Specifically, the promoter is a CAT1 or AOD promoter.

Specifically, the promoter comprises the nucleotide sequence of any of SEQ ID NO:4, or SEQ ID NO:5, or a functional variant characterized by a sequence identity of at least 60% of any of SEQ ID NO:4, or SEQ ID NO:5, specifically at least any of 70%, 80%, 90%, or 95% sequence identity.

Specifically, the episomal plasmid comprises a selection marker that is based on auxotrophy or chemical resistance, preferably wherein the selection marker is based on glycerol utilization, sucrose utilization, inulin utilization, cellobiose utilization, amino acid auxotrophy, thymidine auxotrophy, nitrogen source utilization, resistance to fluoracetamide, resistance to deoxyglucose, resistance to Zeocin or other antibiotics, resistance to a gene encoding a toxin. The selection marker provides for cultivating a host cell that comprises the episomal plasmid described herein, under selective pressure or under selective conditions, e.g., to select those host cells or plasmid comprising the GOI.

Specifically, the selection marker confers resistance to zeocin, geneticin or glycerol utilization.

According to one aspect, the present disclosure provides an episomal plasmid comprising an expression cassette, which expression cassette comprises a gene of interest (GOI) and regulatory sequences required for expressing said GOI, and an autonomously replicating sequence (ARS) which is not operably linked to the GOI, which ARS comprises or consists of a nucleotide sequence identified as any of SEQ ID NO:5-11, or a functionally active variant of any of the foregoing which is characterized by at least 60% sequence identity thereto.

According to a specific aspect, a eukaryotic host cell is provided which comprises the episomal plasmid described herein.

Specifically, the host cell is a yeast cell, preferably of the Pichia genus, preferably of *Pichia pastoris*, which is a wild-type strain or any mutated strain capable of being cultivated in a cell culture. Specifically, the mutated strain is a ku70 deletion strain of *P. pastoris*, wherein the ku70 gene (SEQ ID NO:87, identified in Figure 33) or an analogous ku70 gene is inactivated or inhibited. Specifically, mutated *P. pastoris* strains which are deficient in the ku70 gene expression are preferred for biosynthesis and production of episomal plasminds and libraries of episomal plasmids as described herein.

Specifically, the method further comprises cultivating the selected host cell and producing a POI encoded by said GOI.

According to a further aspect, the present disclosure provides for a method of screening promoter variants from a repertoire of promoter variants, and selecting a promoter, the method comprising:

i. contacting a plurality of host cells comprising the library of episomal plasmids described herein, wherein the library comprises a repertoire of promoter variants and a reporter protein wherein any of the expression level of said reporter protein, transformation efficiency or clonal uniformity is a function of said promoter variants;

ii. determining changes in any of the expression level, transformation efficiency or clonal uniformity in individual host cells of said plurality;

iii. selecting a host cell which is characterized by the desired expression level, transformation efficiency or clonal uniformity; and

iv. identifying the promoter variant from the selected host cell.

According to a further aspect, the present disclosure provides for a method of screening variants of a POI encoded by a repertoire of GOI variants, the method comprising

i. contacting a plurality of host cells comprising the library of episomal plasmids described herein, wherein the library comprises a repertoire of GOI variants and wherein any of the GOI expression level, transformation efficiency or clonal uniformity is a function of GOI variants;

ii. determining changes in any of the expression level, transformation efficiency or clonal uniformity in individual host cells of said plurality;

iii. selecting a host cell which is characterized by the desired expression level, transformation efficiency or clonal uniformity; and

iv. identifying the GOI variant from the selected host cell.

According to a further aspect, the present disclosure provides for a method of biosynthesis of an episomal plasmid in a host cell which is of a KU70 deletion strain of *Pichia pastoris*, comprising

i. providing a linear vector backbone comprising recombination sites at its 5' and 3' ends, an ARS, wherein the ARS comprises or consists of a nucleotide sequence identified as any of SEQ ID NO:5-11, or a functionally active variant of any of the foregoing which is

Specifically, the variants are produced by mutagenesis of the vector inserts, in particular mutagenesis of the region of mutagenesis.

According to a further aspect, the present disclosure provides for a library of episomal plasmids obtained by the method described herein, which is characterized by a diversity of at least any of 10E2, 10E3, 10E4, 10E5, or 10E6 different variants. In certain embodiments, even a higher diversity can be produced, e.g. at least any of 10E7, 10E8, 10E9, 10E10, or 10E11 different variants.

