RECOMBINANT HOST CELL FOR EXPRESSING PROTEINS OF INTEREST

Field of Invention

[001] The present invention is in the field of recombinant biotechnology, in particular in the field of protein expression. The invention generally relates to a method of expressing a protein of interest (POI) from a host cell. The invention relates particularly to improving a host cell's capacity to express and/or secrete a protein of interest and use of the host cell for protein expression. The invention also relates to cell culture technology, and more specifically to culturing cells to produce desired molecules for medical purposes or food products.

Background of the Invention

[002] Successful production of proteins of interest (POI) has been accomplished both with prokaryotic and eukaryotic hosts. The most prominent examples are bacteria like *Escherichia coli*, yeasts like *Saccharomyces cerevisiae*, *Pichia pastoris* or *Hansenula polymorpha*, filamentous fungi like *Aspergillus awamori* or *Trichoderma reesei*, or mammalian cells like CHO cells. While the yield of some proteins is readily achieved at high rates, many other proteins are only produced at comparatively low levels.

[003] To improve the secretion of a recombinant protein, one strategy is to target on the host´s secretory pathway involving the folding and processing of proteins.

[004] Co-expression of a cDNA encoding protein disulfide isomerase (PDI) and a cDNA encoding a heterologous disulphide-bonded protein was first suggested in WO 93/25676 as a means to increase the yield of the heterologous protein. WO 93/25676 reported that the recombinant expression of antistasin and tick anticoagulant protein can be increased by co-expression with PDI.

[005] WO 94/08012 provided methods for increasing the secretion of overexpressed protein in yeast by increasing expression of an Hsp70 chaperone protein, i.e. KAR2 /BiP, or a PDI chaperone protein.

[006] WO 05/0617818 and WO 06/067511 provided methods for producing a desired heterologous protein in yeast by using a 2µm-based expression plasmid. It was demonstrated that the yield of a heterologous protein is substantially increased when the

genes for one or more chaperone protein(s) and a heterologous protein are co-expressed on the same plasmid.

[007] WO 2008/128701A2 described an expression system to increase the secretion of a POI from a eukaryotic cell by employing one of the following proteins BMH2, BFR2, COG6, C0Y1, CUP5, IMH 1, KIN2, SEC31, SSA4 and SSE1.

[008] Another approach to increase protein production is based on the overexpression of HAC1, a transcription factor that activates the unfolded protein response (UPR). Transcriptional analyses revealed that more than 330 genes are regulated by HAC1, most of them being involved in secretion or in the biogenesis of secretory organelles. WO 01/72783 describes methods for increasing the amount of a heterologous protein secreted from a eukaryotic cell by inducing an elevated unfolded protein response (UPR), wherein the UPR is modulated by co-expression of a protein selected from the group consisting of HAC1, PTC2 and IRE1.

[009] Wentz et al. employed a *Saccharomyces cerevisiae* yeast surface display gene library to identify improved secretion strains by applying an appropriate selection pressure. The yeast cDNAs CCW12, SED1, CWP2, RPP0 were found to enhance the display of scTCR in a temperature-dependent manner. ERO1 enhanced protein secretion when induced at 20°C (Wentz et al., Appl. Environ. Microbiol. (2007) 73(4):1189-1198).

[0010] Liu et al. (*Biotechnol. Prog.* (2006), 22:1090-1095) showed that co-overexpression of Kar2 in *Pichia pastoris* increases rhG-CSF expression 5.6 fold. Combining KAR2 with Sec63, PDI and YDJ resulted in an increase of 2.8, 6.5 and 5.94 fold. In the experiments performed by Blatt et al, co-overexpression of KAR2 (BiP) increased A33scFv expression in *Pichia pastoris* two-fold. Combining KAR2 with PDI almost eliminated the positive KAR2 effect (Blatt et al., Appl. Microbiol. Biotechnol., 2007, 74:381-389).

[0011] Guerfall et al. examined the effect of overexpressing endogenous HAC1 in *P. pastoris*. Furthermore, HAC1 was overexpressed constitutively and inducibly. In all cases, an increased KAR2 expression as a result of induced UPR was identified. Constitutive overexpression of full-length HAC1 had little or no effect, while overexpression of the induced form of HAC1 led to an increase of protein secretion in one out of four cases, and a decrease in three out of four cases (Guerfall et al., Microbial Cell Factories, (2010), 9:49).

[0012] Sleep et al. showed that co-overexpression of Lhs1 increases the concentration of rHA. LHS1 was combined with SIL1, JEM1 and SCJ1, but titers were lower than with JEM1 co-overexpressed alone. Co-overexpression of SIL1, LHS1 and JEM1 at the same time increased GM-CSF expression by 1.45 fold and the rHA expression by approximately

1.1 and 2 fold, dependent on the cultivation media (Sleep et al. Applied and Environmental Microbiology, (2008) 74(24):7759-7766).

[0013] US 2009221030 A1 described the co-expression of various helper proteins, inter alia, BiP1, alone and in combination with other helper proteins, inter alia, LHS1, in *Trichoderma reesei*. The highest expression values were obtained with BiP1 alone, while the combination of BiP1 and LHS1 lead to 8% lower secreted protein titers.

[0014] US 8,440,456 provided the genome sequence of *Pichia pastoris* and disclosed nucleic acid sequences encoding signal peptides, chaperons and promoters. It disclosed expression vectors comprising the nucleic acid sequences and genetically engineered yeast capable of overexpression of 14 chaperones. ROT1, SHR3 and SIL1 were specifically selected for testing, however, no expression was observed for SIL1, and overexpression of ROT1 or SHR3 failed to lead to any significant enhancement of the secretion of heterologous proteins. While overexpression of genes encoding helper proteins is usually applied to enhance expression of a protein of interest, underexpression of a gene encoding a protein or even a knock-out of the gene encoding it is not or only rarely applied.

[0015] High level of protein yield in host cells may be limited at one or more different steps, like folding, disulfide bond formation, glycosylation, transport within the cell, or release from the cell. Many of the mechanisms involved are still not fully understood and cannot be predicted on the basis of the current knowledge of the state-of-the-art, even when the DNA sequence of the entire genome of a host organism is available.

[0016] There is a constant need for methods to improve a host cell's capacity to produce and/or secret proteins of interest. One object of the invention is to provide new methods to increase the yield of proteins in host cells which are simple and efficient and suitable for use in industrial methods. It is another object to provide host cells to achieve this purpose.

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[0017] It must be noted that as used herein, the singular forms "a", "an" and "the" include plural references and vice versa unless the context clearly indicates otherwise. Thus, for example, a reference to "a host cell" or "a method" includes one or more of such host cells or methods, respectively, and a reference to "the method" includes equivalent steps and methods that could be modified or substituted known to those of ordinary skill in the art. Similarly, for example, a reference to "methods" or "host cells" includes "a host cell" or "a method", respectively.

[0018] Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the present invention.

[0019] The term "and/or" wherever used herein includes the meaning of "and", "or" and "all or any other combination of the elements connected by said term". For example, A, B and/or C means A, B, C, A+B, A+C, B+C and A+B+C.

[0020] The term "about" or "approximately" as used herein means within 20%, preferably within 10%, and more preferably within 5% of a given value or range. It includes also the concrete number, e.g., about 20 includes 20.

[0021] The term “less than”, “more than” or “larger than” includes the concrete number. For example, less than 20 means ≤20 and more than 20 means ≥20.

[0022] Throughout this specification and the claims or items, unless the context requires otherwise, the word “comprise” and variations such as “comprises” and “comprising” will be understood to imply the inclusion of a stated integer (or step) or group of integers (or steps). It does not exclude any other integer (or step) or group of integers (or steps). When used herein, the term “comprising” can be substituted with “containing”, "composed of", “including”, “having” or "carrying." When used herein, “consisting of" excludes any integer or step not specified in the claim/item. When used herein, "consisting essentially of" does not exclude integers or steps that do not materially affect the basic and novel characteristics of the claim/item. In each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms.

[0023] Further, in describing representative embodiments of the present invention, the specification may have presented the method and/or process of the present invention as a particular sequence of steps. However, to the extent that the method or process does not rely on the particular order of steps set forth herein, the method or process should not be limited to the particular sequence of steps described. As one of ordinary skill in the art would appreciate, other sequences of steps may be possible. Therefore, the particular order of the steps set forth in the specification should not be construed as limitations on the claims. In addition, the claims directed to the method and/or process of the present invention should not be limited to the performance of their steps in the order written, and one skilled in the art can readily appreciate that the sequences may be varied and still remain within the spirit and scope of the present invention.

[0024] It should be understood that this invention is not limited to the particular methodology, protocols, material, reagents, and substances, etc., described herein. The terminologies used herein are for the purpose of describing particular embodiments only and are not intended to limit the scope of the present invention, which is defined solely by the claims/items.

[0025] All publications and patents cited throughout the text of this specification (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, etc.), whether supra or infra, are hereby incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material.

Summary

[0026] The present invention is partly based on the surprising findings of several proteins (herein referred to as knockout (KO) proteins), including KO1, KO2, KO3 (also referred to herein as KO protein 1, KO protein 2, and KO protein 3, respectively) and functional homologues thereof, whose expression was observed to have a negative impact on the yield of POI from a host cell. The meaning of functional homologue is defined in the latter part of the application. Furthermore, it has been discovered that a modification of the genes such as mutation or deletion is able to increase the yield of POI. This disclosure provides methods and materials useful for improving the yield of POI by engineering host cells such that they underexpress the genes identified by the inventors. The term ""yield" refers to the amount of POI or model protein(s) as described herein, in particular SDZ-Fab (SEQ ID NO: 25 and 26) and HyHEL-Fab (SEQ ID NO: 29 and 30), respectively, which is, for example, harvested from the engineered host cell, and increased yields can be due to increased amounts of production or secretion of the POI by the host cell. Yield may be presented by mg POI/g biomass (measured as dry cell weight or wet cell weight) of a host cell. The term "titer" when used herein refers similarly to the amount of produced POI or model protein, presented as mg POI/L culture supernatant. An increase in yield can be determined when the yield obtained from an engineered host cell is compared to the yield obtained from a host cell prior to engineering, i.e., from a non-engineered host cell.

[0027] Preferably, "yield" when used herein in the context of a model protein as described herein, is determined as described in Example 5c. Accordingly, the "yield" when used herein in the context of a model protein as described herein is also referred to as "Fab

yield" or "Fab titer". A Fab titer is given as mg/L and a Fab yield as mg/biomass (measured as dry cell weight or wet cell weight).

