METHOD FOR GENERATING A GENETICALLY MODIFIED MICROBE

[0001] This application claims benefit of U.S. Provisional Application No. 61/183,031, filed June 1, 2009, which is hereby incorporated by reference in its entirety.

1. FIELD OF THE INVENTION

[0002] The methods and compositions provided herein generally relate to the industrial use of microorganisms such as yeast. Provided herein are genetically modified microorganisms that are modified to significantly reduce the risk of their dissemination in nature, and in particular, dissemination in nature of their recombinant DNA sequences. Also provided are methods for making and using such genetically modified microorganisms.

2. BACKGROUND

[0003] Advances in recombinant DNA technology have allowed for the production of industrially useful substances in large amounts through the utilization of prokaryotes or eukaryotes. Among eukaryotes, yeasts (in particular, yeasts belonging to the genus *Saccharomyces*), have been widely used for the production of fermented products. Generally, yeasts can grow rapidly and can be cultivated at higher density as compared with bacteria, and do not require an aseptic environment in the industrial setting. Furthermore, yeast cells can be easily separated from culture medium as compared with bacteria, greatly simplifying the process for product extraction and purification. Because of these characteristics, yeasts (in particular, genetically modified yeasts harboring recombinant DNA sequences) have been employed as hosts for the production of useful products, and the utility of such yeasts has been established. However, the use of genetically modified yeasts in industry carries a potential environmental risk, because the dispersal of such yeasts, and/or the recombinant DNA sequences contained in such yeasts, may have unpredictable consequences on the ecosystem. Thus, there exists a need for yeasts that are suitable for industrial applications but pose a reduced risk of being disseminated and propagated in nature, and in particular, pose a reduced risk of disseminating their recombinant DNA sequences in nature.

3. SUMMARY OF THE INVENTION

[0004] ~~Provided~~Described herein are methods for generating genetically modified microorganisms, *e.g.*, genetically modified yeast strains, that are sporulation and/or endogenous mating impaired. The methods ~~provided~~described herein comprise functionally disrupting one or more sporulation genes and/or one or more pheromone response genes in genetically

modified haploid microbial cells, *e.g.*, yeast cells, and inducing said genetically modified haploid microbial cells to form stable diploids that are effectively sexually sterile and constrained to the diploid state of their life cycle due to their lack of sporulation and/or mating capability. Microorganisms, *e.g.*, yeast strains, genetically modified in accordance with the methods provided herein find use in industrial applications, *e.g.*, industrial fermentation applications, and can provide the advantage of posing a significantly reduced risk of being disseminated and propagated in nature through mating with wild-type microorganisms.

[0004a] In a first aspect, the present invention refers to a genetically modified yeast cell comprising:

(a) a functional disruption in at least one sporulation gene;

(b) a functional disruption in at least one pheromone response gene; and

(c) a chromosomally integrated heterologous nucleotide sequence encoding a protein of interest;

wherein said genetically modified diploid yeast cell is sporulation impaired and endogenous mating impaired.

[0004b] In a second aspect, the present invention refers to a genetically modified heterothallic (*ho*) haploid yeast cell comprising:

(a) a functional disruption in at least one sporulation gene;

(b) a functional disruption in at least one pheromone response gene; and

(c) a chromosomally integrated heterologous nucleotide sequence encoding a protein of interest;

wherein said genetically modified heterothallic (*ho*) haploid yeast cell is sporulation impaired and endogenous mating impaired.

[0005] In one exampleaspect, disclosedprovided herein is a genetically modified yeast cell comprising: a functional disruption in one or more sporulation genes, wherein said yeast cell lacks sporulation capability as a result of said disruption of the one or more sporulation genes; a functional disruption in one or more pheromone response genes, wherein said yeast cell lacks endogenous mating capability as a result of said disruption of the one or more pheromone response genes; and one or more integrated heterologous nucleotide sequences of interest. In some embodimentsexamples, the genetically modified yeast cell is heterothallic (*ho*). In some embodimentsexamples, the genetically modified yeast cell is a diploid cell in which both copies of one or more sporulation genes and/or both copies of one or more pheromone response genes are functionally disrupted. In some embodimentsexamples, the genetically modified diploid cell is homozygous other than for its mating type allele. In some embodimentsexamples, the genetically modified yeast cell is a haploid cell. In some embodimentsexamples, the genetically modified haploid cell further comprises a recombinant plasmid encoding a homothallism (HO) protein. In some embodimentsexamples, the genetically modified haploid cell comprises one or more recombinant plasmids encoding the one or more pheromone response genes that is functionally disrupted in said yeast cell.

