, said engineering host cell comprising at least three different expression cassettes integrated into the genome of the engineered host cell wherein;
 - a. a first expression cassette comprising a first promoter operably linked to a heterologous gene sequence encoding the heterologous protein
 - b. a second expression cassette comprising a second promoter operably linked to a heterologous gene sequence encoding the heterologous protein;
 - c. a third expression cassette comprising a third promoter operably linked to a helper factor sequence; and
 - d. a copy number ratio of the helper factor encoding sequence to the heterologous protein encoding sequence is at most 1:2.
3. The engineered host cell of claims 1 or 2, wherein the copy number ratio of the helper factor encoding sequence to the heterologous protein encoding sequence is at least 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4 or 1:3.
4. The engineered host cell of claims 1 or 2, wherein the copy number ratio of the helper factor encoding sequence to the heterologous protein encoding sequence is at most 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3 or 1:2.

5. The engineered host cell of claims 1 or 2, wherein at least one promoter is an inducible promoter.
6. The engineered host cell of claims 1 or 2, wherein all of the promoters are inducible promoters.
7. The engineered host cell of claim 5 or claim 6, wherein the inducible promoter is a methanol inducible promoter.
8. The engineered host cell of claim 7, wherein each methanol inducible promoter is independently selected from the group consisting of AOX1, AOX2, DAK2, DAS2, FDH1, FGH1, FLD1, and PEX11 or a methanol inducible fragment thereof.
9. The engineered host cell of any one of claims 1 to 3, wherein at least one promoter is a constitutive promoter.
10. The engineered host cell of claim 9, wherein each constitutive promoter is independently selected from the group consisting of GAP and GCW14.
11. The engineered host cell of any one of claims 1 to 10, wherein the host cell comprises at least 2 copies of the first expression cassette.
12. The engineered host cell of any one of claims 1 to 11, wherein the host cell comprises at least 2 copies of the second expression cassette.
13. The engineered host cell of any one of claims 1 to claim 12, wherein the host cell comprises at least 1 copy of a fourth expression cassette comprising a fourth promoter operably linked to the heterologous gene sequence.
14. The engineered host cell of any one of claims 1 to 13, wherein the first cassette and the second cassette are integrated into the genome in the same 5' to 3' orientation.
15. The engineered host cell of any one of claims 1 to 13, wherein the first cassette and the second cassette are integrated into the genome in an opposite 5' to 3' orientation.
16. The engineered host cell of any one of the previous claims, wherein the host cell comprises at least 2 copies of the helper factor encoding sequence in 1 or 2 expression cassettes.
17. The engineered host cell of any one of the previous claims, wherein the host cell comprises at least 3, 4 or 5 copies of the helper factor encoding sequence in 1, 2, 3, 4, or 5 expression cassettes.
18. The engineered host cell of any one of the previous claims, wherein the host cell comprises at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 copies of the heterologous encoding sequence in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 expression cassettes.

19. The engineered host cell of any one of the previous claims, wherein the heterologous protein is a food-related protein.
20. The engineered host cell of claim 19, wherein the food-related protein comprises an enzyme, a nutritive protein, a food ingredient or a food additive.
21. The engineered host cell of claim 20, wherein the food-related protein is a pepsinogen protein.
22. The engineered host cell of claim 21, wherein a copy number ratio of the helper factor encoding sequence to pepsinogen encoding sequence is from 1:2 to 1:5.
23. The engineered host cell of claim 20, wherein the food-related protein comprises an egg-white protein.
24. The engineered host cell of claim 23, wherein the egg-white protein is ovomucoid.
25. The engineered host cell of claim 24, wherein a copy number ratio of the helper factor encoding sequence to ovomucoid encoding sequence is from 1:3 to 1:6.
26. The engineered host cell of claim 23, wherein the egg-white protein is ovalbumin.
27. The engineered host cell of claim 26, wherein a copy number ratio of the helper factor encoding sequence to ovalbumin encoding sequence is from 1:3 to 1:8.
28. The engineered host cell of any one of the previous claims, wherein the engineered host cell is capable of producing at least about 5 g per liter of the heterologous protein under fermentation conditions.
29. The engineered host cell of any one of the previous claims, wherein the engineered host cell is capable of producing at least about 10 g per liter of the heterologous protein under fermentation conditions.
30. The engineered host cell of any one of the previous claims, wherein the engineered host cell is capable of producing at least about 20 g per liter of the heterologous protein under fermentation conditions.
31. The engineered host cell of any one of the previous claims, wherein at least one of the expression cassettes comprises a secretion signal.
32. The engineered host cell of any one of the previous claims, wherein at least one of the expression cassettes comprises a terminator sequence.
33. The engineered host cell of any of the previous claims, wherein each of the helper factor gene sequences encodes for a protein independently selected from the group consisting of HAC1, Serine/threonine protein kinase 2 (Kin2), squalene synthase (ERG9), protein disulfide isomerase 1 (PDI1), SSA1, SSA4, SSB1, SSE1, BiP, ER Membrane Protein Complex Subunit