Briefly, *P. pastoris* strains CBS7435mut^S pPM2d_pAOX HyHEL and/or CBS7435mut^S pPM2d_pAOX SDZ (see Example 1 for their generation) which express the model protein HyHEL-Fab and SDZ-Fab, respectively, are engineered with a polynucleotide encoding a helper protein or functional homologue thereof as described herein. For co-overexpression, the gene encoding a helper protein is cloned under control of the *P. pastoris* GAP promoter and transformed into the Fab producing strains as described in example 4. For underexpression the gene encoding a KO target or its functional homologue is knocked out from the genome of the Fab producing strain (see example 6). Engineered cells are grown in YP-medium containing 10 g/L glycerol and 50 μg/mL Zeocin overnight at 25°C (see Example 5a). Aliquots of such a culture (corresponding to a final OD_{600} of 2.0) are transferred to synthetic medium M2 containing 20 g/L glucose and a glucose feed tablet (described in Example 5a) and incubated for 25 h at 25°C. Cultures are washed and resuspended in synthetic medium M2 and aliquots (corresponding to a final OD_{600} of 4.0) are transferred into synthetic medium M2 supplemented with 5 g/L methanol. Methanol (5g/L) is added 3 more times every 12 hours. After 48 h, cells are harvested by centrifugation. Biomass is determined by measuring the weight of the cell pellet derived from 1 mL cell suspension. The supernatant is used for quantification of SDZ-Fab or HyHEL-Fab, respectively, by ELISA (described in Example 5c). Specifically, an anti-human IgG antibody (e.g. ab7497, Abcam) is used as coating antibody and a e.g. goat anti-human anti-human IgG (Fab specific) antibody (e.g. Sigma A8542, alkaline phosphatase conjugated) is used as detection antibody. Commercial Human Fab/Kappa, IgG fragment is used as standard with a starting concentration of 100 ng/mL, supernatant samples are diluted accordingly. An increase in the yield may be determined based on a comparison of POI yield before and after the cell is engineered to underexpress the polypeptide encoding the KO protein. A standard test as shown in the example may be used to determine the yield difference.

[0028] The present invention is partly based on, but not limited to, the following knockout proteins which are discovered by the inventors:

Table 1

KO proteins	(Designation(in analogy to S. cerevisiae))	Amino acid sequences	Polynucleotides	Designation in the Examples (see Table 7)
KO1	FLO8	SEQ ID NO: 10	SEQ ID NO: 22	PP7435_Chr4-0252
KO2	HCH1	SEQ ID NO: 11	SEQ ID NO: 23	PP7435_Chr3-1062
KO3	SCJ1	SEQ ID NO: 12	SEQ ID NO: 24	PP7435_Chr1-0176

[0029] The present invention provides genetically engineered recombinant host cells having one or more underexpressed genes encoding the KO protein. As used herein, "engineered" host cells are host cells which have been manipulated using genetic engineering, i.e. by human intervention. When a host cell is "engineered to underexpress" a given protein, the host cell is manipulated such that the expression level of a functional KO protein is decreased compared to the host cell under the same condition prior to the genetic manipulation to achieve underexpression. The host cell which is to be subjected to genetic engineering comprises at least one, such as at least 2, or at least 3 polynucleotide sequence encoding a KO protein or functional homologues thereof. Genetic engineering results in the underexpression of genes encoding the KO protein. Preferably, the engineering will result in a cell having no detectable level for the KO protein(s). Underexpression can be observed by comparing the engineered host cell with a "corresponding parent strain" which refers to the host cell prior to aforementioned engineering. As used herein, KO proteins are referred to in the present invention interchangeably in plural or singular forms, which however should be understood as in singular form unless expressly stated otherwise.

[0030] As to the genes that have to be knocked-out – from a chaperone (KO2, KO3) one would have expected that overexpression is beneficial, but it is in fact the deletion that has a positive effect.

[0031] KO1 which plays a role in flocculation in baker's yeast, one would not have expected that its deletion enhances protein secretion. Flocculation in baker's yeast is a phenomenon when cells aggregate reversibly in a vegetative growth phase, not related to mating.

[0032] In a first aspect, the present invention provides a recombinant host cell for manufacturing a protein of interest, wherein the host cell is engineered to underexpress at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12.

[0033] The recombinant host cell is obtained by first providing a host cell which comprises at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39,

40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12, and then engineering the host cell to underexpress the polynucleotide.

[0034] In a preferred embodiment, the host cell is a yeast cell, preferably a host cell of the genus *Pichia* and more preferably *Pichia pastoris* which does not comprise a KO protein of the present invention and/or which does not comprise a polynucleotide encoding the KO protein(s). In one embodiment, the host cell is one obtained by deleting a polynucleotide sequence as shown in SEQ ID NO: 22, 23, 24, or homologous thereof.

[0035] As used herein, a "homologue" or "functional homologue" of a polypeptide shall mean that a polypeptide has the same or conserved residues at a corresponding position in their primary, secondary or tertiary structure. The term also extends to two or more nucleotide sequences encoding homologous polypeptides. In particular, polypeptides homologous to the KO1, KO2 and KO3 described in the examples may have at least about 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, amino acid sequence identity with regard to a full-length native sequence or any fragment thereof. Preferably, a homologous polypeptide will have at least about 35% amino acid sequence identity, more preferably at least about 40% amino acid sequence identity, more preferably at least about 45% amino acid sequence identity, more preferably at least about 50% amino acid sequence identity, more preferably at least about 55% amino acid sequence identity, more preferably at least about 60% amino acid sequence identity, more preferably at least about 65% amino acid sequence identity, more preferably at least about 70% amino acid sequence identity, more preferably at least about 75% amino acid sequence identity, more preferably at least about 80% amino acid sequence identity, more preferably at least about 85% amino acid sequence identity, more preferably at least about 90%, such as 91, 92, 93, 94, 95, 96, 97, 98 or 99% amino acid sequence identity, more preferably at least about 95% amino acid sequence identity to a native compound, or any other specifically defined fragment of a full-length compound. When the function as a KO1, KO2, and KO3 protein is proven with such a homologue, the homologue is called "functional homologue". A functional homologue performs the same or substantially the same function as the KO protein or helper protein from which it is derived from, i.e. it increases the yield of the model protein SDZ-Fab and/or HyHEL-Fab as described herein. The function can be tested by assay known in the art or preferably with the assay using the model proteins as described in

Example 7 or Example 5c, particularly as described herein above in the context of "Fab titer" or "Fab yield".

[0036] The function of a potential functional homologue can be tested by providing expression cassettes into which the functionally equivalent variant sequences have been inserted, transforming host cells that carry the sequence encoding a test protein such as one of the model proteins used in the Example or a specific POI, and determining the difference in the yield of the model protein or POI under identical conditions.

[0037] In one embodiment, the host cell is engineered to underexpress KO protein 1. The host cell is preferably engineered to underexpress a polynucleotide encoding a protein having an amino acid sequence as shown in SEQ ID NO: 10 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 10.

[0038] In another embodiment, the host cell is engineered to underexpress KO2. The host cell is preferably engineered to underexpress a polynucleotide encoding a protein having an amino acid sequence as shown in SEQ ID NO: 11 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 11.

[0039] In a further embodiment, the host cell is engineered to underexpress KO3. The host cell is preferably engineered to underexpress a polynucleotide encoding a protein having an amino acid sequence as shown in SEQ ID NO: 12 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 12.

[0040] The present invention provides an isolated polynucleotide sequence comprising, consisting essentially of, or consisting of SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24, Furthermore, the present invention provides an isolated polynucleotide sequence which

encodes a polypeptide sequence comprising any one of SEQ ID NO: 10, 11, 12, or functional homologues thereof. In some embodiments, the present invention provides an isolated polynucleotide sequence having at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO: 22, 23, 24. More preferably, the isolated polynucleotide sequence has 100% sequence identity with SEQ ID NO: 22, SEQ ID NO23, SEQ ID NO: 24.

[0041] Furthermore, the present invention provides an isolated polypeptide comprising the polypeptide sequence of any one of SEQ ID NO: 10, 11, 12, or functional homologues thereof. Preferably, the present invention provides an isolated polypeptide comprising the polypeptide sequence of SEQ ID NO: 10 or functional homologues thereof. Preferably, the present invention provides an isolated polypeptide comprising the polypeptide sequence of SEQ ID NO: 11 or functional homologues thereof. Preferably, the present invention provides an isolated polypeptide comprising the polypeptide sequence of SEQ ID NO: 12 or functional homologues thereof. In a further aspect, the present invention provides an isolated polypeptide with a polypeptide sequence having at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 10, 11, or 12.

[0042] The term "nucleotide sequence" or “nucleic acid sequence” used herein refers to either DNA or RNA. "Nucleic acid sequence" or "polynucleotide sequence" or simply “polynucleotide” refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes both self-replicating plasmids, infectious polymers of DNA or RNA, and non-functional DNA or RNA.

[0043] The term “expressing a polynucleotide” means when a polynucleotide is transcribed to mRNA and the mRNA is translated to a polypeptide. The term "underexpress" generally refers to any amount less than an expression level exhibited by a reference standard, which is the host cell prior to the engineering to underexpress the KO protein. The terms "underexpress," "underexpressing," "underexpressed" and "underexpression" in the present invention refer an expression of a gene product or a polypeptide at a level less than the expression of the same gene product or polypeptide prior to a genetic alteration of the host cell or in a comparable host which has not been genetically altered. “Less than” includes,

e.g., 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80, 90% or more. No expression of the gene product or a polypeptide is also encompassed by the term "underexpression."

[0044] The host cell may be obtained by providing a cell which expresses KO protein 1 or functional homologues thereof and engineering the cell to underexpress the gene which encodes KO protein 1.The host cell may also be obtained by providing a cell which expresses KO protein 2 or functional homologues thereof and engineering the cell to underexpress the gene which encodes KO protein 2. Furthermore, the host cell may be obtained by providing a cell which expresses KO protein 3 or functional homologues thereof and engineering the cell to underexpress the gene encoding KO protein 3.

[0045] In a preferred embodiment, the KO protein 1, when preferably underexpressed, may increase the production of the model protein SDZ-Fab or HyHEL-Fab in the host cell by at least 1%, such as at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or at least 200% or more, compared to the host cell prior to the engineering to underexpress the polynucleotide encoding the KO protein. It has been surprisingly found that exemplary recombinant cells described in the Examples were all able to increase the yield of the model protein SDZ-Fab or HyHEL-Fab by at least 20% (1.2 fold change).

[0046] In a preferred embodiment, the KO protein 2, when preferably underexpressed, may increase the yield of the model protein SDZ-Fab or HyHEL-Fab in the host cell by at least 1%, such as at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or at least 200% or more, compared to the host cell prior to engineering. It has been surprisingly found that exemplary recombinant cells described in the Examples were all able to increase the yield of the model protein SDZ-Fab or HyHEL-Fab by at least 20% (1.2 fold change).

[0047] In a preferred embodiment, the KO protein 3, when preferably underexpressed, may increase the yield of the model protein SDZ-Fab or HyHEL-Fab in the host cell by at least 1%, such as at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or at least 200% or more, compared to the host cell prior to engineering. It has been surprisingly found that exemplary recombinant cells described in the Examples were all able to increase the yield of the model protein SDZ-Fab or HyHEL-Fab by at least 20% (1.2 fold change).

[0048] In a third aspect, the present invention provides use of any of the recombinant host cells described in the present for manufacturing a protein of interest.

[0049] In a fourth aspect, the present invention provides a method of increasing the yield of a protein of interest in a host cell, comprising underexpressing at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12.

[0050] Preferably, the method comprises underexpressing a polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10 or a functional homologue thereof, wherein the functional homologue has having at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 10.

[0051] Preferably, the method comprises underexpressing a polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 11 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 11.

[0052] Preferably, the method comprises underexpressing a polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 12 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 12.

[0053] In a fifth aspect, the present invention provides a method of increasing the yield of a protein of interest in a host cell comprising:

- engineering the host cell to underexpress at least one, such as at least 2, or at least 3, polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12,
- recombining in said host cell a heterologous polynucleotide encoding a protein of interest, and
- culturing said host cell under suitable conditions to express the protein of interest.

[0054] In the context of methods for increasing the yield of a protein the order of the "engineering step" and "recombining step" can alternatively be reversed such that the "recombining step" precedes the "engineering step". Notably, as described herein, the yield of a protein of interest is increased when a KO protein is underexpressed and/or a helper protein is overexpressed.