Specifically, the library is incorporated into at least 10E6 transformed cells, preferably at least 10E7, 10E8, or 10E9 transformants. In certain embodiments, even a higher number of transformants can be produced, such as to cover the repertoire of episomal plasmid variants.

Specifically, the transformed cells are of the Pichia genus, preferably *P. pastoris*, or a KU70 deletion strain of *P. pastoris*.

According to the present disclosure, the GOI is encoding an antibody, an antibody fragment or antigen-binding sequence thereof, such as a scFv, Fab, VH and/or VL, a single CDR sequence or a set of CDR sequences, e.g. 3 CDR sequences. Specifically, GOI variants are produced by mutagenesis of one or more variable regions or antigen-binding sequences, such as to produce a repertoire of antigen-binding molecules which differ in at least one of epitope specificity or affinity of binding. Alternatively, GOI variants are produced by mutagenesis of one or more constant or framework sequences, such as to improve the stability or Fc function of the antigen-binding molecules.

Specifically, an antibody library is provided, which is characterized by the repertoire of GOI variants, each encoding an antibody or antigen-binding sequence thereof.

Specifically, the library is expressing a repertoire of POI variants, each representing an antibody or antigen-binding sequence thereof. The library is suitably expressed by in vitro, ex vivo or in vivo expression systems, e.g. in a suitable host cell culture. Upon expression, the POI variants can be screened for selecting a POI variant with desired binding or other features.

Claims

1. An episomal plasmid comprising an expression cassette, which expression cassette comprises a gene of interest (GOI) and regulatory sequences required for expressing said GOI, and an autonomously replicating sequence (ARS) which is not operably linked to the GOI, which ARS comprises or consists of a nucleotide sequence identified as any of SEQ ID NO:5-11, or a functionally active variant of any of the foregoing which is characterized by at least 60% sequence identity thereto.

2. The episomal plasmid of claim 1, which further comprises a selection marker and optionally one or more regulatory sequences.

3. The episomal plasmid of any one of the claims 1 to 2, which comprises a promoter which is operably linked to the GOI.

4. The episomal plasmid of claim 3, wherein the promoter is a regulatable or constitutive promoter, preferably a promoter selected from the group consisting of AOX1, GAP, AOD, AOX2, DAS1, DAS2, ENO1, FLD1, FMD, GPM1, HSP82, ICL1, ILV5, KAR2, KEX2, PET9, PEX8, PGK1, PHO89/NSP, SSA4, TEF1, THI11, TPI1, YPT1, GTH1, GCW14, and GUT1, which promoter is preferably of *Pichia pastoris*, or a functional variant of any of the foregoing which is characterized by at least 60% sequence identity and functional as a promoter in a *P. pastoris* strain.

5. The episomal plasmid of any one of claims 1 to 4, which comprises a selection marker that is based on auxotrophy or chemical resistance, preferably wherein the selection marker is based on glycerol utilization, sucrose utilization, inulin utilization, cellobiose utilization, amino acid auxotrophy, thymidine auxotrophy, nitrogen source utilization, resistance to fluoracetamide, resistance to deoxyglucose, resistance to Zeocin or other antibiotics, resistance to a gene encoding a toxin.

6. A eukaryotic host cell comprising the episomal plasmid of any one of claims 1 to 5.

7. The host cell of claim 6, wherein the host cell is a yeast cell, preferably of the Pichia genus, preferably of a *Pichia pastoris* strain, which is a wild-type strain or any mutated strain capable of being cultivated in a cell culture.

8. The host cell of claim 6 or 7 in a cell culture wherein the host cells are characterized by a genomic stability regarding the content of the episomal plasmid during at least 20 generations.

9. A method of producing a protein of interest (POI) that is encoded by a GOI by cultivating the host cell of any of claims 6 to 8 under conditions to express said GOI.

10. The method of claim 9, wherein the POI expression is increased at least 1.5 fold, preferably at least 3 fold, more preferably at least 5 fold or at least 10 fold, compared to the POI expression employing genomic integration of the comparable expression cassette expressing said GOI, which does not contain a functional ARS.

11. The method of claim 9 or 10, wherein transformation efficiency of the episomal plasmid is increased at least 20 fold or at least 50 fold, preferably at least 100 fold, at least 200 fold, or at least 300 fold, more preferably at least 500 fold compared to transformation employing genomic integration of the comparable expression cassette expressing said GOI, which does not contain a functional ARS.