[0006] In some embodimentsexamples, the genetically modified yeast cell useful for the practice of the methods provided herein is a *Saccharomyces cerevisiae* cell. In some embodimentsexamples, the *Saccharomyces cerevisiae* cell is of the Baker's yeast, Mauri, Santa Fe, CBS 7959, CBS 7960, CBS 7961, CBS 7962, CBS 7963, CBS 7964, IZ-1904, TA, BG-1, CR-1, SA-1, M-26, Y-904, PE-2, PE-5, VR-1, BR-1, BR-2, ME-2, VR-2, MA-3, MA-4, CAT-1, CB-1, NR-1, BT-1 or AL-1 strain. In particular embodimentsexamples, the *Saccharomyces cerevisiae* cell is of the PE-2 strain. In other particular embodimentsexamples, the *Saccharomyces cerevisiae* cell is of the CAT-1 strain.

[0007] In some embodimentsexamples, the one or more sporulation genes disrupted in the genetically modified yeast cell is selected from the group consisting of *IME1, IME2, NDT80,*

SPO11, SPO20, AMA1, HOP2, and *SPO21*. In particular ~~embodiments~~examples, the *IME1* gene is disrupted. In some ~~embodiments~~examples, the one or more pheromone response genes disrupted in the genetically modified yeast cell is selected from the group consisting of *STE5, STE4, STE18, STE12, STE7,* and *STE11*. In particular ~~embodiments~~examples, the *STE5* gene is disrupted. In particular ~~embodiments~~examples, the genetically modified yeast cell comprises a functional disruption of both the *STE5* and *IME1* gene. In some ~~embodiments~~examples, a haploid cell of the genetically modified yeast cell comprises a functional disruption of the *STE5* gene and a recombinant plasmid encoding a *STE5* protein.

[0007a] In a third aspect, the present invention refers to a method of generating a genetically modified diploid yeast cell that is sporulation and endogenous mating impaired, the method comprising:

(a) transforming each of a first and a second genetically modified haploid yeast cell with at least one plasmid encoding a protein capable of complementing an endogenous mating impairment of said first and second genetically modified haploid yeast cells,

wherein said first genetically modified haploid yeast cell is sporulation and endogenous mating impaired and comprises a chromosomally integrated heterologous nucleotide sequence encoding a protein of interest, and wherein said second genetically modified haploid yeast cell is sporulation and endogenous mating impaired, is of the opposite mating type as the first genetically modified haploid yeast cell, and comprises a chromosomally integrated heterologous nucleotide sequence encoding said protein of interest;

(b) mating the first genetically modified haploid yeast cell with the second genetically modified haploid yeast cell, thereby forming a genetically modified diploid yeast cell; and,

(c) eliminating the one or more plasmids from the genetically modified diploid yeast cell,

wherein the resulting genetically modified diploid yeast cell is sporulation and endogenous mating impaired and comprises two copies of a chromosomally integrated heterologous nucleotide sequence encoding said protein of interest;

wherein the first genetically modified haploid yeast cell and the second genetically modified haploid yeast cell are endogenous mating impaired due to a functional disruption of at least one pheromone response gene.

[0008] In another exampleaspect, provided herein is a method for generating a diploid yeast strain having impaired sporulation and mating capabilities. In some embodimentsexamples, the method comprises: (a) obtaining a first genetically modified haploid yeast cell, wherein the first genetically modified haploid yeast cell is sporulation and endogenous mating impaired and comprises a chromosomally integrated heterologous nucleotide sequence encoding a protein of interest; (b) obtaining a second genetically modified haploid yeast cell, wherein the second genetically modified haploid yeast cell is sporulation and endogenous mating impaired, of the opposite mating type as the first genetically modified haploid yeast cell, and comprises a chromosomally integrated heterologous nucleotide sequence encoding said protein of interest; (c) transforming each of the first and second genetically modified haploid yeast cells with one or more plasmids encoding a protein capable of complementing the endogenous mating impairment of said first and second genetically modified haploid yeast cells; (d) mating the first genetically modified haploid yeast cell with the second genetically modified haploid yeast cell, thereby forming a genetically modified diploid yeast cell; and (e) eliminating the one or more plasmids from the genetically modified diploid yeast cell, wherein the resulting genetically modified diploid yeast cell is sporulation and endogenous mating impaired.