[0055] It should be noted that the engineering step and the recombining step do not have to be performed in the recited sequence. It is possible to perform the steps in different order.

[0056] Preferably, the present invention provides a method of increasing the yield of a protein of interest in a host cell comprising:

- engineering the host cell to underexpress at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 10,
- recombining in said host cell a heterologous polynucleotide encoding a protein of interest, and
- culturing said host cell under suitable conditions to express the protein of interest.

[0057] Preferably, the present invention provides a method of increasing the yield of a protein of interest in a host cell comprising:

- engineering the host cell to underexpress at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 11,
- recombining in said host cell a heterologous polynucleotide encoding a protein of interest, and
- culturing said host cell under suitable conditions to express the protein of interest.

[0058] Preferably, the present invention provides a method of increasing the yield of a protein of interest in a host cell comprising:

- engineering the host cell to underexpress at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 12,
- recombining in said host cell a heterologous polynucleotide encoding a protein of interest, and
- culturing said host cell under suitable conditions to express the protein of interest.

[0059] In a sixth aspect, the present invention provides a method of producing a protein of interest in host cell comprising:

- providing a host cell engineered to underexpress at least one, such as at least 2, or at least 3 polynucleotide encoding a KO protein sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has an amino acid having at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an

amino acid sequence as shown in SEQ ID NO: 10, 11 or 12, wherein said host cell comprises a heterologous polynucleotide encoding a protein of interest; and

- culturing the host cell under suitable conditions to express the protein of interest.

[0060] Preferably, the present invention provides a method of producing a protein of interest in a host cell comprising:

- providing a host cell engineered to underexpress at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, wherein said host cell comprises a heterologous polynucleotide encoding a protein of interest; and
- culturing the host cell under suitable conditions to express the protein of interest.

[0061] Preferably, the present invention provides a method of producing a protein of interest in a host cell comprising:

- providing a host cell engineered to underexpress at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 11, wherein said host cell comprises a heterologous polynucleotide encoding a protein of interest; and
- culturing the host cell under suitable conditions to express the protein of interest.

[0062] Preferably, the present invention provides a method of producing a protein of interest in host cell comprising:

- providing a host cell engineered to underexpress at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68,

69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 12, wherein said host cell comprises a heterologous polynucleotide encoding a protein of interest; and

- culturing the host cell under suitable conditions to express the protein of interest.

[0063] The present invention encompasses the underexpression of 2 or all of KO1, KO2 and KO3 genes. The present invention also encompasses the recombinant host cell for manufacturing a protein of interest, wherein the host cell is engineered to underexpress at least 2, 3 or more polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12. In some embodiments 2, 3, 4, 5, 6 or more of KO proteins selected from SEQ ID NO: 10, 11, 12 or functional homologues thereof are underexpressed in the host cell. A functional homologue may be a biologically active fragment of the KO proteins. Generally, biologically active fragment of a protein shall mean a fragment that exerts a biological effect similar or comparable to that of the full length protein. Such fragments or variants can be produced e.g. by amino- and/or carboxy- terminal deletions as well as by internal deletions.

Brief Description of the Drawings

FIG. 1 shows the amino acid and polynucleotide sequences of the HP1, HP2, HP 3, HP 4, HP5, HP6, HP7, HP8, HP9, HP10, KO1, KO2 and KO3.

FIG. 2 shows the amino acid and polynucleotide sequences of the heavy chain and light chain of the model proteins SDZ-Fab and HyHEL-Fab, respectively.

Items of the Invention

1. A recombinant host cell for manufacturing a protein of interest, wherein the host cell is engineered to underexpress at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or12.

2. The host cell of item 1, wherein said polynucleotide encoding the KO protein or a functional homologue thereof, preferably when underexpressed, increases the yield of the model protein SDZ-Fab (SEQ ID NO: 25 and 26) and/or HyHEL-Fab (SEQ ID NO: 29 and 30), preferably by at least 20% compared to the host cell prior to engineering.

3. The host cell in item 1 or 2, wherein underexpression is achieved by knocking out the polynucleotide encoding the KO protein or a functional homologue thereof from the genome of said the host cell.

4. The host cell in item 1 or 2, wherein underexpression is achieved by disrupting the polynucleotide encoding the KO protein or a functional homologue thereof in the host cell.

5. The host cell in item 1 or 2, wherein underexpression is achieved by disrupting a promoter which is operably linked with said polypeptide encoding the KO protein or a functional homologue thereof.

6. The host cell in item 1 or 2, wherein underexpression is achieved by post-transcriptional gene silencing.

7. The host cell in any one of the preceding items, wherein the host cell is a *Pichia pastoris, Hansenula polymorpha, Trichoderma reesei, Saccharomyces cerevisiae, Kluyveromyces lactis, Yarrowia lipolytica, Pichia methanolica, Candida boidinii, and Komagataella, and Schizosaccharomyces pombe*.

8. The host cell of any of the preceding items, wherein the protein of interest is an enzyme, a therapeutic protein, a food additive or feed additive, preferably a detoxifying enzyme.

9. The host cell of item 8, wherein the therapeutic protein comprises an antibody or antibody fragment

10. The host cell in any of the preceding items, wherein said polynucleotide encoding the KO protein, preferably when underexpressed, increases the yield of the model protein SDZ-Fab (SEQ ID NO: 25 and 26) and/or HyHEL-Fab (SEQ ID NO: 29 and 30) by preferably at least 20% compared to the host cell prior to engineering.

11. The host cell in any one of the preceding items, wherein the host cell underexpresses said at least one polynucleotide encoding the KO protein or a functional homologue thereof and expresses the protein of interest.

12. The host cell in any one of the preceding items, wherein the host cell comprises at least one polynucleotide encoding a helper protein.

13. .The host cell of item 12, wherein the helper protein has an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162.

14. The host cell of item 12 or 13, wherein the host cell is engineered to express or overexpress the helper protein or a functional homologue thereof.

15. The host cell of any one of the preceding claims, wherein the host cell is engineered to

    (i) underexpress a polynucleotide encoding a protein having an amino acid as shown in SEQ ID NO: 10 or a functional homologue thereof and further engineered to overexpress a polynucleotide encoding a helper protein having an amino acid sequence as shown in SEQ ID NO: 1 or a functional homologue thereof and a helper protein having an amino acid sequence as shown in SEQ ID NO: 2 or a functional homologue thereof, or

    (ii) underexpress a polynucleotide encoding a protein having an amino acid as shown in SEQ ID NO: 10 or a functional homologue thereof and further engineered to overexpress a polynucleotide encoding a helper protein having an

amino acid sequence as shown in SEQ ID NO: 4 or a functional homologue thereof and SEQ ID NO: 1 or a functional homologue thereof.

16. A host cell obtained by providing a cell which expresses KO protein 1 or functional homologues thereof and engineering the cell to underexpress the gene which encodes KO1.

17. A host cell obtained by providing a cell which expresses KO protein 2 or functional homologues thereof and engineering the cell to underexpress the gene which encodes KO2.

18. A host cell obtained by providing a cell which expresses KO protein 3 or functional homologues thereof and engineering the cell to underexpress the gene encoding KO3.

19. Use of the host cell in any one of the preceding items for manufacturing a protein of interest.

20. A method of increasing the yield of a protein of interest in a host cell, comprising underexpressing at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12.

21. A method of increasing the yield of a protein of interest in a host cell comprising:

- engineering the host cell to underexpress at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12,

- recombining in said host cell a heterologous polynucleotide encoding a protein of interest,

- culturing said host cell under suitable conditions to express the protein of interest, and optionally

- isolating the protein of interest from the cell culture.

22. A method of producing a protein of interest in a host cell comprising:

- providing a host cell engineered to underexpress at least one polynucleotide encoding an KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12, wherein said host cell comprises a heterologous polynucleotide encoding a protein of interest;

- culturing the host cell under suitable conditions to express protein of interest, and optionally

- -isolating the protein of interest from the cell culture.

23. The method in any one of items 20-22, wherein underexpression is achieved by knocking out the polynucleotide encoding the KO protein or a functional homologue thereof from the genome of said the host cell.

24. The method in any one of items 20-22, wherein underexpression is achieved by disrupting the polynucleotide encoding the KO protein or a functional homologue thereof in the host cell.

25. The method in any one of items 20-23, wherein underexpression is achieved by disrupting a promoter which is operably linked with said polypeptide encoding the KO protein or a functional homologue thereof.

26. The method in any one of items 20-24, wherein underexpression is achieved by post-transcriptional gene silencing, preferably by expression of a heterologous RNA sequence which binds to transcripts from the polynucleotide encoding the KO protein.

27. The method in any one of items 20-26, wherein the host cell is a *Pichia pastoris, Hansenula polymorpha, Trichoderma reesei, Saccharomyces cerevisiae, Kluyveromyces lactis, Yarrowia lipolytica, Pichia methanolica, Candida boidinii, and Komagataella, and Schizosaccharomyces pombe*.

28. The method in any of items 20-27, wherein the protein of interest is an enzyme, a therapeutic protein, a food additive or feed additive, preferably a detoxifying enzyme.

29. The method of item 28, wherein the therapeutic protein comprises an antibody, or antibody fragment.

30. The method of any of items 20-29, wherein said polynucleotide encoding the KO protein, preferably when underexpressed, increases the yield of the model protein

SDZ-Fab (SEQ ID NO: 25 and 26) and/or HyHEL-Fab (SEQ ID NO: 29 and 30) by preferably at least 20% compared to the host cell prior to engineering

31. The method in any one of items 20-30, wherein the host cell comprises a polynucleotide encoding at least one helper protein.

32. The method of item 31, wherein the helper protein has an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162.

33. The method of any one of items 31 or 32, wherein the host cell is engineered to express or overexpress a helper protein having an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162.

34. A composition comprising at least 10%, 20%, 30%, 40%, or 50% of a protein of interest and a polynucleotide encoding a helper protein as defined in item 13, wherein said polynucleotide is operably linked with a heterologous promoter.

Detailed Description of the Invention

[0064] The present invention is concerned with the manipulation of KO1, KO2 and/or KO3 genes in host cells. It has been surprisingly found that the manipulated host has an increased capacity to produce an expressed protein of interest.

[0065] In a first aspect, the preset invention provides a recombinant host cell for manufacturing a protein of interest, wherein the host cell is engineered to underexpress at least one, such as at least 2, or at least 3, polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12.

[0066] For the purpose of the present invention the term “KO protein” is also meant to encompass functional homologues of KO1, KO2, or KO3.

KO proteins 1

[0067] The function of FLO8 in baker’s yeast is presumably a transcriptional activator that is required for diploid filamentous growth and haploid invasive growth and flocculation. It

has never been suggested to lead to an increased yield of protein production in a yeast or other host cell.

KO protein 2

[0068] KO2 encodes in baker's yeast the co-chaperone HCH1. HCH1 in some yeasts were found to regulate Hsp90 function (Armstrong et al., PLoS One. 2012;7(11):e49322.). It has never been suggested before that the underexpression of this protein leads to an increased yield of POI in a host cell.

KO protein 3

[0069] KO protein 3 encodes in baker's yeast one of several homologs of the bacterial chaperone DnaJ. It has never been suggested before that the underexpression of this protein leads to an increased yield of POI in the host cell.

