with little effort and high success rates and does not require extensive transformation combined with high throughput screening of state of the art *P. pastoris* strain transformants.

## Summary

[0008] It is an objective of the present disclosure to provide improved means of producing recombinant proteins in yeast strains. It is a specific objective of the present disclosure to provide improved yeast strains of the genus *Komagataella*, which allow production of recombinant proteins in high yields.

[0009] The objective is solved by the subject matter of the present disclosure.

[0010] According to the disclosure there is provided a genetically modified *Komagataella phaffii* yeast cell for expression of a Protein or Polypeptide of Interest (POI), comprising in its genome a recombinant nucleic acid sequence encoding a POI, and a genetic modification in the open reading frame at position 949930 on chromosome 1 (genbank LT962476.1), position 65654 on chromosome 2 (genbank LT962477.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1) and/or position 1491140 on chromosome 4 (genbank LT962479.1).

[0011] Specifically, said genetic modification is an inactivating modification.

[0011a] In one aspect, the present disclosure refers to a genetically modified Komagataella phaffii yeast cell for expression of a Protein or Polypeptide of Interest (POI), comprising in its genome a recombinant nucleic acid sequence encoding a POI, and a genetic modification in the open reading frame at any one or more of position 949930 on chromosome 1 (genbank LT962476.1), position 65654 on chromosome 2 (genbank LT962477.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1) and/or position 1491140 on chromosome 4 (genbank LT962479.1),
wherein said genetic modification is an inactivating modification.

[0012] Specifically, the yeast cells provided herein comprise a genetic modification in any one or all of the open reading frames at position 949930 on chromosome 1 (genbank LT962476.1), position 65654 on chromosome 2 (genbank LT962477.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1) and/or position 1491140 on chromosome 4 (genbank LT962479.1). Specifically, the yeast cells described herein comprise a genetic modification in at least 1, 2, 3, or 4 or all 5 of said reading frames.

[0013] In a specific example, the genetically modified yeast cell described herein comprises a genetic modification around, *e.g.* within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 100, 200, 300, 400 or 500 nucleotides upstream or downstream, position 65654 on chromosome 2 (genbank LT962477.1), position 949930 on chromosome 1 (genbank LT962476.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1) or position 1491140 on chromosome 4 (genbank LT962479.1).

[0014] In a further specific example, the genetically modified yeast cell described herein comprises a genetic modification in the gene at any one or more

or all of the positions selected from the group consisting of position 65654 on chromosome 2 (genbank LT962477.1), position 949930 on chromosome 1 (genbank LT962476.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1) and position 1491140 on chromosome 4 (genbank LT962479.1).

[0015] In a further specific example, the genetically modified yeast cell described herein comprises a genetic modification at any one or more or all of the positions selected from the group consisting of position 65654 on chromosome 2 (genbank LT962477.1), position 949930 on chromosome 1 (genbank LT962476.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1) and position 1491140 on chromosome 4 (genbank LT962479.1).

[0016] Specifically, the yeast cell described herein has a genetic modification as described herein, specifically an inactivating mutation, in an endogenous gene selected from the group consisting of ALG9 (SEQ ID NO:14), SRB8 (SEQ ID NO:15), ACIB2EUKG772803 (SEQ ID NO:16), KAP123 (SEQ ID NO:17) and FLO400 (SEQ ID NO:18).

[0017] Specifically, the yeast cell described herein has a genetic modification as described herein, specifically an inactivating mutation, in an endogenous gene encoding a protein selected from the group consisting of ALG9 (SEQ ID NO:21), SRB8 (SEQ ID NO:20), ACIB2EUKG772803 (SEQ ID NO:22), KAP123 (SEQ ID NO:23) and FLO400 (SEQ ID NO:24). Specifically, said genetic modification prevents expression of functional ALG9 (SEQ ID NO:21), SRB8 (SEQ ID NO:20), ACIB2EUKG772803 (SEQ ID NO:22), KAP123 (SEQ ID NO:23) and/or FLO400 (SEQ ID NO:24).

[0018] Specifically, the genetic modification is a deletion and/or insertion of one or more bases, and/or a fusion of a chromosomal DNA sequence with a sequence of another chromosome.