[0009] In some embodimentsexamples, the first genetically modified haploid yeast cell and the second genetically modified haploid yeast cell are endogenous mating impaired due to a functional disruption of one or more pheromone response genes. In some such embodimentsexamples, step (c) comprises transforming each of the first and second genetically modified haploid yeast cells with one or more plasmids encoding a functional copy of the pheromone response gene that is functionally disrupted in said first and second genetically modified haploid yeast cells. In some embodimentsexamples, the one or more pheromone response genes is selected from the group consisting of *STE5, STE4, STE18, STE12, STE7,* and *STE11*. In certain embodimentsexamples,

the first genetically modified haploid yeast cell and the second genetically modified haploid yeast cell are endogenous mating impaired due to a functional disruption of the *STE5* gene.

[0010] In some ~~embodiments~~examples, the first genetically modified haploid yeast cell and the second genetically modified haploid yeast cell is sporulation impaired due to a functional disruption of one or more sporulation genes. In some such ~~embodiments~~examples, the one or more sporulation genes is selected from the group consisting of *IME1, IME2, NDT80, SPO11, SPO20, AMA1, HOP2,* and *SPO21*. In some ~~embodiments~~examples, the first genetically modified haploid yeast cell and the second genetically modified haploid yeast cell is sporulation impaired due to a functional disruption of the *IME1* gene. In particular ~~embodiments~~examples, the first genetically modified haploid yeast cell and the second genetically modified haploid yeast cell are mating impaired due to a functional disruption of the *STE5* gene, and are sporulation impaired due to a functional disruption of the *IME1* gene.

[0011] In some ~~embodiments~~examples, the second genetically modified haploid yeast cell is obtained by inducing a mating type switch in a population of the first genetically modified haploid yeast cell. In some such ~~embodiments~~examples, the first genetically modified haploid yeast cell is heterothallic (ho), and said population is induced to switch mating type by transforming the genetically modified haploid yeast cell with a recombinant plasmid encoding a homothallism (HO) protein, wherein expression of the HO protein induces a mating type switch of the genetically modified haploid yeast cell.

[0012] In other ~~embodiments~~examples, the second genetically modified haploid yeast cell is obtained by changing the mating type locus in the first genetically modified haploid yeast cell using recombinant DNA technology. In some ~~embodiments~~examples, the first genetically modified haploid yeast cell is transformed with an integration construct that comprises as an integrating sequence a nucleotide sequence that encodes a mating type other than the mating type of the first genetically modified haploid yeast cell, flanked by homologous sequences that are homologous to nucleotide sequences that flank the mating type locus in the first genetically modified haploid yeast cell. Upon integration of the integrating sequence via homologous recombination the mating type locus of the first genetically modified haploid yeast cell is replaced by the mating type locus encoded by the inserting sequence, resulting in the generation of the second genetically modified haploid yeast cell. In some ~~embodiments~~examples, the integration construct is used to switch the mating type of the first genetically modified haploid yeast cell from **a** to alpha using an integration construct encoding encoding the alpha mating type (MAT alpha). In some ~~embodiments~~examples, the integration construct comprises SEQ ID NO: 155. In other ~~embodiments~~examples, the integration construct is used to switch the mating type of

the first genetically modified haploid yeast cell from alpha to a using an integration construct encoding encoding the a mating type (MAT A). In some ~~embodiments~~examples, the integration construct comprises SEQ ID NO: 156.

[0012a] In a fourth aspect, the present invention refers to a sporulation and endogenous mating impaired diploid yeast cell generated by the method as defined above.