12. A library of episomal plasmids of any one of claims 1 to 5, which comprises a repertoire of promoter variants and/or a repertoire of GOI variants, which are optionally coexpressed in a host cell culture.

13. A method of selecting a host cell for a desired yield of a GOI expression, the method comprising:

i. contacting a plurality of host cells comprising the library of episomal plasmids of claim 12, wherein the library comprises a repertoire of promoter variants and wherein the expression level of said GOI is a function of said promoter variants;
ii. determining the expression level in individual host cells of said plurality; and
iii. selecting a host cell which is characterized by the desired expression level of said GOI.

14. The method of claim 13, which further comprises cultivating the selected host cell and producing a POI encoded by said GOI.

15. A method of screening promoter variants from a repertoire of promoter variants, and selecting a promoter, the method comprising:

i. contacting a plurality of host cells comprising the library of episomal plasmids of claim 12, wherein the library comprises a repertoire of promoter variants and a reporter protein wherein any of the expression level of said reporter protein, transformation efficiency or clonal uniformity is a function of said promoter variants;

ii. determining changes in any of the expression level, transformation efficiency or clonal uniformity in individual host cells of said plurality;

iii. selecting a host cell which is characterized by the desired expression level, transformation efficiency or clonal uniformity; and

iv. identifying the promoter variant from the selected host cell.

16. A method of screening variants of a POI encoded by a repertoire of GOI variants, the method comprising

i. contacting a plurality of host cells comprising the library of episomal plasmids of claim 12, wherein the library comprises a repertoire of GOI variants and wherein any of the GOI expression level, transformation efficiency or clonal uniformity is a function of GOI variants;

ii. determining changes in any of the expression level, transformation efficiency or clonal uniformity in individual host cells of said plurality;

iii. selecting a host cell which is characterized by the desired expression level, transformation efficiency or clonal uniformity; and

iv. identifying the GOI variant from the selected host cell.

17. A method of biosynthesis of an episomal plasmid in a host cell which is of a KU70 deletion strain of *Pichia pastoris*, comprising

i. providing a linear vector backbone comprising recombination sites at its 5' and 3' ends, an ARS, wherein the ARS comprises or consists of a nucleotide sequence identified as any of SEQ ID NO:5-11, or a functionally active variant of any of the foregoing which is characterized by at least 60% sequence identity thereto, and optionally further regulatory sequences;

ii. providing a vector insert comprising a GOI and 5' and 3' homologous sequences which are homologous to said recombination sites;

iii. introducing said linear vector backbone and said insert into said host cell and recombining said vector insert with said recombination sites by homologous recombination, thereby producing the episomal plasmid comprising said GOI.

18. The method of claim 17, wherein said linear vector backbone and said insert are introduced into said host cell at a molar ratio between 1: 1 and 1:10.

19. The method of any one of claims 17 to 18, wherein the vector backbone further comprises a selection marker, which is based on auxotrophy or chemical resistance, preferably wherein the selection marker is based on glycerol utilization, sucrose utilization, inulin utilization, cellobiose utilization, amino acid auxotrophy, thymidine auxotrophy, nitrogen source utilization, resistance to fluoracetamide, resistance to deoxyglucose, resistance to Zeocin or other antibiotics, resistance to a gene encoding a toxin.

20. The method of any one of claims 17 to 19, wherein the 5' and 3' homologous sequences each comprise or consist of at least any one of 30, 50, 70, 100, 300 base pairs.

21. Use of the method of any of claim 17 to 20 for producing an episomal plasmid of any of claims 1 to 5.

22. An episomal plasmid obtained by any one of the methods of claim 17 to 20.

23. A method of producing a library of episomal plasmids using the method of any of claims 17 to 20, wherein the library comprises a repertoire of plasmid variants which differ in at least one point mutation in a predefined region of the GOI.

24. The method of claim 23, wherein the variants are produced by mutagenesis of the vector inserts.

25. A library of episomal plasmids obtained by the method of claim 23 or 24, which is characterized by a diversity of at least any of 10E2, 10E3, 10E4, 10E5, or 10E6 different variants.

26. The library of claim 25, which is incorporated into at least 10E6 transformed cells, preferably at least 10E7, 10E8, or 10E9 transformants.

27. The library of claim 26, wherein the transformed cells are of the Pichia genus, preferably *P. pastoris*, or a KU70 deletion strain of *P. pastoris*.

28. The library of any of claims 25 to 27, wherein the GOI is encoding an antibody, an antibody fragment, or antigen-binding sequence thereof.