[0019] In a specific example, the genetic modification is a fusion of chromosomal sequences. Specifically, it is a fusion of at least two chromosomes. Specifically, it is a fusion of a DNA sequence at or around, *e.g.* within about 10, 50 or 100 bases, of any one of the positions described herein with a DNA sequence of a different chromosome.

[0020] According to a specific example, it is a fusion between chromosome 1 and chromosome 4 of *K. phaffii*. Specifically, a fusion of the chromosomal sequence at or around, *e.g.* within about 10, 50 or 100 bases, position 1323758 of chromosome 1 (genbank LT962476.1) and the chromosomal sequence at or around, *e.g.* within about 10, 50 or 100 bases, position 1491140 of chromosome 4 (genbank LT962479.1).

[0021] In a specific example, the genetic modification is a knock-out, specifically of a part of the gene or of the whole gene.

[0022] In a specific example, the genetic modification is at least one point mutation.

[0023] In a specific example, the genetic modification is a modification, specifically an inactivating mutation, in the *ALG9* gene, the *SRB8* gene, and/or the ACIB2EUKG772803 gene. Specifically, the genetic modification is a modification preventing expression of a functional protein from the *ALG9* gene, the *SRB8* gene, and/or the ACIB2EUKG772803 gene.

[0024] In a specific example, the genetic modification is caused by a genomic rearrangement within a chromosome or exchange of DNA sequences between different chromosomes.

[0025] In a further specific example, the genetic modification is a deletion of 1 or more bases. Specifically, it is a deletion of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400 or 500 nucleotides or more.

[0026] In yet a further specific example, the deletion is a deletion of at least 50%, preferably at least 90%, of the gene at position 65654 on chromosome 2 (genbank LT962477.1), position 949930 on chromosome 1 (genbank LT962476.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1) or position 1491140 on chromosome 4 (genbank LT962479.1).

[0027] In a specific example, the genetic modification is an insertion or replacement of 1 or more bases. Specifically, it is an insertion or replacement of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400 or 500 nucleotides or more.

[0028] In a further specific example, the genetic modification is integration of the recombinant nucleic acid sequence encoding the POI.

[0029] In a specific example, the sequence encoding the POI is comprised in an expression cassette, preferably comprising the following functional regions:

a. a promoter active in yeast of the genus *Komagataella*,
b. the nucleic acid sequence encoding the POI, operably linked to said promoter,
c. transcription termination sequences, and optionally
d. a selection marker, preferably an antibiotics resistance gene or carbon source utilization marker.

[0030] In another aspect, the present disclosure refers to a method of producing a recombinant Protein or Polypeptide of Interest (POI) comprising the steps of:

a. providing the genetically modified yeast cell described herein,
b. cultivating said genetically modified yeast cell in a culture medium under conditions that allow for expression of the POI, and
c. isolating the POI from the cells or the culture medium.

[0031] Further provided herein is a genetically modified *Komagataella phaffi* yeast cell for expression of a variety of Proteins or Polypeptides of Interest (POIs), comprising in its genome

a. a landing pad, comprising an empty expression cassette comprising target sequences for homologous recombination, and optionally any one or more of a selection marker or reporter protein, a stuffer fragment, a promoter 5' of said stuffer fragment, and a transcription terminator 3' of said stuffer fragment; and
b. a genetic modification in the open reading frame at position 949930 on chromosome 1 (genbank LT962476.1), position 65654 on chromosome 2 (genbank LT962477.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1), and/or position 1491140 on chromosome 4 (genbank LT962479.1).

[0031a] In one aspect, the present disclosure refers to a genetically modified *Komagataella phaffi* yeast cell for expression of a variety of Proteins or Polypeptides of Interest (POIs), comprising in its genome

a. a landing pad, comprising an empty expression cassette comprising target sequences for homologous recombination, and optionally any one or more of a selection marker, a stuffer fragment, a promoter 5' of said stuffer fragment, and a transcription terminator 3' of said stuffer fragment; and
b. a genetic modification in the open reading frame at position 949930 on chromosome 1 (genbank LT962476.1), position 65654 on chromosome 2 (genbank LT962477.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1), and/or position 1491140 on chromosome 4 (genbank LT962479.1).