[0012b] In a fifth aspect, the present invention refers to a method for generating a sporulation and endogenous mating impaired genetically modified heterothallic (*ho*) diploid yeast cell, the method comprising:

(a) transforming a population of a first genetically modified heterothallic haploid yeast cell with a plasmid encoding a functional homothallism (HO) protein to yield a first genetically modified haploid yeast cell, wherein expression of the HO protein is capable of inducing a mating-type switch of the first genetically modified haploid yeast cell, whereby a second genetically modified haploid yeast cell of the opposite mating type as the first genetically modified haploid yeast cell is obtained,

wherein the first genetically modified heterothallic haploid yeast cell comprises a chromosomally integrated heterologous nucleotide sequence encoding a protein of interest and functional disruptions in the *STE5* gene and the *IME1* gene;

(b) transforming each of the first and the second genetically modified haploid yeast cells with a plasmid encoding a *STE5* protein, whereby said transforming results in mating of the first genetically modified haploid yeast cell with the second genetically modified haploid yeast cell, thereby forming a genetically modified diploid yeast cell; and,

(c) eliminating any plasmids from the genetically modified diploid yeast cell to yield a genetically modified heterothallic diploid yeast cell,

wherein the resulting genetically modified heterothallic diploid yeast cell is sporulation and endogenous mating impaired and comprises two copies of a chromosomally integrated heterologous nucleotide sequence encoding said protein of interest.

[0012c] In a sixth aspect, the present invention refers to a sporulation and endogenous mating impaired heterothallic (*ho*) diploid yeast cell generated by the method as defined above.

[0013] In another exampleaspect, describedprovided herein is a method for generating a sporulation and endogenous mating impaired heterothallic (*ho*) diploid yeast cell, the method comprising: (a) obtaining a first genetically modified heterothallic haploid yeast cell, wherein the first genetically modified heterothallic haploid yeast cell comprises: (i) a chromosomally integrated heterologous nucleotide sequence encoding a protein of interest; and (ii) functional disruptions in the *STE5* gene and the *IME1* gene; (b) transforming a population of the first genetically modified heterothallic haploid yeast cell with a plasmid comprising a polynucleotide encoding a homothallism (HO) protein, wherein expression of the HO protein induces a mating-type switch of the first genetically modified heterothallic haploid yeast cell, whereby a second genetically modified heterothallic haploid yeast cell is obtained, wherein the second genetically modified haploid yeast cell is of the opposite mating type as the first genetically modified haploid yeast cell, and comprises: (i) a chromosomally integrated heterologous nucleotide sequence encoding said protein of interest; and (ii) functional disruptions in the *STE5* gene and the *IME1* gene; (c) transforming each of the first and second genetically modified heterolthallic haploid yeast cells with a plasmid encoding a *STE5* gene; (d) mating the first genetically modified haploid yeast cell with the second genetically modified haploid yeast cell, thereby forming a genetically modified diploid yeast cell; and (e) eliminating any plasmids from the genetically modified diploid yeast cell, wherein the resulting genetically modified heterothallic diploid yeast cell is sporulation and endogenous mating impaired.

[0014] Also describedprovided herein is a genetically modified heterothallic (*ho*) yeast cell that lacks sporulation and endogenous mating capability generated by the present methods.

[0015] In a seventh aspect, the present invention refers toAlso provided herein is a *MATα*/**a** *ste5*/*ste5ime1*/*ime1* yeast cell.

4. BRIEF DESCRIPTION OF THE FIGURES

[0016] FIG. 1 provides the structure of the Phase I disruption construct and of the target locus after integration of the disrupting sequence by homologous recombination.

[0017] FIG. 2 provides the structure of the Phase II disruption construct and of the target locus after integration of the disrupting sequence by homologous recombination.

[0018] FIG. 3 provides the structure of the Phase III disruption construct and of the target locus after integration of the disrupting sequence by homologous recombination.

~~AMENDED~~ CLAIMS ~~(clean version)~~

1. A genetically modified diploid yeast cell comprising:

(a) a functional disruption in at least one sporulation gene;

(b) a functional disruption in at least one pheromone response gene; and

(c) a chromosomally integrated heterologous nucleotide sequence encoding a protein of interest;

wherein said genetically modified diploid yeast cell is sporulation impaired and endogenous mating impaired.

2. The genetically modified yeast cell of claim 1 that is heterothallic (*ho*).

3. The genetically modified diploid yeast cell of claim 2 that is homozygous other than for its mating type allele.

4. A genetically modified heterothallic (*ho*) haploid yeast cell comprising:

(a) a functional disruption in at least one sporulation gene;

(b) a functional disruption in at least one pheromone response gene; and

(c) a chromosomally integrated heterologous nucleotide sequence encoding a protein of interest;

wherein said genetically modified heterothallic (*ho*) haploid yeast cell is sporulation impaired and endogenous mating impaired.