[0070] From a chaperone (KO2, KO3) one would have expected that overexpression is beneficial, but it is in fact the deletion that has a positive effect. For KO1 which plays a role in flocculation in baker's yeast, one would not have expected that its deletion enhances protein secretion. Flocculation in baker's yeast is a phenomenon when cells aggregate reversibly in a vegetative growth phase, not related to mating. However, a skilled person would most likely not have assumed that knocking-out a flocculation gene enhances protein secretion.

[0071] "Sequence identity" or "% identity" refers to the percentage of residue matches between at least two polypeptide or polynucleotide sequences aligned using a standardized algorithm. Such an algorithm may insert, in a standardized and reproducible way, gaps in the sequences being compared in order to optimize alignment between two sequences, and therefore achieve a more meaningful comparison of the two sequences. For purposes of the present invention, the sequence identity between two amino acid sequences or nucleotide is determined using the NCBI BLAST program version 2.2.29 (Jan-06-2014) (Altschul et al., Nucleic Acids Res. (1997) 25:3389-3402). Sequence identity of two amino acid sequences can be determined with blastp set at the following parameters: Matrix: BLOSUM62, Word Size: 3; Expect value: 10; Gap cost: Existence = 11, Extension = 1; Filter = low complexity activated; Filter String: L; Compositional adjustments: Conditional compositional score matrix adjustment. For purposes of the present invention, the sequence identity between two nucleotide sequences is determined using the NCBI BLAST program version 2.2.29 (Jan-06-2014) with blastn set at the following parameters: Word Size: 11; Expect value: 10; Gap costs: Existence = 5 , Extension = 2; Filter = low complexity activated; Match/Mismatch Scores: 2,-3; Filter String: L; m.

Host cell

[0072] As used herein, a "host cell" refers to a cell which is capable of protein expression and optionally protein secretion. Such host cell is applied in the methods of the present invention. For that purpose, for the host cell to express a polypeptide, a nucleotide sequence encoding the polypeptide is present or introduced in the cell. Host cells provided by the present invention can be prokaryotes or eukaryotes. As will be appreciated by one of skill in the art, a prokaryotic cell lacks a membrane-bound nucleus, while a eukaryotic cell has a membrane-bound nucleus. Examples of eukaryotic cells include, but are not limited to, vertebrate cells, mammalian cells, human cells, animal cells, invertebrate cells, plant cells, nematodal cells, insect cells, stem cells, fungal cells or yeast cells.

[0073] Examples of yeast cells include but are not limited to the *Saccharomyces* genus (e.g. *Saccharomyces cerevisiae, Saccharomyces kluyveri, Saccharomyces uvarum*), the *Komagataella* genus (*Komagataella pastoris, Komagataella pseudopastoris or Komagataella phaffii), Kluyveromyces* genus (e.g. *Kluyveromyces lactis, Kluyveromyces marxianus), the Candida genus (e.g. Candida utilis, Candida cacaoi), the Geotrichum* genus (e.g. *Geotrichum fermentans*), as well as *Hansenula polymorpha and Yarrowia lipolytica*.

[0074] The genus *Pichia* is of particular interest. *Pichia* comprises a number of species, including the species *Pichia pastoris*, *Pichia methanolica*, *Pichia kluyveri*, and *Pichia angusta*. Most preferred is the species *Pichia pastoris*.

[0075] The former species *Pichia pastoris* has been divided and renamed to *Komagataella pastoris* and *Komagataella phaffii*. Therefore *Pichia pastoris* is synonymous for both *Komagataella pastoris* and *Komagataella phaffii*.

[0076] Examples for *Pichia pastoris* strains useful in the present invention are X33 and its subtypes GS115, KM71, KM71H; CBS7435 (mut+) and its subtypes CBS7435 mut^s, CBS7435 mut^s ΔArg, CBS7435 mut^s ΔHis, CBS7435 mut^s ΔArg, ΔHis, CBS7435 mut^s PDI^+, CBS 704 (=NRRL Y-1603 = DSMZ 70382), CBS 2612 (=NRRL Y-7556), CBS 9173-9189 and DSMZ 70877 as well as mutants thereof.

[0077] Examples of *E. coli* include those derived from *Escherichia coli* K12 strain, specifically, HMS 174, HMS174 (DE3), NM533, XL1-Blue, C600, DH1, HB101, JM109, as well as those derived from B-strains, specifically BL-21, BL21 (DE3) and the like.

[0078] According a further preferred embodiment, the host cell is a *Pichia pastoris, Hansenula polymorpha*, *Trichoderma reesei*, *Saccharomyces cerevisiae, Kluyveromyces*

lactis, Yarrowia lipolytica, Pichia methanolica, Candida boidinii, and Komagataella, and Schizosaccharomyces pombe. It may also be a host cell from *Ustilago maydis*.

[0079] Preferably, the helper proteins expressed by the host cell is from the same cell or recombined from a cell of the same species, genus or family. As used herein, "recombinant" refers to the alteration of genetic material by human intervention. Typically, recombinant refers to the manipulation of DNA or RNA in a virus, cell, plasmid or vector by molecular biology (recombinant DNA technology) methods, including cloning and recombination. A recombinant cell, polypeptide, or nucleic acid can be typically described with reference to how it differs from a naturally occurring counterpart (the "wild-type"). A "recombinant cell" or "recombinant host cell" refers to a cell or host cell that has been genetically altered to comprise a nucleic acid sequence which was not native to said cell.

[0080] The term "manufacture" or "manufacturing" as used presently refers to the process in which the protein of interest is expressed. A "host cell for manufacturing a protein of interest" refers to a host cell in which nucleic acid sequences encoding a protein of interest may be introduced. The recombinant host cell within the present invention does not necessarily contain the nucleic acid sequences encoding a protein of interest. It is appreciated by a skilled person in the art that the host cells can be provided for inserting desired nucleotide sequences into the host cell, for example, in a kit.

[0081] The terms "polypeptide" and "protein" are interchangeably used. The term "polypeptide" refers to a protein or peptide that contains two or more amino acids, typically at least 3, preferably at least 20, more preferred at least 30, such as at least 50 amino acids. Accordingly, a polypeptide comprises an amino acid sequence, and, thus, sometimes a polypeptide comprising an amino acid sequence is referred to herein as a "polypeptide comprising a polypeptide sequence". Thus, herein the term "polypeptide sequence" is interchangeably used with the term "amino acid sequence".

[0082] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an a carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to

chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

[0083] In a preferred embodiment, the present invention provides a host cell engineered to underexpress the polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12, wherein when the underexpression takes place, the yield of the model protein SDZ-Fab (SEQ ID NO: 25 for heavy chain and SEQ ID NO: 26 for light chain; Fig. 2) and/or HyHEL-Fab (SEQ ID NO: 29 for heavy chain and SEQ ID NO: 30 for light chain; Fig. 2) is increased by at least 10% compared to the host cell prior to engineering.

[0084] As used herein, "engineered" host cells are host cells which have been manipulated using genetic engineering, i.e. by human intervention. When a host cell is "engineered to underexpress" a given protein, the host cell is manipulated such that the host cell has no longer the capability to express a KO protein or functional homologue such as a non-engineered host cell with, thereby expression of a given protein, e.g. POI or model protein is increased compared to the host cell under the same condition prior to manipulation.

[0085] "Prior to engineering" when used in the context of host cells of the present invention means that such host cells are not engineered such that a polynucleotide encoding a KO protein or functional homologue thereof is underexpressed. Said term thus also means that host cells do not underexpress a polynucleotide encoding a KO protein or functional homologue thereof or are not engineered to underexpress a polynucleotide encoding a KO protein or functional homologue thereof.

[0086] The term "underexpression" includes any method that prevents or reduces the functional expression of one or more of KO1, KO2, KO3 or functional homologues thereof. This results in the incapability or reduction to exert its known function. Means of underexpression may include gene silencing (e.g. RNAi genes antisense), knocking-out, altering expression level, altering expression pattern, by mutagenizing the gene sequence, disrupting the sequence, insertions, additions, mutations, modifying expression control sequences, and the like. As mentioned herein, a host cell of the present invention is preferably engineered to underexpress a polynucleotide encoding a protein having an amino acid as defined herein. This includes that, if a host cell may have more than one copy of such a polynucleotide, also the other copies of such a polynucleotide are underexpressed. For example, a host cell of the present invention may not only be haploid, but it may be diploid,

tetraploid or even more –ploid. Accordingly, in a preferred embodiment all copies of such a polynucleotide are underexpressed, such as two, three, four, five, six or even more copies.

Method of Underexpression

[0087] Preferably, underexpression is achieved by knocking-out the polynucleotide encoding the KO protein in the host cell. A gene can be knocked out by deleting the entire or partial coding sequence. Methods of making gene knockouts are known in the art, e.g., see Kuhn and Wurst (Eds.) Gene Knockout Protocols (Methods in Molecular Biology) Humana Press (March 27, 2009). A gene can also be knocked out by removing part or all of the gene sequence. Alternatively, a gene can be knocked-out or inactivated by the insertion of a nucleotide sequence, such as a resistance gene. Alternatively, a gene can be knocked-out or inactivated by inactivating its promoter.

[0088] In an embodiment, underexpression is achieved by disrupting the polynucleotide encoding the gene in the host cell.

[0089] A “disruption” is a change in a nucleotide or amino acid sequence, which resulted in the addition, deleting, or substitution of one or more nucleotides or amino acid residues, as compared to the original sequence prior to the disruption.

[0090] An “insertion” or “addition” is a change in a nucleic acid or amino acid sequence in which one or more nucleotides or amino acid residues have been added as compared to the original sequence prior to the disruption.

[0091] A “deletion” is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, have been removed (i.e., are absent). A deletion encompasses deletion of the entire sequence, deletion of part of the coding sequence, or deletion of single nucleotides or amino acid residues.

[0092] A "substitution" generally refers to replacement of nucleotides or amino acid residues with other nucleotides or amino acid residues. “Substitution” can be performed by site-directed mutation, generation of random mutations, and gapped-duplex approaches (See e.g., U.S. Pat. No. 4,760,025; Moring et al., Biotech. (1984) 2:646 ; and Kramer et al., Nucleic Acids Res., (1984) 12:9441). Site-directed mutagenesis can be accomplished in vitro by PCR involving the use of oligonucleotide primers containing the desired mutation. Site-directed mutagenesis can also be performed in vitro by cassette mutagenesis involving the cleavage by a restriction enzyme at a site in the plasmid comprising a polynucleotide encoding the parent and subsequent ligation of an oligonucleotide containing the mutation in

the polynucleotide. Usually the restriction enzyme that digests the plasmid and the oligonucleotide is the same, permitting sticky ends of the plasmid and the insert to ligate to one another. See, e.g., Scherer and Davis, 1979, Proc. Natl. Acad. Sci. USA 76: 4949-4955; and Barton et ai, 1990, Nucleic Acids Res. 18: 7349-4966. Site-directed mutagenesis can also be accomplished in vivo by methods known in the art. See, e.g., U.S. Patent Application Publication No. 2004/0171 154; Storici et ai, 2001 , Nature Biotechnol. 19: 773-776; Kren et ai, 1998, Nat. Med. 4: 285-290; and Calissano and Macino, 1996, Fungal Genet. Newslett. 43: 15-16. Synthetic gene construction entails in vitro synthesis of a designed polynucleotide molecule to encode a polypeptide of interest. Gene synthesis can be performed utilizing a number of techniques, such as the multiplex microchip-based technology described by Tian et al. (2004, Nature 432: 1050-1054) and similar technologies wherein oligonucleotides are synthesized and assembled upon photo-programmable microfluidic chips. Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, Science 241:53-57; Bowie and Sauer, 1989, Proc. Natl. Acad. Sci. USA 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (e.g., Lowman et al, 1991, Biochemistry 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204) and region-directed mutagenesis (Derbyshire et al., 1986, Gene 46: 145; Ner et al., 1988, DNA 7:127). Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness et a/., 1999, Nature Biotechnology 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide. Semi-synthetic gene construction is accomplished by combining aspects of synthetic gene construction, and/or site-directed mutagenesis, and/or random mutagenesis, and/or shuffling. Semisynthetic construction is typified by a process utilizing polynucleotide fragments that are synthesized, in combination with PCR techniques. Defined regions of genes may thus be synthesized de novo, while other regions may be amplified using site-specific mutagenic primers, while yet other regions may be subjected to error-prone PCR or non-error prone PCR amplification. Polynucleotide subsequences may then be shuffled. Alternatively, homologues can be obtained from a natural source such as by screening cDNA libraries of closely or distantly related microorganisms.

