Ia Pla et al. (Biotechnol Prog, 2006, pp 881-888) describe Mut^S and Mut+ *P. pastoris* strains for expressing scFv using AOX promoters and induction by methanol.

EP1905836A1 discloses P. pastoris strains for producing recombinant human interferon alpha, and suggests using an AOX1-disturbed clone which comprises a mutated AOX1 gene, yet no complete deletion of the AOX1 locus.

Ching-Hsiang Chang et al. (BMC Biotechnology 2018, 18(1):81) suggest a flexible pAOX1 induction system in *P. pastoris* using methanol expression regulator 1 (Mxr1)-reprogrammed cells.

Russmayer H. et al. (BMC Biology 2015, 13(1):80) describe the importance of AOX in regulating *P. pastoris* expression systems.

Tomas-Gamisans M. et al. (Microbial Biotechnology 2018, 11(1):224-237) describe a *P. pastoris* genome-scale metabolic model for improved prediction on methanol or glycerol as sole carbon sources.

Moser et al. (Microbial Cell Factories 2017, 16(1):49) describe adaptive laboratory evolution to improve growth and recombinant protein production in *P. pastoris*.

Recombinant protein production in *P. pastoris* requires an intense process scheme, leading to high oxygen demand, and heat production, demanding a high biomass concentration and methanol consumption. Oxygen transfer, cooling and biomass separation in downstream processing is expensive. It is thus desirable to develop processes of low oxygen demand and heat production. It is further desirable to increase the yield of protein production, in particular by efficient use of a carbon source.

SUMMARY OF THE INVENTION

It is the objective of the invention to improve recombinant protein production in methylotrophic yeast.

The objective is solved by the subject of the claims and as further described herein.

The invention provides for a recombinant methanol utilization pathway deficient methylotrophic yeast (Mut-) host cell which is engineered:

a) by one or more genetic modifications to render the host cell methanol utilization pathway deficient, by reduc~~e~~ing expression of a first and a second endogenous gene compared to the host cell prior to said one or more genetic modifications, wherein

i. the first endogenous gene encodes alcohol oxidase 1 (AOX1) comprising the amino acid sequence identified as SEQ ID NO:1 or a homologue thereof that is endogenous to the respective wild-type host cell, and

ii. the second endogenous gene encodes alcohol oxidase 2 (AOX2) comprising the amino acid sequence identified as SEQ ID NO:3 or a homologue thereof that is endogenous to the respective wild-type host cell,

and

b) by one or more genetic modifications to increase expression of an alcohol dehydrogenase (*ADH2*) gene compared to the host cell prior to said one or more genetic modifications, wherein the *ADH2* gene encodes an alcohol dehydrogenase (ADH2).

Specifically, the ADH2 protein is an alcohol dehydrogenase classified as EC 1.1.1.1.

As described herein, the term “ADH2” shall refer to either a native alcohol dehydrogenase, such as *P. pastoris* alcohol dehydrogenase comprising or consisting of the amino acid sequence identified as SEQ ID NO:50 (UniProtKB - F2QSX6_KOMPC; FR839629 Genomic DNA Translation: CCA38504.1; gene: PP7435_Chr2-0821), or a sequence which has a certain homology (or sequence identity) to SEQ ID NO:50.

Specifically, the ADH2 may originate from a *P. pastoris* strain or may be a homologue or ortholog thereof which is naturally-occurring originating from or endogenous to a wild-type cell of an organism, such as eukaryotes, including *e.g*. yeast, in particular of a methylotrophic yeast strain, species, or genus, or which is a mutant of such naturally-occurring ADH2.

Specifically, the ADH2 protein is naturally-occurring in or endogenous to the species of the host cell, or a mutant thereof.

Specifically, the gene encoding the *P. pastoris* alcohol dehydrogenase, herein referred to as *ADH2* gene, comprises or consists of the nucleotide sequence identified as SEQ ID NO:51, or a homologous polynucleotide (gene) encoding ADH2, which ADH2 has a certain homology (or sequence identity) to SEQ ID NO:50.

Specifically, the *ADH2* gene is endogenous or heterologous to the Mut- host cell. Specifically, the Mut- host cell comprises one or more copies of said *ADH2* gene.

Specifically, the ADH2 is any one of:

a) an ADH2, which is *P. pastoris* ADH2 comprising the amino acid sequence identified as SEQ ID NO:50, or a homologue thereof that is endogenous to a yeast species, in particular methylotrophic yeast; or

cell under conditions that permit the Mut- host cell to use methanol as a substrate for alcohol dehydrogenase (ADH2), and to produce the fermentation product.