5. The genetically modified haploid yeast cell of claim 4, further comprising a recombinant plasmid encoding a functional homothallism (HO) protein.

6. The genetically modified haploid yeast cell of claim 5, further comprising at least one recombinant plasmid encoding a functional copy of the at least one pheromone

response gene that is functionally disrupted in said genetically modified haploid yeast cell.

7. The genetically modified yeast cell of any one of claims 1-6 that is a *Saccharomyces cerevisiae* cell.

8. The genetically modified yeast cell of claim 7, wherein the *Saccharomyces cerevisiae* cell is of the Baker's yeast, Mauri, Santa Fe, IZ-1904, TA, BG-1, CR-1, SA-1, M-26, Y-904, PE-2, PE-5, VR-1, BR-1, BR-2, ME-2, VR-2, MA-3, MA-4, CAT-1, CB-1, NR-1, BT-1 or AL-1 strain.

9. The genetically modified yeast cell of claim 7, wherein the *Saccharomyces cerevisiae* cell is of the PE-2 strain.

10. The genetically modified yeast cell of claim 7, wherein the *Saccharomyces cerevisiae* cell is of the CAT-1 strain.

11. The genetically modified yeast cell of claim 1 or 4, wherein said at least one sporulation gene is selected from the group consisting of *IME1, IME2, NDT80, SPO11, SPO20, AMA1, HOP2,* and *SPO21*.

12. The genetically modified yeast cell of claim 1 or 4, wherein said at least one pheromone response gene is selected from the group consisting of *STE5, STE4, STE18, STE12, STE7,* and *STE11*.

13. The genetically modified yeast cell of claim 11 comprising a functional disruption of the *IME1* gene.

14. The genetically modified yeast cell of claim 12 comprising a functional disruption of the *STE5* gene.

15. The genetically modified yeast cell of claim 1 or 4 comprising a functional disruption of the *IME1* gene and the *STE5* gene.

16. The genetically modified haploid yeast cell of claim 6 that comprises a functional disruption of the *STE5* gene and a recombinant plasmid encoding a functional *STE5* protein.

17. A method of generating a genetically modified diploid yeast cell that is sporulation and endogenous mating impaired, the method comprising:

(a) transforming each of a first and a second genetically modified haploid yeast cell with at least one plasmid encoding a protein capable of complementing an endogenous mating impairment of said first and second genetically modified haploid yeast cells,

wherein said first genetically modified haploid yeast cell is sporulation and endogenous mating impaired and comprises a chromosomally integrated heterologous nucleotide sequence encoding a protein of interest, and wherein said second genetically modified haploid yeast cell is sporulation and endogenous mating impaired, is of the opposite mating type as the first genetically modified haploid yeast cell, and comprises a chromosomally integrated heterologous nucleotide sequence encoding said protein of interest;

(b) mating the first genetically modified haploid yeast cell with the second genetically modified haploid yeast cell, thereby forming a genetically modified diploid yeast cell; and,

(c) eliminating the one or more plasmids from the genetically modified diploid yeast cell,

wherein the resulting genetically modified diploid yeast cell is sporulation and endogenous mating impaired and comprises two copies of a chromosomally integrated heterologous nucleotide sequence encoding said protein of interest;

wherein the first genetically modified haploid yeast cell and the second genetically modified haploid yeast cell are endogenous mating impaired due to a functional disruption of at least one pheromone response gene.

18. The method of claim 17, wherein step (a) comprises transforming each of the first and second genetically modified haploid yeast cells with at least one plasmid encoding a functional copy of the at least one pheromone response gene that is

functionally disrupted in said first and second genetically modified haploid yeast cells.

19. The method of claim 17, wherein the pheromone response gene is selected from the group consisting of *STE5*, *STE4*, *STE18*, *STE12*, *STE7*, and *STE11*.

20. The method of claim 18, wherein the pheromone response gene is selected from the group consisting of *STE5*, *STE4*, *STE18*, *STE12*, *STE7*, and *STE11*.

21. The method of claim 17, wherein the pheromone response gene is *STE5*.

22. The method of claim 18, wherein the pheromone response gene is *STE5*.

23. The method of claim 17, wherein the first genetically modified haploid yeast cell and the second genetically modified haploid yeast cell are sporulation impaired due to a functional disruption of at least one sporulation gene.