[0093] Preferably, disruption results in a frame shift mutation, early stop codon, point mutations of critical residues, translation of a nonsense or otherwise non-functional protein product.

[0094] In another embodiment, underexpression is achieved by disrupting the promoter which is operably linked with said polypeptide encoding the KO protein. A promoter directs the transcription of a downstream gene. The promoter is necessary, together with other expression control sequences such as ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences, to express a given gene. Therefore, it is also possible to disrupt any of the expression control sequence to hinder the expression of the polypeptide encoding the KO protein.

[0095] A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence on the same nucleic acid molecule. For example, a promoter is operably linked with a coding sequence of a recombinant gene when it is capable of effecting the expression of that coding sequence.

[0096] In another embodiment, underexpression is achieved by post-transcriptional gene silencing (PTGS). A technique commonly used in the art, PTGS reduces the expression level of a gene via expression of a heterologous RNA sequence, frequently antisense to the gene requiring disruption (Lechtreck et al., J. Cell Sci (2002). 115:1511-1522; Smith et al., Nature (2000). 407:319-320; Furhmann et al., J. Cell Sci (2001). 114:3857-3863; Rohr et al., Plant J (2004). 40(4):611-21. Post-transcriptional gene silencing is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules using small RNAs including microRNA (miRNA), small interfering RNA (siRNA) siRNA, or antisense RNA. Gene silencing can occur either through the blocking of transcription (in the case of gene-binding), the degradation of the mRNA transcript (e.g. by small interfering RNA (siRNA) or RNase-H dependent antisense), or through the blocking of either mRNA translation, pre-mRNA splicing sites, or nuclease cleavage sites used for maturation of other functional RNAs, including miRNA (e.g. by Morpholino oligos or other RNase-H independent antisense). These small RNAs can bind to other specific messenger RNA (mRNA) molecules and decrease their activity, for example by preventing an mRNA from producing a protein. Exemplary siRNA molecules have a length from about 10-50 or more nucleotides. The small RNA molecules comprise at least one strand that has a sequence that is "sufficiently complementary" to a target mRNA sequence to direct target-specific RNA interference (RNAi). Small interfering RNAs can originate from inside the cell or can be exogenously introduced into the cell. Once introduced into the cell, exogenous siRNAs are processed by the RNA-induced silencing complex (RISC). The siRNA is complementary to the target mRNA to be silenced, and the RISC uses the siRNA as a template for locating the target mRNA. After the RISC localizes to the target mRNA, the RNA can be cleaved by a ribonuclease. The strand has a sequence sufficient to trigger the destruction of the target mRNA by the RNAi machinery or process is commonly referred to

as an antisense strand in the context of a ds-siRNA molecule. The siRNA molecule can be designed such that every residue is complementary to a residue in the target molecule. PTGS is found in many organisms. For yeast cells, the fission yeast, *Schizosaccharomyces pombe*, has an active RNAi pathway involved in heterochromatin formation and centromeric silencing (Raponi et al., Nucl. Acids Res. (2003) 31 (15): 4481-4489). Some budding yeasts, including *Saccharomyces cerevisiae*, *Candida albicans and Kluyveromyces polysporus* were also found to have such RNAi pathway (Bartel et la., Science Express doi:10.1126/ science.1176945, published online 10 September 2009)."Underexpression" can be achieved with any known techniques in the art which lowers gene expression. For example, the promoter which is operably linked with the polypeptide encoding the KO protein can be replaced with another promoter which has lower promoter activity. Promoter activity may be assessed by its transcriptional efficiency. This may be determined directly by measurement of the amount of mRNA transcription from the promoter, e.g. by Northern Blotting, quantitative PCR or indirectly by measurement of the amount of gene product expressed from the promoter.

[0097] Underexpression may in another embodiment be achieved by intervening in the folding of the expressed KO protein so that the KO protein is not properly folded to become functional. For example, mutation can be introduced to remove a disulfide bond formation of the KO protein or to disruption the formation of an alpha helices and beta sheets.

Protein of Interest

[0098] The term "protein of interest" (POI) as used herein refers to a protein that is produced by means of recombinant technology in a host cell. More specifically, the protein may either be a polypeptide not naturally occurring in the host cell, i.e. a heterologous protein, or else may be native to the host cell, i.e. a homologous protein to the host cell, but is produced, for example, by transformation with a self-replicating vector containing the nucleic acid sequence encoding the POI, or upon integration by recombinant techniques of one or more copies of the nucleic acid sequence encoding the POI into the genome of the host cell, or by recombinant modification of one or more regulatory sequences controlling the expression of the gene encoding the POI, e.g. of the promoter sequence. In general, the proteins of interest referred to herein may be produced by methods of recombinant expression well known to a person skilled in the art.

[0099] There is no limitation with respect to the protein of interest (POI). The POI is usually a eukaryotic or prokaryotic polypeptide, variant or derivative thereof. The POI can be any eukaryotic or prokaryotic protein. The protein can be a naturally secreted protein or an

intracellular protein, i.e. a protein which is not naturally secreted. The present invention also includes biologically active fragments of proteins. In another embodiment, a POI may be an amino acid chain or present in a complex, such as a dimer, trimer, hetero-dimer, multimer or oligomer.

[00100] The protein of interest may be a protein used as nutritional, dietary, digestive, supplements, such as in food products, feed products, or cosmetic products. The food products may be, for example, bouillon, desserts, cereal bars, confectionery, sports drinks, dietary products or other nutrition products. Preferably, the protein of interest is a food additive.

[00101] In another embodiment, the protein of interest may be used in animal feeds. The POI may be a detoxifying enzyme such as a mycotoxin degrading enzyme. A detoxifying enzyme means an enzyme which breaks down a toxin such to reduce its toxicity. Mycotoxins are toxic secondary metabolites produced by fungi that readily colonize crops and are often characterized by the ability to harm crops and cause health problems for people and animals. Mycotoxin degrading enzymes include aflatoxin detoxizyme, zearalenone esterases, zearalenone lactonases, zearalenone hydrolase, fumonisin carboxylesterases, fumonisin aminotransferases, aminopolyol amine oxidases, deoxynivalenol expoxide hydrolases. The POI may also be an enzyme which degrades ochratoxin derivatives or ergot alkaloid. Ochratoxins are a group of mycotoxins produced as secondary metabolites by several fungi of the *Aspergillus* or *Penicillium* families and are weak organic acids consisting of a derivative of an isocoumarin. There are three generally recognized ochratoxins, designated A, B and C. Ochratoxin A is the most abundant member of the ochratoxin family and hence the most commonly detected, but is also the most toxic. Ochratoxin A (ochratoxin A) is a nephrotoxic, teratogenic, hepatotoxic, and carcinogenic mycotoxin present in cereals and other starch rich foods. Ergot alkaloids are compounds containing amide bonds and include, for example, ergocornine, ergocorninine, ergocristine, ergocristinine, ergocryptine, ergocryptinine, ergometrine, ergosine, ergotamine and ergotaminine. These compounds are toxic to living organisms including humans and farm animals. Examples of such enzymes include ochratoxin amidase, ergotamine hydrolase, ergotamine amylase. Mycotoxin degrading enzymes in animal feed is useful in controlling mycotoxin contamination of feed.

[00102] Further examples of POI include anti-microbial proteins, such as lactoferrin, lysozyme, lactoferricin, lactohedrin, kappa-casein, haptocorrin, lactoperoxidase, a milk protein, acute-phase proteins, e.g., proteins that are produced normally in production animals in response to infection, and small anti-microbial proteins such as lysozyme and lactoferrin. Other examples include bactericidal protein, antiviral proteins, acute phase proteins (induced

in production animals in response to infection), probiotic proteins, bacteriostatic protein, and cationic antimicrobial proteins.

[00103] "Feed" means any natural or artificial diet, meal or the like or components of such meals intended or suitable for being eaten, taken in, digested, by a non-human animal. A “feed additive” generally refers to substances added in a feed. It typically include one or more compounds such as vitamins, minerals, enzymes and suitable carriers and/or excipient. For the present invention, a food additive may be an enzyme or other proteins. Examples of enzymes which can be used as feed additive include phytase, xylanase and β-glucanase.A "food" means any natural or artificial diet meal or the like or components of such meals intended or suitable for being eaten, taken in, digested, by a human being.

[00104] A “food additive” is generally refers to substances added in a food. It typically includes one or more compounds such as vitamins, minerals, enzymes and suitable carriers and/or excipient. For the present invention, a food additive may be an enzyme or other proteins. Examples of enzymes which can be used as food additive include protease, lipase, lactase, pectin methyl esterase, pectinase, transglutaminase, amylase, β-glucanase, acetolactate decarboxylase and laccase.

[00105] In some embodiments, the food additive is an anti-microbial protein, which includes, for example, (i) anti-microbial milk proteins (either human or non-human) lactoferrin, lysozyme, lactoferricin, lactohedrin, kappa-casein, haptocorrin, lactoperoxidase, alpha- 1-antitrypsin, and immunoglobulins, e.g., IgA, (ii) acute-phase proteins, such as C-reactive protein (CRP); lactoferrin; lysozyme; serum amyloid A (SAA); ferritin; haptoglobin (Hp); complements 2-9, in particular complement-3; seromucoid; ceruloplasmin (Cp); 15-keto-13,14-dihydro- prostaglandin F2 alpha (PGFM); fibrinogen (Fb); alpha(1)-acid glycoprotein (AGP); alpha(1)-antitrypsin; mannose binding protein; lipoplysaccharide binding protein; alpha-2 macroglobulin and various defensins, (iii) antimicrobial peptides, such as cecropin, magainin, defensins, tachyplesin, parasin I.buforin I, PMAP-23, moronecidin, anoplin, gambicin, and SAMP-29, and (iv) other anti-microbial protein(s), including CAP37, granulysin, secretory leukocyte protease inhibitor, CAP18, ubiquicidin, bovine antimicrobial protein-1 , Ace- AMP1 , tachyplesin, big defensin, Ac-AMP2, Ah-AMP1 , and CAP18.