Specifically, said Mut- host cell is deficient of alcohol oxidase 1 (AOX1) and alcohol oxidase 2 (AOX2).

Specifically, in said Mut- host cell, the genes encoding alcohol oxidase 1 (AOX1) and alcohol oxidase 2 (AOX2) are knocked out or deleted.

According to a specific aspect, the invention provides for the use of a recombinant methanol utilization pathway deficient methylotrophic yeast (Mut-) host cell in a method of producing a fermentation product which method comprises culturing said Mut- host cell under conditions that permit the Mut- host cell to produce the fermentation product using methanol as a carbon source, which Mut- host cell is engineered by one or more genetic modifications

a) to render the host cell methanol utilization pathway deficient, by reduceing expression of a first and a second endogenous gene compared to the host cell prior to said one or more genetic modifications, wherein

i. the first endogenous gene encodes alcohol oxidase 1 (AOX1) comprising the amino acid sequence identified as SEQ ID NO:1 or a homologue thereof that is endogenous to the respective wild-type host cell, and

ii. the second endogenous gene encodes alcohol oxidase 2 (AOX2) comprising the amino acid sequence identified as SEQ ID NO:3 or a homologue thereof that is endogenous to the respective wild-type host cell, and

b) to increase expression of an alcohol dehydrogenase (*ADH2*) gene, wherein the *ADH2* gene encodes an alcohol dehydrogenase (ADH2).

According to a specific aspect, the invention provides for a method for producing a protein of interest (POI) in a host cell, comprising the steps:

a) genetically engineering the host cell to reduce expression (underexpress) of said first and second genes encoding the AOX1 and AOX2, respectively;

b) genetically engineering the host cell to increase expression (overexpress) of a gene encoding ADH2;

c) introducing into the host cell a heterologous expression cassette comprising a gene of interest (GOI) encoding said POI under the control of an expression cassette promoter (ECP);

d) culturing said host cell under conditions to produce said POI using methanol as a carbon source, thereby particularly providing energy for growth and/or POI production;

CLAIMS

1. A recombinant methanol utilization pathway deficient methylotrophic yeast (Mut-) host cell which is engineered:

a) by one or more genetic modifications to render the host cell methanol utilization pathway deficient, by reduc~~e~~ing expression of a first and a second endogenous gene compared to the host cell prior to said one or more genetic modifications, wherein

i. the first endogenous gene encodes alcohol oxidase 1 (AOX1) comprising the amino acid sequence identified as SEQ ID NO:1 or a homologue thereof that is endogenous to the respective wild-type host cell, and

ii. the second endogenous gene encodes alcohol oxidase 2 (AOX2) comprising the amino acid sequence identified as SEQ ID NO:3 or a homologue thereof that is endogenous to the respective wild-type host cell,

and

b) by one or more genetic modifications to increase expression of an alcohol dehydrogenase (*ADH2*) gene compared to the host cell prior to said one or more genetic modifications, wherein the *ADH2* gene encodes an alcohol dehydrogenase (ADH2).

2. The Mut- host cell of claim 1, wherein said one or more genetic modifications comprise a disruption, substitution, deletion, knockin or knockout of (i) one or more polynucleotides, or a part thereof; or (ii) an expression control sequence.

3. The Mut- host cell of claim 2, wherein said expression control sequence is selected from the group consisting of a promoter, a ribosomal binding site, transcriptional or translational start and stop sequences, an enhancer and activator sequence.

4. The Mut- host cell of any one of claims 1 to 3, wherein said first and/or second endogenous gene is knocked out by said one or more genetic modifications.

5. The Mut- host cell of any one of claims 1 to 4, wherein the *ADH2* gene is endogenous or heterologous to the Mut- host cell.

6. The Mut- host cell of any one of claims 1 to 5, wherein the ADH2 is any one of:

a) an ADH2, which is *P. pastoris* ADH2 comprising the amino acid sequence identified as SEQ ID NO:50, or a homologue thereof that is endogenous to a yeast species;

b) a mutant of the ADH2 of a), which is at least 60% identical to SEQ ID NO:50.

7. The Mut- host cell of any one of claims 1 to 6, wherein said one or more genetic modifications include a gain-of-function alteration in the *ADH2* gene resulting in increasing the level or activity of ADH2.

8. The Mut- host cell of claim 7, wherein said gain-of-function alteration includes a knockin of the *ADH2* gene.

9. The Mut- host cell of claim 7 or 8, wherein said gain-of-function alteration up-regulates the *ADH2* gene expression in said cell.