24. The method of claim 23, wherein the sporulation gene is selected from the group consisting of *IME1*, *IME2*, *NDT80*, *SPO11*, *SPO20*, *AMA1*, *HOP2*, and *SPO21*.

25. The method of claim 24, wherein the sporulation gene is *IME1*.

26. The method of claim 17, wherein the first genetically modified haploid yeast cell and the second genetically modified haploid yeast cell are endogenous mating impaired due to a functional disruption of the *STE5* gene, and are sporulation impaired due to a functional disruption of the *IME1* gene.

27. The method of claim 17, wherein the second genetically modified haploid yeast cell is obtained by inducing a mating type switch in a population of the first genetically modified haploid yeast cell.

28. The method of claim 27, wherein the population of the first genetically modified haploid yeast cell is heterothallic (*ho*) and is induced to switch mating type by

transforming the first genetically modified haploid yeast cell with a recombinant plasmid encoding a functional homothallism (HO) protein, wherein expression of the HO protein is capable of inducing a mating type switch of said first genetically modified haploid yeast cell.

29. The method of claim 17, wherein the genetically modified diploid yeast cell is a *Saccharomyces cerevisiae* cell.

30. The method of claim 29, wherein the *Saccharomyces cerevisiae* cell is of the Baker's yeast, IZ-1904, TA, BG-1, CR-1, SA-1, M-26, Y-904, PE-2, PE-5, VR-1, BR-1, BR-2, ME-2, VR-2, MA-3, MA-4, CAT-1, CB-1, NR-1, BT-1 or AL-1 strain.

31. The method of claim 29, wherein the *Saccharomyces cerevisiae* cell is of the PE-2 strain.

32. The method of claim 29, wherein the *Saccharomyces cerevisiae* cell is of the CAT-1 strain.

33. A sporulation and endogenous mating impaired diploid yeast cell generated by the method of any one of claims 17-32.

34. A method for generating a sporulation and endogenous mating impaired genetically modified heterothallic (*ho*) diploid yeast cell, the method comprising:

(a) transforming a population of a first genetically modified heterothallic haploid yeast cell with a plasmid encoding a functional homothallism (HO) protein to yield a first genetically modified haploid yeast cell, wherein expression of the HO protein is capable of inducing a mating-type switch of the first genetically modified haploid yeast cell, whereby a second genetically modified haploid yeast cell of the opposite mating type as the first genetically modified haploid yeast cell is obtained,

wherein the first genetically modified heterothallic haploid yeast cell comprises a chromosomally integrated heterologous nucleotide sequence encoding a protein of interest and functional disruptions in the *STE5* gene and the *IME1* gene;

(b) transforming each of the first and the second genetically modified haploid yeast cells with a plasmid encoding a *STE5* protein, whereby said transforming results in mating of the first genetically modified haploid yeast cell with the second genetically modified haploid yeast cell, thereby forming a genetically modified diploid yeast cell; and,

(c) eliminating any plasmids from the genetically modified diploid yeast cell to yield a genetically modified heterothallic diploid yeast cell,

wherein the resulting genetically modified heterothallic diploid yeast cell is sporulation and endogenous mating impaired and comprises two copies of a chromosomally integrated heterologous nucleotide sequence encoding said protein of interest.

35. The method of claim 34, wherein the sporulation and endogenous mating impaired heterothallic (*ho*) diploid yeast cell is a *Saccharomyces cerevisiae* cell.

36. The method of claim 35, wherein the *Saccharomyces cerevisiae* cell is of the Baker's yeast, Mauri, Santa Fe, IZ-1904, TA, BG-1, CR-1, SA-1, M-26, Y-904, PE-2, PE-5, VR-1, BR-1, BR-2, ME-2, VR-2, MA-3, MA-4, CAT-1, CB-1, NR-1, BT-1 or AL-1 strain.

37. The method of claim 35, wherein the *Saccharomyces cerevisiae* cell is of the PE-2 strain.

38. The method of claim 35, wherein the *Saccharomyces cerevisiae* cell is of the CAT-1 strain.

39. A sporulation and endogenous mating impaired heterothallic (*ho*) diploid yeast cell generated by the method of any one of claims 34-38.

40. A *MATα/***a** *ste5/ste5 ime1/ime1* yeast cell.