[0032] Specifically, the yeast cells comprising a landing pad provided herein for expression of a variety of POIs comprise a genetic modification in any one or all of the open reading frames, or genes, at position 949930 on chromosome 1 (genbank LT962476.1), position 65654 on chromosome 2 (genbank LT962477.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1) and/or position 1491140 on chromosome 4

(genbank LT962479.1). Specifically, the yeast cells described herein comprise a genetic modification in at least 1, 2, 3, or 4 or all 5 of said reading frames.

[0033] In a specific example, the yeast cells provided herein for expression of a variety of POIs comprises a genetic modification around, *e.g.* within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides upstream or downstream, position 65654 on chromosome 2 (genbank LT962477.1), position 949930 on chromosome 1 (genbank LT962476.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1) or position 1491140 on chromosome 4 (genbank LT962479.1).

[0034] In a further specific example, the yeast cells comprising a landing pad provided herein for expression of a variety of POIs comprises a genetic modification at position 65654 on chromosome 2 (genbank LT962477.1), position 949930 on chromosome 1 (genbank LT962476.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1) or position 1491140 on chromosome 4 (genbank LT962479.1).

[0035] Specifically, the genetic modification is a deletion and/or insertion of one or more bases.

[0036] In a specific example, the genetic modification is at least one point mutation.

[0037] In a further specific example, the genetic modification is a deletion of 1 or more bases. Specifically, it is a deletion of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400 or 500 nucleotides or more.

[0038] In yet a further specific example, the deletion is a deletion of at least 50%, preferably at least 90%, of the gene at position 65654 on chromosome 2 (genbank LT962477.1), position 949930 on chromosome 1 (genbank LT962476.1), position 1303485 on chromosome 4 (genbank LT962479.1), position 1323758 on chromosome 1 (genbank LT962476.1) or position 1491140 on chromosome 4 (genbank LT962479.1).

[0039] In a specific example, the genetic modification is an insertion or replacement of 1 or more bases. Specifically, it is an insertion or replacement of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400 or 500 nucleotides or more.

[0040] In a further specific example, the genetic modification is integration of the landing pad.

[0041] In a specific example, the promoter comprised in the landing pad is a PDF or PDC promoter.

[0042] In a specific example, the promoter comprised in the landing pad is a DAS1, DAS2, AOX1 or GAP (*e.g.* Qin et al, 2011) promoter. Further preferred promoters are for example the promoters as published by Vogl and Glieder 2013 and Vogl et al. 2016.

[0043] In a specific example, the promoter comprised in the landing pad is a GCW14 (Liang et al. 2013), UPP (US20160097053A1) or pCS1 (US9150870B2) promoter.

[0044] In a specific example, the promoter comprised in the landing pad is a bidirectional promoter.

[0045] In a further specific example, the transcription terminator comprised in the landing pad is a pUC origin genetic element.

[0046] Specifically, the stuffer fragment has a length of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400 or 500 nucleotides or more.

[0047] Further provided herein is a method of producing a recombinant Protein or Polypeptide of Interest (POI) comprising the steps of:

a. providing the genetically modified yeast cell comprising a landing pad as described herein,
b. replacing the stuffer fragment with a nucleic acid sequence encoding a POI, preferably using homologous recombination, or integrating the POI as an insertion at one of the homologous sequences
c. cultivating said genetically modified yeast cells in a culture medium under conditions that allow for expression of the POI, and
d. isolating the POI from the cells or the culture medium.

[0047a] In one aspect, the present disclosure refers to a method of producing a recombinant Protein or Polypeptide of Interest (POI) comprising the steps of:

a. providing a genetically modified yeast cell described herein,
b. replacing the stuffer fragment with a nucleic acid sequence encoding a POI, preferably using homologous recombination,
c. cultivating said genetically modified yeast cell in a culture medium under conditions that allow for expression of the POI, and
d. isolating the POI from the cells or the culture medium.

[0048] In one aspect, the present disclosure refers to use of the yeast cell described herein, for producing a recombinant protein or polypeptide of interest (POI). Further provided herein is the use of the genetically modified yeast cells described herein, for producing a recombinant protein or polypeptide of interest (POI). In one aspect, the present disclosure refers to use the yeast cell described herein, for producing a variety of POIs. Specifically, the genetically modified yeast strains described herein are used for the production of a variety of different POIs.