[00106] A POI may be an enzyme. Preferred enzymes are those which can be used for industrial application, such as in the manufacturing of a detergent, starch, fuel, textile, pulp and paper, oil, personal care products, or such as for baking, organic synthesis, and the like. Examples of such enzymes include protease, amylase, lipase, mannanase and cellulose for stain removal and cleaning; pullulanase amylase and amyloglucosidase for starch liquefaction and saccharification; glucose isomerase for glucose to fructose conversion;

cyclodextrin-glycosyltransferase for cyclodextrin production; xylanase for xiscosity reduction in fuel and starch; amylase, xylanase, lipase, phospholipase, glucose, oxidase, lipoxygenase, transglutaminase for dough stability and conditioning in baking; cellulase in textile manufacturing for denim finishing and cotton softening; amylase for de-sizing of texile; pectate lyase for scouring; catalase for bleach termination; laccase for bleaching; peroxidase for excess dye removal; lipase, protease, amylase, xylanase, cellulose, in pulp and paper production; lipase for transesterification and phospholipase for de-gumming in fat processing fats and oils; lipase for resolution of chiral alcohols and amides in organic synthesis; acylase for synthesis of semisynthetic penicillin, nitrilase for the synthesis of enantiopure carboxylic acids; protease and lipase for leather production; amyloglucosidase, glucose oxidase, and peroxidase for the making personal care products (see Kirk et al., Current Opinion in Biotechnology (2002) 13:345-351)

Therapeutic protein

[00107] A POI may be a therapeutic protein. A POI may be but is not limited to a protein suitable as a biopharmaceutical substance like an antibody or antibody fragment, growth factor, hormone, enzyme, vaccine, or.

[00108] The POI may be a naturally secreted protein or an intracellular protein, i.e. a protein which is not naturally secreted. The present invention also provides for the recombinant production of functional homologues, functional equivalent variants, derivatives and biologically active fragments of naturally secreted or not naturally secreted proteins. Functional homologues are preferably identical with or correspond to and have the functional characteristics of a sequence.

[00109] The POI may be structurally similar to the native protein and may be derived from the native protein by addition of one or more amino acids to either or both the C- and N-terminal end or the side-chain of the native protein, substitution of one or more amino acids at one or a number of different sites in the native amino acid sequence, deletion of one or more amino acids at either or both ends of the native protein or at one or several sites in the amino acid sequence, or insertion of one or more amino acids at one or more sites in the native amino acid sequence. Such modifications are well known for several of the proteins mentioned above.

[00110] Preferably, the protein of interest is a mammalian polypeptide or even more preferably a human polypeptide. Especially preferred therapeutic proteins, which refer to any polypeptide, protein, protein variant, fusion protein and/or fragment thereof which may be administered to a mammal. It is envisioned but not required that therapeutic protein

according to the present invention is heterologous to the cell. Examples of proteins that can be produced by the cell of the present invention are, without limitation, enzymes, regulatory proteins, receptors, peptide hormones, growth factors, cytokines, scaffold binding proteins (e.g. anticalins), structural proteins, lymphokines, adhesion molecules, receptors, membrane or transport proteins, and any other polypeptides that can serve as agonists or antagonists and/or have therapeutic or diagnostic use. Moreover, the proteins of interest may be antigens as used for vaccination, vaccines, antigen-binding proteins, immune stimulatory proteins. It may also be an antigen-binding fragment of an antibody, which can include any suitable antigen-binding antibody fragment known in the art. For example, an antibody fragment may include but not limited to Fv (a molecule comprising the VL and VH), single-chain Fv (scFV) (a molecule comprising the VL and VH connected with by peptide linker), Fab, Fab', $F(ab')_2$, single domain antibody (sdAb) (molecules comprising a single variable domain and 3 CDR), and multivalent presentations thereof. The antibody or fragments thereof may be murine, human, humanized or chimeric antibody or fragments thereof. Examples of therapeutic proteins include an antibody, polyclonal antibody, monoclonal antibody, recombinant antibody, antibody fragments, such as Fab', $F(ab')_2$, Fv, scFv, di-scFvs, bi-scFvs, tandem scFvs, bispecific tandem scFvs, sdAb, nanobodies, V_H, and V_L, or human antibody, humanized antibody, chimeric antibody, IgA antibody, IgD antibody, IgE antibody, IgG antibody, IgM antibody, intrabody, minibody or monobody.

[00111] Such therapeutic proteins include, but are not limited to, insulin, insulin-like growth factor, hGH, tPA, cytokines, e.g. interleukines such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, interferon (IFN) alpha, IFN beta, IFN gamma, IFN omega or IFN tau, tumor necrosisfactor (TNF) TNF alpha and TNF beta, TRAIL; G-CSF, GM-CSF, M-CSF, MCP-1 and VEGF.

[00112] In a preferred embodiment, the protein is an antibody. The term "antibody" is intended to include any polypeptide chain-containing molecular structure with a specific shape that fits to and recognizes an epitope, where one or more non-covalent binding interactions stabilize the complex between the molecular structure and the epitope. The archetypal antibody molecule is the immunoglobulin, and all types of immunoglobulins, IgG, IgM, IgA, IgE, IgD, etc., from all sources, e.g. human, rodent, rabbit, cow, sheep, pig, dog, other mammals, chicken, other avians, etc., are considered to be "antibodies." Numerous antibody coding sequences have been described; and others may be raised by methods well-known in the art.

[00113] For example, antibodies or antigen binding fragments may be produced by methods known in the art. Generally, antibody-producing cells are sensitized to the desired antigen or immunogen. The messenger RNA isolated from antibody producing cells is used

as a template to make cDNA using PCR amplification. A library of vectors, each containing one heavy chain gene and one light chain gene retaining the initial antigen specificity, is produced by insertion of appropriate sections of the amplified immunoglobulin cDNA into the expression vectors. A combinatorial library is constructed by combining the heavy chain gene library with the light chain gene library. This results in a library of clones which co-express a heavy and light chain (resembling the Fab fragment or antigen binding fragment of an antibody molecule). The vectors that carry these genes are co-transfected into a host cell. When antibody gene synthesis is induced in the transfected host, the heavy and light chain proteins self-assemble to produce active antibodies that can be detected by screening with the antigen or immunogen.

[00114] Antibody coding sequences of interest include those encoded by native sequences, as well as nucleic acids that, by virtue of the degeneracy of the genetic code, are not identical in sequence to the disclosed nucleic acids, and variants thereof. Variant polypeptides can include amino acid (aa) substitutions, additions or deletions. The amino acid substitutions can be conservative amino acid substitutions or substitutions to eliminate non-essential amino acids, such as to alter a glycosylation site, or to minimize misfolding by substitution or deletion of one or more cysteine residues that are not necessary for function. Variants can be designed so as to retain or have enhanced biological activity of a particular region of the protein (e.g., a functional domain, catalytic amino acid residues, etc). Variants also include fragments of the polypeptides disclosed herein, particularly biologically active fragments and/or fragments corresponding to functional domains. Techniques for in vitro mutagenesis of cloned genes are known. Also included in the subject invention are polypeptides that have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a therapeutic agent.

[00115] Chimeric antibodies may be made by recombinant means by combining the variable light and heavy chain regions (VK and VH), obtained from antibody producing cells of one species with the constant light and heavy chain regions from another. Typically, chimeric antibodies utilize rodent or rabbit variable regions and human constant regions, in order to produce an antibody with predominantly human domains. The production of such chimeric antibodies is well known in the art, and may be achieved by standard means (as described, e.g., in U.S. Patent No. 5,624,659.

[00116] Humanized antibodies are engineered to contain even more human-like immunoglobulin domains, and incorporate only the complementarity-determining regions of the animal-derived antibody. This is accomplished by carefully examining the sequence of the hyper-variable loops of the variable regions of the monoclonal antibody, and fitting them

to the structure of the human antibody chains. Although facially complex, the process is straightforward in practice. See, e.g., U.S. Patent No. 6,187,287.

[00117] In addition to entire immunoglobulins (or their recombinant counterparts), immunoglobulin fragments comprising the epitope binding site (e.g., Fab', F(ab')2, or other fragments) may be synthesized. "Fragment" or minimal immunoglobulins may be designed utilizing recombinant immunoglobulin techniques. For instance "Fv" immunoglobulins for use in the present invention may be produced by synthesizing a variable light chain region and a variable heavy chain region. Combinations of antibodies are also of interest, e.g. diabodies, which comprise two distinct Fv specificities.

[00118] Immunoglobulins may be modified post-translationally, e.g. to add chemical linkers, detectable moieties, such as fluorescent dyes, enzymes, substrates, chemiluminescent moieties and the like, or specific binding moieties, such as streptavidin, avidin, or biotin, and the like may be utilized in the methods and compositions of the present invention.

[00119] Further examples of therapeutic proteins include blood coagulation factors (VII, VIII, IX), alkaline protease from Fusarium, calcitonin, CD4 receptor darbepoetin, DNase (cystic fibrosis), erythropoetin, eutropin (human growth hormone derivative), follicle stimulating hormone (follitropin), gelatin, glucagon, glucocerebrosidase (Gaucher disease), glucosamylase from A. niger, glucose oxidase from A. niger, gonadotropin, growth factors (GCSF, GMCSF), growth hormones (somatotropines),hepatitis B vaccine, hirudin, human antibody fragment, human apolipoprotein AI, human calcitonin precursor ,human collagenase IV, human epidermal growth factor, human insulin-like growth factor, human interleukin 6, human laminin, human proapolipoprotein AI, human serum albumininsulin, insulin and muteins, insulin, interferon alpha and muteins, interferon beta, interferon gamma (mutein), interleukin 2, luteinization hormone, monoclonal antibody 5T4, mouse collagen,OP-1 (osteogenic, neuroprotective factor), oprelvekin (interleukin 11-agonist), organophosphohydrolase, PDGF-agonist, phytase, platelet derived growth factor (PDGF), recombinant plasminogen-activator G, staphylokinase, stem cell factor, tetanus toxin fragment C, tissue plasminogen-activator, and tumor necrosis factor (see Schmidt, Appl Microbiol Biotechnol (2004) 65:363-372).

Leader sequence

[00120] The protein of interest may be linked with a leader sequence which causes secretion of the POI from the host cell. The presence of such a secretion leader sequence in the expression vector is required when the POI intended for recombinant expression and

secretion is a protein which is not naturally secreted and therefore lacks a natural secretion leader sequence, or its nucleotide sequence has been cloned without its natural secretion leader sequence. In general, any secretion leader sequence effective to cause secretion of the POI from the host cell may be used in the present invention. The secretion leader sequence may originate from yeast source, e.g. from yeast α-factor such as MFa of *Saccharomyces cerevisiae*, or yeast phosphatase, from mammalian or plant source, or others. The selection of the appropriate secretion leader sequence is apparent to a skilled person. Alternatively, the secretion leader sequence can be fused to the nucleotide sequence encoding a POI intended for recombinant expression by conventional cloning techniques known to a skilled person prior to cloning of the nucleotide sequence in the expression vector or the nucleotide sequence encoding a POI comprising a natural secretion leader sequence is cloned in the expression vector. In these cases the presence of a secretion leader sequence in the expression vector is not required.

Promoter

[00121] The sequence encoding POI may be operably linked to a promoter. The term "promoter" as used herein refers to a region that facilitates the transcription of a particular gene. A promoter typically increases the amount of recombinant product expressed from a nucleotide sequence as compared to the amount of the expressed recombinant product when no promoter exists. A promoter from one organism can be utilized to enhance recombinant product expression from a sequence that originates from another organism. The promoter can be integrated into a host cell chromosome by homologous recombination using methods known in the art (e.g. Datsenko et al, Proc. Natl. Acad. Sci. U.S.A., 97(12): 6640-6645 (2000)). In addition, one promoter element can increase the amount of products expressed for multiple sequences attached in tandem. Hence, one promoter element can enhance the expression of one or more recombinant products.