1 (EMC1), YNL181W oxidoreductase, integral membrane protein zinc metalloprotease Ste24, 14-3-3 protein Bmh2 and ER oxidoreductin 1 (Ero1).

34. The engineered host cell of any one of the previous claims, wherein the host cell is engineered to favor non-homologous integration over homologous integration and/or the host cell is selected based on a greater number of non-homologous integrations than homologous integrations.

35. The engineered host cell of any one of the previous claims, wherein at least two of the expression cassettes comprising the heterologous gene sequence integrate at different integration sites.

36. The engineered host cell of any of the previous claims, wherein the host cell is a yeast cell.

37. The engineered host cell of claim 36, wherein the yeast cell is *Pichia pastoris*.

38. The engineered host cell of claims 1-37, wherein the copy number is measured by sequencing the host cell genome.

39. A method of producing a recombinant heterologous protein in a host cell, the method comprising:

- a. transforming a first vehicle into the host cell; wherein said first vehicle comprises one or more first expression cassettes; wherein each of the first expression cassettes comprises at least a first promoter operably linked to a heterologous gene sequence encoding the heterologous protein;
- b. allowing random integration of the one or more first expression cassettes into the host cell;
- c. identifying the integration of the one or more first expression cassettes into the host cell;
- d. transforming a second vehicle into the host cell; wherein said second vehicle comprises one or more second expression cassettes; wherein each of the second expression cassettes comprises at least a second promoter operably linked to the heterologous gene sequence; wherein the second promoter is different from the first promoter; and
- e. allowing random integration of the one or more second expression cassettes into the host cell;

wherein the host cell is a yeast or filamentous fungi, and wherein the engineered cell is capable of producing at least 5 g per liter of the heterologous protein under fermentation conditions.

40. The method of claim 39, wherein step (d) further comprises transformation of a plurality of vehicles in addition to the second vehicle, wherein each of the plurality of vehicles comprise one or more expression cassettes each comprising one or more promoter driving expression of the heterologous gene sequence encoding the heterologous protein.

41. The method of claim 40, wherein the one or more promoters comprise the first promoter, the second promoter or a combination thereof.

42. The method of claim 40, wherein the one or more promoters comprise the first promoter, the second promoter, promoters other than the first or second promoter or a combination thereof.

43. The method of claim 39, wherein identifying the integration of the one or more first expression cassettes comprise sequencing a nucleic acid obtained from the host cell.

44. The method of claims 39-43, wherein identifying the integration of the one or more first expression cassettes comprises identifying the presence or absence of a resistance marker; wherein the first expression cassette or the first plasmid comprises a sequence encoding the resistance marker.

45. The method of any one of claims 39 to 44, wherein the heterologous protein is secreted into the medium during fermentation and wherein the heterologous recombinant protein is harvested from the fermentation media.

46. The method of any one of claims 39 to 45, wherein the first expression cassette and the second expression cassette are linear molecules, and where the first expression cassette and the second expression cassette comprise less than 700 bp at the 5' end with homology to a native host cell genomic locus.