10. The Mut- host cell of any one of claims 7 to 9, wherein said gain-of-function alteration includes an insertion of a heterologous expression cassette to overexpress the *ADH2* gene in said cell.

11. The Mut- host cell of claim 10, wherein said heterologous expression cassette comprises a heterologous polynucleotide comprising an *ADH2* gene under the control of a promoter sequence.

12. The Mut- host cell of any one of claims 1 to 11, which comprises a heterologous gene of interest expression cassette (GOIEC) comprising an expression cassette promoter (ECP) operably linked to a gene of interest (GOI) encoding a protein of interest (POI).

13. The Mut- host cell of claim 12, wherein the ECP is a methanol-inducible promoter.

14. The Mut- host cell of claim 13, wherein the ECP is any one of the following:

a) a pAOX1 promoter comprising at least ~~60~~90% sequence identity to SEQ ID NO:5;

b) a pAOX2 promoter comprising at least ~~60~~90% sequence identity to SEQ ID NO:6; or

c) a promoter comprising a nucleotide sequence selected from the group consisting of SEQ ID NO:36-49.

15. The Mut- host cell of any one of claims 12 to 14, wherein the GOIEC further comprises a nucleotide sequence encoding a signal peptide enabling the secretion of the POI.

16. The Mut- host cell of claim 15, wherein the nucleotide sequence encoding the signal peptide is fused adjacent to the 5'-end of the GOI.

17. The Mut- host cell of any one of claims 12 to 16, wherein the POI is heterologous to the Mut- host cell or the ECP.

18. The Mut- host cell of any one of claims 12 to 17, wherein the POI is a peptide or protein selected from the group consisting of an antigen-binding protein, a therapeutic protein, an enzyme, a peptide, a protein antibiotic, a toxin fusion protein, a carbohydrate - protein conjugate, a structural protein, a regulatory protein, a vaccine antigen, a growth factor, a hormone, a cytokine, a process enzyme.

19. The Mut- host cell of any one of claims 1 to 18, wherein the Mut- host cell is a yeast cell of the genus Pichia, Komagataella, Hansenula, Ogataea or Candida.

20. A method of producing a protein of interest (POI) comprising culturing a Mut- host cell of any one of claims 1 to ~~18~~ 19 using methanol as a carbon source to produce the POI.

21. The method of claim 20, wherein a fermentation product is isolated from the cell culture, which fermentation product comprises the POI or a host cell metabolite obtained from the Mut- host cell.

22. The method of claim 20 or 21, wherein

a) a growing phase, during which the Mut- host cell is cultured using a basal carbon source as a source of energy; is followed by

b) a production phase, during which the Mut- host cell is cultured using a methanol feed thereby producing the POI.

23. The method of claim 22, wherein an average methanol concentration of 0.5-2.0% (v/v) is used in the host cell culture during the production phase of at least 24 hours.

24. The method of claim 22 or 23, wherein the methanol feed is at an average feed rate of at least 2 mg methanol/ (g dry biomass*h) during the production phase of at least 24 hours.

25. The method of any one of claims 22 to 24, wherein the Mut- host cell is cultured during the production phase under conditions limiting the host cell growth to less than 10% (w/w biomass).

26. Use of a recombinant methanol utilization pathway deficient methylotrophic yeast (Mut-) host cell in a method of producing a fermentation product which method comprises culturing said Mut- host cell under conditions that permit the Mut- host cell to use methanol as a substrate for alcohol dehydrogenase (ADH2), and to produce the fermentation product, wherein the method is of any one of claims 20 to 25.

27. Use of a recombinant methanol utilization pathway deficient methylotrophic yeast (Mut-) host cell in a method of producing a fermentation product which method comprises culturing said Mut- host cell under conditions that permit the Mut- host cell to produce the fermentation product using methanol as a carbon source, which Mut- host cell is engineered by one or more genetic modifications

a) to render the host cell methanol utilization pathway deficient, by ~~reduce~~reducing expression of a first and a second endogenous gene compared to the host cell prior to said one or more genetic modifications, wherein

i. the first endogenous gene encodes alcohol oxidase 1 (AOX1) comprising the amino acid sequence identified as SEQ ID NO:1 or a homologue thereof that is endogenous to the respective wild-type host cell, and

ii. the second endogenous gene encodes alcohol oxidase 2 (AOX2) comprising the amino acid sequence identified as SEQ ID NO:3 or a homologue thereof that is endogenous to the respective wild-type host cell, and

b) to increase expression of an alcohol dehydrogenase (*ADH2*) gene, wherein the *ADH2* gene encodes an alcohol dehydrogenase (ADH2).