[00122] The promoter could be an "inducible promoter" or "constitutive promoter." "Inducible promoter" refers to a promoter which can be induced by the presence or absence of certain factors, and "constitutive promoter" refers to an unregulated promoter that allows for continuous transcription of its associated gene or genes.

[00123] In a preferred embodiment, the nucleotide sequences encoding the POI is driven by an inducible promoter.

[00124] Many inducible promoters are known in the art. Many are described in a review by Gatz, Curr. Op. Biotech., 7: 168 (1996) (see also Gatz, Ann. Rev. Plant. Physiol. Plant Mol. Biol., 48:89 (1997)). Examples include tetracycline repressor system, Lac repressor

system, copper-inducible systems, salicylate-inducible systems (such as the PR1 a system), glucocorticoid-inducible (Aoyama et al., 1997), alcohol-inducible systems, e.g., AOX promoters, and ecdysome-inducible systems. Also included are the benzene sulphonamide-inducible (U.S. Patent No. 5,364,780) and alcohol-inducible (WO 97/06269 and WO 97/06268) inducible systems and glutathione S-transferase promoters.

[00125] Suitable promoter sequences for use with yeast host cells are described in Mattanovich et al., Methods Mol. Biol. (2012) 824:329-58 and include glycolytic enzymes like triosephosphate isomerase (TPI), phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH or GAP) and variants thereof, lactase (LAC) and galactosidase (GAL), *P. pastoris* glucose-6-phosphate isomerase promoter (PPGI), the 3-phosphoglycerate kinase promoter (PPGK), the glycerol aldehyde phosphate dehydrogenase promoter (PGAP), translation elongation factor promoter (PTEF), and the promoters of *P. pastoris* enolase 1 (PENO1), triose phosphate isomerase (PTPI), ribosomal subunit proteins (PRPS2, PRPS7, PRPS31, PRPL1), alcohol oxidase promoter (PAOX) or variants thereof with modified characteristics, the formaldehyde dehydrogenase promoter (PFLD), isocitrate lyase promoter (PICL), alpha-ketoisocaproate decarboxylase promoter (PTHI), the promoters of heat shock protein family members (PSSA1, PHSP90, PKAR2), 6-Phosphogluconate dehydrogenase (PGND1), phosphoglycerate mutase (PGPM1), transketolase (PTKL1), phosphatidylinositol synthase (PPIS1), ferro-O2-oxidoreductase (PFET3), high affinity iron permease (PFTR1), repressible alkaline phosphatase (PPHO8), N-myristoyl transferase (PNMT1), pheromone response transcription factor (PMCM1), ubiquitin (PUBI4), single-stranded DNA endonuclease (PRAD2), the promoter of the major ADP/ATP carrier of the mitochondrial inner membrane (PPET9) (WO2008/128701) and the formate dehydrogenase (FMD) promoter. The GAP promoter, AOX promoter or a promoter derived from GAP or AOX promoter is particularly preferred. AOX promoters can be induced by methanol and are repressed by glucose.

[00126] Further examples of suitable promoters include Saccharomyces cerevisiae enolase (ENO-1), Saccharomyces cerevisiae galactokinase (GAL1), Saccharomyces cerevisiae alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), Saccharomyces cerevisiae triose phosphate isomerase (TPI), Saccharomyces cerevisiae metallothionein (CUP1), and Saccharomyces cerevisiae 3-phosphoglycerate kinase (PGK), and the maltase gene promoter (MAL).

[00127] Other useful promoters for yeast host cells are described by Romanos et al, 1992, Yeast 8:423-488.

[00128] Suitable promoter sequences for use with E. coli include T7 promoter, T5 promoter, tryptophan (trp) promoter, lactose (lac) promoter, tryptophan/lactose (tac) promoter, lipoprotein (lpp) promoter, and λ phage PL promoter in plasmids.

[00129] As shown in the examples, the inventors have discovered that when the genes are underexpressed, the yield of the POI (SDZ-Fab or HyHEL-Fab) by at least 20% compared to the host cell without the underexpression.

In a preferred embodiment, underexpression of KO1 gene increases the yield of the model protein SDZ-Fab or HyHEL-Fab in the host cell by at least 1%, such as at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or at least 200% or more, compared to the host cell prior to engineering.

In a preferred embodiment, underexpression of KO2 gene increases the yield of the model protein SDZ-Fab or HyHEL-Fab in the host cell by at least 1%, such as at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or at least 200% or more, compared to the host cell prior to engineering.

In a preferred embodiment, underexpression of KO3 gene increases the yield of the model protein SDZ-Fab or HyHEL-Fab in the host cell by at least 1%, such as at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or at least 200% or more, compared to the host cell prior to engineering.

[00130] It is envisioned that in preferred embodiments, when the host cell are cultured to produce the protein of interest, the cell underexpresses at least one the polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12 and expresses the protein of interest.

[00131] In a further aspect, the present invention provides the use of the engineered host cell for manufacturing a protein of interest. The host cells can be advantageously used for introducing polynucleotides encoding one or more POI(s), and thereafter can be cultured under suitable conditions to express the POI. Details of such use are described in the later section concerning methods of the present invention.

[00132] The POI encoding polynucleotide may be recombined in to the host cell by ligating the relevant genes into a vector, and transforming the host cell with the vectors. More teaching can be found in the following sections of the application.

[00133] Generally, proteins of interest can be produced by culturing the host cell in an appropriate medium, isolating the expressed POI from the culture, and purifying it by a method appropriate for the expressed product, in particular to separate the POI from the cell.

[00134] In another aspect, the present invention provides a method of increasing the yield of a protein of interest in a host cell, comprising underexpressing at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12.

[00135] As used herein, the term "increasing the yield of a protein of interest in a host cell" means that the yield of the protein of interest is increased when compared to the same cell expressing the same POI under the same culturing conditions, however, without the underexpression of the knockout protein(s).

[00136] As will be appreciated by a skilled person in the art, the underexpression of the present KO protein(s) have been shown to increase product yield of POI. Therefore, for a given host cell which expressed a POI with a level that should be increased, it is possible to apply the present invention by underexpressing the present KO protein(s).

[00137] In yet a further aspect, the present invention provides a method of increasing the yield of a protein of interest in a host cell. The method comprises (i) engineering the host cell to underexpress at least one, polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12, (ii) recombining in said host cell a heterologous polynucleotide encoding a protein of interest, and (iii) culturing said host cell under suitable conditions to express the protein of interest.

[00138] As used herein, the term "recombining" as used herein means that a host cell of the present invention is equipped with a heterologous polynucleotide encoding a protein of interest, i.e., a host cell of the present invention is engineered to contain a heterologous polynucleotide encoding a protein of interest. This can be achieved, e.g., by transformation or transfection or any other suitable technique known in the art for the introduction of a polynucleotide into a host cell.

[00139] Procedures used to manipulate polynucleotide sequences, e.g. coding for the POI, the promoter, enhancers, leaders, etc., are well known to persons skilled in the art, e.g. described by J. Sambrook et al., Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York (2001).

[00140] A foreign or target polynucleotide such as the polynucleotides encoding the POI or helper protein can be inserted into the chromosome by various means, e.g., by homologous recombination or by using a hybrid recombinase that specifically targets sequences at the integration sites. The foreign or target polynucleotide described above is typically present in a vector ("inserting vector"). These vectors are typically circular and linearized before used for homologous recombination. As an alternative, the foreign or target polynucleotides may be DNA fragments joined by fusion PCR or synthetically constructed DNA fragments which are then recombined into the host cell. In addition to the homology arms, the vectors may also contain markers suitable for selection or screening, an origin of replication, and other elements

[00141] Polynucleotides encoding the target sequence may also be present on an expression vector. Such vectors are known in the art and already described above. In expression vectors, a promoter is placed upstream of the gene encoding the heterologous protein and regulates the expression of the gene. Multi-cloning vectors are especially useful due to their multi-cloning site. For expression, a promoter is generally placed upstream of the multi-cloning site. A vector for integration of the polynucleotides encoding the POI may be constructed either by first preparing a DNA construct containing the entire DNA sequence coding for the POI and subsequently inserting this construct into a suitable expression vector, or by sequentially inserting DNA fragments containing genetic information for the individual elements, such as the leader sequence, the target DNA sequence, followed by ligation. As an alternative to restriction and ligation of fragments, recombination methods based on attachemtn (att) sites and recombination enzymes may be used to insert DNA sequences into a vector. Such methods are described, for example, by Landy (1989) Ann. Rev. Biochem. 58:913-949; and are known to those of skill in the art.

[00142] Host cells according to the present invention can be obtained by introducing a vector or plasmid comprising the target polynucleotide sequences into the cells. Techniques for transfecting or transforming eukaryotic cells or transforming prokaryotic cells are well known in the art. These can include lipid vesicle mediated uptake, heat shock mediated uptake, calcium phosphate mediated transfection (calcium phosphate/DNA co-precipitation), viral infection, particularly using modified viruses such as, for example, modified adenoviruses, microinjection and electroporation. For prokaryotic transformation, techniques can include heat shock mediated uptake, bacterial protoplast fusion with intact cells,

microinjection and electroporation. Techniques for plant transformation include *Agrobacterium* mediated transfer, such as by *A. tumefaciens*, rapidly propelled tungsten or gold microprojectiles, electroporation, microinjection and polyethylyne glycol mediated uptake. The DNA can be single or double stranded, linear or circular, relaxed or supercoiled DNA. For various techniques for transfecting mammalian cells, see, for example, Keown et al. (1990) Processes in Enzymology 185:527-537.

[00143] In a further aspect, the present invention provides a method of manufacturing a protein of interest in a host cell comprising (i) providing a host cell engineered to underexpress at least one polynucleotide encoding a protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11, 12, wherein said host cell comprises a heterologous polynucleotide encoding a protein of interest; and (ii) culturing the host cell under suitable conditions to express the protein of interest. It is understood that the methods disclosed herein may further include cultivating said recombinant host cells under conditions permitting the expression of the POI. A recombinantly produced POI can then be isolated from the cell or the cell culture medium, depending on the nature of the expression system and the expressed protein, e.g. whether the protein is fused to a signal peptide and whether the protein is soluble or membrane-bound. As will be understood by the skilled artisan, cultivation conditions will vary according to factors that include the type of host cell, in particular the expression vector employed. Signal peptides generally contain a positively charged N-terminus followed by a hydrophobic core, followed by a recognition site for an enzyme known as signal peptidase. This enzyme cleaves the signal peptide from the protein during translocation. The protein is transported from the endoplasmic reticulum to the Golgi apparatus, and then follows one of a number of routes in the secretory pathway, depending on the nature of the protein. The protein may be secreted into the culture medium or may be retained on the cell surface, for example. Certain receptors that comprise extracellular, transmembrane, and cytoplasmic domains are examples of proteins that may be retained on the cell membrane, with only the extracellular domain located outside the cell. The leader sequences of certain secreted proteins comprise peptides that are located C-terminal to the signal peptide and are processed from the mature protein of interest subsequent to cleavage of the signal peptide. Such leaders often are referred to as prepro peptides, wherein the pre region is the signal sequence and the pro region designates the remainder of the leader.