47. The method of any one of claims 39 to 46, wherein the host cell is engineered to favor non-homologous integration over homologous integration and/or the host cell is selected based on a greater number of non-homologous integrations than homologous integrations.

48. The method of any one of claims 39 to 47, wherein the method further comprises transformation of a helper vehicle into the host cell; wherein said helper vehicle comprises one or more helper expression cassettes; wherein each of the helper expression cassettes comprises at least one promoter operably linked to a gene sequence encoding a helper factor protein.

49. The method of claim 48, wherein a promoter in the one or more helper expression cassettes is the same as the first or second promoter.
50. The method of claim 48, wherein a promoter in the one or more helper expression cassettes is different from the first or second promoter.
51. The method of any one of claims 39-50, wherein the vehicle is a plasmid.
52. The method of any one of claims 39-50, wherein the vehicle is a linearized plasmid.
53. A method of producing a recombinant heterologous protein in a host cell, the method comprising:
- a. transforming a plurality of plasmids into the host cell; wherein the plurality of plasmids comprises at least three plasmids, each one of the at least three plasmids comprising a different expression cassette; wherein each of the different expression cassettes comprises a different promoter operably linked to a heterologous gene sequence;
 - b. allowing the integration of at least one copy of each of the at least three different expression cassettes into the host cell;
- wherein at least one of the at least three expression cassettes comprises a promoter operably linked to a helper factor gene sequence;
- wherein a copy number ratio of the helper factor gene to the heterologous gene is at least 1:10.
54. A method of producing a recombinant heterologous protein in a host cell, the method comprising:
- a. transforming a plurality of plasmids into the host cell; wherein the plurality of plasmids comprises at least three plasmids, each one of the at least three plasmids comprising a different expression cassette; wherein each of the different expression cassettes comprises a different promoter operably linked to a heterologous gene sequence;
 - b. allowing the integration of at least one copy of each of the at least three different expression cassettes into the host cell;
- wherein at least one of the at least three expression cassettes comprises a promoter operably linked to a helper factor gene sequence;
- wherein a copy number ratio of the helper factor gene to the heterologous gene is at most 1:2.

55. The method of claims 53 or 54, wherein the copy number ratio of the helper factor encoding sequence to the heterologous protein encoding sequence is at least 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4 or 1:3.
56. The method of claims 53 or 54, wherein the copy number ratio of the helper factor encoding sequence to the heterologous protein encoding sequence is at most 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3 or 1:2.
57. The method of claims 53 or 54, wherein at least one promoter is an inducible promoter.
58. The method of claims 53 or 54, wherein all of the promoters are inducible promoters.
59. The method of claim 57 or claim 58, wherein the inducible promoter is a methanol inducible promoter.
60. The method of claim 59, wherein each methanol inducible promoter is independently selected from the group consisting of AOX1, AOX2, DAK2, DAS2, FDH1, FGH1, FLD1, and PEX11 or a methanol inducible fragment thereof.
61. The method of any one of claims 53 to 60, wherein at least one promoter is a constitutive promoter.
62. The method of claim 61, wherein each constitutive promoter is independently selected from the group consisting of GAP and GCW14.
63. The method of any one of claims 53 to 62, wherein the host cell comprises at least 2 copies of a first expression cassette.
64. The method of any one of claims 53 to 63, wherein the host cell comprises at least 2 copies of a second expression cassette.
65. The method of any one of claims 53 to 64, wherein the host cell comprises at least 1 copy of a third expression cassette comprising a third promoter operably linked to the heterologous gene sequence.
66. The method of any one of claims 53 to 65, wherein the host cell comprises at least 1 copy of a fourth expression cassette comprising a fourth promoter operably linked to the heterologous gene sequence.
67. The method of any one of claims 63 to 66, wherein the first cassette and the second cassette are integrated into the genome in the same 5' to 3' orientation.
68. The method of any one of claims 63 to 66, wherein the first cassette and the second cassette are integrated into the genome in an opposite 5' to 3' orientation.
69. The method of any one of claims 53-68, wherein the host cell comprises at least 2 copies of a helper factor encoding sequence in 1 or 2 expression cassettes.

70. The method of any one of claims 53-69, wherein the host cell comprises at least 3, 4 or 5 copies of the helper factor encoding sequence in 1, 2, 3, 4, or 5 expression cassettes.
71. The method of any one of claims 53-70, wherein the host cell comprises at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 copies of the heterologous encoding sequence in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 expression cassettes.
72. The method of any one of claims 53-72, wherein the heterologous protein is a food-related protein.
73. The method of claim 72, wherein the food-related protein comprises an enzyme, a nutritive protein, a food ingredient or a food additive.
74. The method of claim 73, wherein the food-related protein is a pepsinogen protein.
75. The method of claim 74, wherein a copy number ratio of the helper factor encoding sequence to pepsinogen encoding sequence is from 1:2 to 1:5.
76. The method of claim 72, wherein the food-related protein comprises an egg-white protein.
77. The method of claim 76, wherein the egg-white protein is ovomucoid.
78. The method of claim 77, wherein a copy number ratio of the helper factor encoding sequence to ovomucoid encoding sequence is from 1:3 to 1:6.
79. The method of claim 76, wherein the egg-white protein is ovalbumin.
80. The method of claim 79, wherein a copy number ratio of the helper factor encoding sequence to ovalbumin encoding sequence is from 1:3 to 1:8.
81. The method of any one of claims 53-80, wherein the engineered host cell is capable of producing at least about 5 g per liter of the heterologous protein under fermentation conditions.
82. The method of any one of claims 53-81, wherein the engineered host cell is capable of producing at least about 10 g per liter of the heterologous protein under fermentation conditions.
83. The method of any one of claims 53-82, wherein the engineered host cell is capable of producing at least about 20 g per liter of the heterologous protein under fermentation conditions.
84. The method of any one of claims 53-83, wherein at least one of the expression cassettes comprises a secretion signal.
85. The method of any one of claims 53-84, wherein at least one of the expression cassettes comprises a terminator sequence.

86. The method of any one of claims 53-85, wherein each of the helper factor gene sequences encodes for a protein independently selected from the group consisting of HAC1, Serine/threonine protein kinase 2 (Kin2), squalene synthase (ERG9), protein disulfide isomerase 1 (PDI1), SSA1, SSA4, SSB1, SSE1, BiP, ER Membrane Protein Complex Subunit 1 (EMC1), YNL181W oxidoreductase, integral membrane protein zinc metalloprotease Ste24, 14-3-3 protein Bmh2 and ER oxidoreductin 1 (Ero1).
87. The method of any one of claims 53-86, wherein the host cell is engineered to favor non-homologous integration over homologous integration and/or the host cell is selected based on a greater number of non-homologous integrations than homologous integrations.
88. The method of any one of claims 53-87, wherein at least two of the expression cassettes comprising the heterologous gene sequence integrate at different integration sites.
89. The method of any one of claims 53-88, wherein the host cell is a yeast cell.
90. The method of claim 89, wherein the yeast cell is *Pichia pastoris*.
91. The method of any one of claims 53 or 54, wherein the method further comprises identifying the integrated expression cassettes.
92. The method of claim 91, wherein the identifying comprises sequencing the host cell genome.
93. The method of claim 91, wherein the identifying comprises determining the presence or absence of promoters operably linked to the heterologous gene sequence.
94. The method of claim 93, wherein the method further comprises transforming at least one plasmid comprising one or more expression cassettes, wherein each of the expression cassettes comprise a promoter operably linked to the heterologous gene sequence, wherein the promoter was identified to be present in the host cell genome.
95. The method of claim 93, wherein the method further comprises transforming at least one plasmid comprising one or more expression cassettes, wherein each of the expression cassettes comprise a promoter operably linked to the heterologous gene sequence, wherein the promoter was identified to be absent in the host cell genome.