[00144] One example is the yeast α-factor leader, which contains a signal peptide (including a C-terminal signal peptidase recognition site AlaLeuAla) followed by a pro region containing a basic amino acid pair LysArg that constitutes a KEX2 protease processing site,

immediately followed by a peptide GluAlaGluAla at the C-terminus of the pro region. Processing of this leader involves removal of the signal peptide by signal peptidase, followed by cleavage between the Lys and Arg residues by KEX2 protease. The GluAlaGluAla residues are subsequently removed by a peptidase that is the product of the STE13 gene (Julius et al., Cell (1983) 32:839). The yeast α-factor leader is described in U.S. Patent 4,546,082. Signal peptides derived from proteins naturally secreted by yeast cells have been employed in recombinant expression systems for production of heterologous proteins in yeast. The use of mammalian signal peptides in yeast expression systems also has been reported, although certain of the mammalian signal peptides were not effective in promoting secretion of heterologous proteins in yeast.

[00145] The phrase culturing under "suitable conditions such that a desired polypeptide is expressed" refers to maintaining and/or growing microorganisms under conditions (e.g., temperature, pressure, pH, duration, etc.) appropriate or sufficient to obtain the desired compound or to obtain desired polypeptide.

[00146] In preferred embodiments the host cell also expresses a helper protein. Helper proteins are described in later sections of the application. Using different promoters and/or copies and/or integration sites for the helper gene(s) and/or the POI(s), the expression of the helper genes can be controlled with respect to time point and strength of induction in relation to the expression of the POI(s). For example, prior to induction of POI expression, the helper protein(s) may be first expressed. This has the advantage that the helper protein(s) are already present at the beginning of POI translation. Alternatively, the helper protein(s) and POI(s) can be induced at the same time.

[00147] A host cell according to the invention obtained by transformation with the POI genes may preferably first be cultivated at conditions to grow efficiently to a large cell number without the burden of expressing a heterologous protein. When the cells are prepared for POI expression, suitable cultivation conditions are selected and optimized to produce the POI.

[00148] An inducible promoter may be used that becomes activated as soon as an inductive stimulus is applied, to direct transcription of the gene under its control. Under growth conditions with an inductive stimulus, the cells usually grow more slowly than under normal conditions, but since the culture has already grown to a high cell number in the previous stage, the culture system as a whole produces a large amount of the heterologous protein. An inductive stimulus is preferably the addition of an appropriate agents (e.g.: methanol for the AOX-promoter) or the depletion of an appropriate nutrient (e.g., methionine

for the MET3-promoter). Also, the addition of ethanol, methylamine, cadmium or copper as well as heat or an osmotic pressure increasing agent can induce the expression.

[00149] It is preferred to cultivate the hosts according to the invention in a bioreactor under optimized growth conditions to obtain a cell density of at least 1 g/L, preferably at least 10 g/L cell dry weight, more preferably at least 50 g/L cell dry weight. It is advantageous to achieve such yields of POI not only on a laboratory scale, but also on a pilot or industrial scale.

[00150] According to the present invention, due to underexpression of the KO protein, the POI is obtainable in high yields, which can be measured in mg POI/g dry biomass, in the range of 1 to 200, such as 50-200, such as 100-200 in the laboratory, pilot and industrial scale is feasible. The specific yield of a production host according to the invention preferably provides for an increase of at least 1.1 fold, more preferably at least 1.2 fold, at least 1.3 or at least 1.4 fold, in some cases an increase of more than 2 fold can be shown, when compared to the expression of the product without the underexpression of helper proteins.

[00151] The host cell according to the invention may be tested for its expression/secretion capacity or yield by standard tests, e.g. ELISA, activity assays, HPLC, Surface Plasmon Resonance (Biacore), Western Blot, capillary electrophoresis (Caliper) or SDS-Page.

[00152] Preferably, the cells are cultivated in a mineral medium with a suitable carbon source, thereby further simplifying the isolation process significantly. By way of example, the mineral medium contains an utilizable carbon source (e.g. glucose, glycerol or methanol), salts containing the macro elements (potassium, magnesium, calcium, ammonium, chloride, sulphate, phosphate) and trace elements (copper, iodide, manganese, molybdate, cobalt, zinc, and iron salts, and boric acid).

[00153] In the case of yeast cells, the cells may be transformed with one or more of the above-described expression vector(s) or plasmid(s), mated to form diploid strains, and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants or amplifying the genes encoding the desired sequences. A number of minimal media suitable for the growth of yeast are known in the art. Any of these media may be supplemented as necessary with salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES, citric acid and phosphate buffer), nucleosides (such as adenosine and thymidine), antibiotics, trace elements, vitamins, and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH and the like, are those previously used with the

host cell selected for expression and are known to the ordinarily skilled artisan. Cell culture conditions for other type of host cells are also known and can be readily determined by the artisan. Descriptions of culture media for various microorganisms are for example contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C, USA, 1981).

[00154] Cells can be cultured (e.g., maintained and/or grown) in liquid media and preferably are cultured, either continuously or intermittently, by conventional culturing methods such as standing culture, test tube culture, shaking culture (e.g., rotary shaking culture, shake flask culture, etc.), aeration spinner culture, or fermentation. In some embodiments, cells are cultured in shake flasks or deep well plates. In yet other embodiments, cells are cultured in a bioreactor (e.g., in a bioreactor cultivation process). Cultivation processes include, but are not limited to, batch, fed-batch and continuous methods of cultivation. The terms "batch process" and "batch cultivation" refer to a closed system in which the composition of media, nutrients, supplemental additives and the like is set at the beginning of the cultivation and not subject to alteration during the cultivation; however, attempts may be made to control such factors as pH and oxygen concentration to prevent excess media acidification and/or cell death. The terms "fed-batch process" and "fed-batch cultivation" refer to a batch cultivation with the exception that one or more substrates or supplements are added (e.g., added in increments or continuously) as the cultivation progresses. The terms "continuous process" and "continuous cultivation" refer to a system in which a defined cultivation media is added continuously to a bioreactor and an equal amount of used or "conditioned" media is simultaneously removed, for example, for recovery of the desired product. A variety of such processes has been developed and is well-known in the art.

[00155] In some embodiments, cells are cultured for about 12 to 24 hours, in other embodiments, cells are cultured for about 24 to 36 hours, about 36 to 48 hours, about 48 to 72 hours, about 72 to 96 hours, about 96 to 120 hours, about 120 to 144 hours, or for a duration greater than 144 hours. In yet other embodiments, culturing is continued for a time sufficient to reach desirable production yields of POI.

[00156] The above mentioned methods may further comprise a step of isolating the expressed POI. If the POI is secreted from the cells, it can be isolated and purified from the culture medium using state of the art techniques. Secretion of the POI from the cells is generally preferred, since the products are recovered from the culture supernatant rather than from the complex mixture of proteins that results when cells are disrupted to release intracellular proteins. A protease inhibitor, such as phenyl methyl sulfonyl fluoride (PMSF) may be useful to inhibit proteolytic degradation during purification, and antibiotics may be

included to prevent the growth of adventitious contaminants. The composition may be concentrated, filtered, dialyzed, etc., using methods known in the art. Alternatively, cultured host cells may also be ruptured sonically or mechanically, enzymatically or chemically to obtain a cell extract containing the desired POI, from which the POI may be isolated and purified.

[00157] As isolation and purification methods for obtaining the POI may be based on methods utilizing difference in solubility, such as salting out and solvent precipitation, methods utilizing difference in molecular weight, such as ultrafiltration and gel electrophoresis, methods utilizing difference in electric charge, such as ion-exchange chromatography, methods utilizing specific affinity, such as affinity chromatography, methods utilizing difference in hydrophobicity, such as reverse phase high performance liquid chromatography, and methods utilizing difference in isoelectric point, such as isoelectric focusing may be used. Specific purification steps are preferably employed to remove any helper protein that is also expressed and would contaminate the POI preparation.

[00158] The isolated and purified POI can be identified by conventional methods such as Western Blotting or specific assays for its activity. The structure of the purified POI can be defined by amino acid analysis, amino-terminal analysis, primary structure analysis, and the like. It is preferred that the POI is obtainable in large amounts and in a high purity level, thus meeting the necessary requirements for being used as an active ingredient in pharmaceutical compositions or as feed or food additive.

Helper Proteins

[00159] The host cell of the present invention may additionally comprise at least one polynucleotide encoding a helper protein. Any helper protein known in the art can be used.

[00160] A “helper proteins” as used in the present invention means a protein which enhances the expression and/or secretion of a protein of interest. This term should be understand broadly and should not be limited to chaperons or chaperon-like proteins.

[00161] A helper protein may for example be a chaperone. A chaperone generally has the function of binding to and stabilising an otherwise unstable conformer of another protein. By controlled binding and release, a chaperone facilitates its correct fate in vivo, be it folding, oligomeric assembly, transport to a particular subcellular compartment, or disposal by degradation. Chaperone proteins of this type are known in the art, for example in the Stanford Genome Database (SGD), http://db.yeastgenome.org or http://www.yeastgenome.org. Preferred chaperones are eukaryotic chaperones, especially

preferred chaperones are yeast chaperones, including AHA1, CCT2, CCT3, CCT4, CCT5, CCT6, CCT1, CCT8, CNS1, CPR3, CPR6, ERO1, EUG1, FMO1, HSP10, HSP12, HSP104, HSP26, HSP30, HSP42, HSP60, HSP78, HSP82, JEM1, MDJ1, MDJ2, MPD1, MPD2, PDI1, PFD1, ABC1, APJ1, ATP11, ATP12, BTT1, CDC37, CPR7, HSC82, KAR2, LHS1, MGE1, MRS11, NOB1, ECM10, SSA1, SSA2, SSA3, SSA4, SSC1, SSE2, SIL1, SLS1, ORM1, ORM2, PER1, PTC2, PSE1, UBI4 and HAC1 or a truncated intronless HAC1 (Valkonen et al. 2003, Applied Environ Micro., 69, 2065), as well as TIM9, PAM18 (also known as TIM14) and TCP1 (also known as CCT1). For example, the host cell may comprise a polynucleotide encoding BMH2, BFR2, COG6, COY1, CUP5, IMH1, KIN2, SEC31, SSA4, SSE1 or KAR2 (WO2008128701).

[00162] Preferably, the host cell comprises at least one polynucleotide encoding a helper protein of the present invention, namely, HP1, HP2, HP3, HP4, HP5, HP6, HP7, HP8, HP9, HP10 or functional homologues thereof. A helper protein of the present invention comprises the amino acid sequences of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162 and functional homologues thereof. A helper protein of the present invention can be encoded by the nucleotide sequences of SEQ ID NO: 13, 14, 15, 16, 17, 18, 19, 20, 21, or 163. Thus, the term "helper protein" when used in the context of HP1-HP10 is meant to encompass HP1, HP2, HP3, HP4, HP5, HP6, HP7, HP8, HP9 and HP 10 as well as functional homologues of HP1, HP2, HP3, HP4, HP5, HP6, HP7, HP8, HP9, and HP10, respectively.

[00163] As used herein, such helper protein has an amino acid sequence having at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162. The amino acid sequences of the helper proteins HP1 to HP10 are listed in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162.

[00164] The helper proteins of the present invention were originally isolated from *Pichia pastoris* CBS7435 strain. The methylotrophic yeast *Pichia pastoris* (*Komagataella phaffii*) CBS7435 is the parental strain of commonly used *P. pastoris* recombinant protein production hosts. Its complete genomic sequence is described in Küberl et al. (J Biotechnol. (2011) 154(4):312-20). These genes encoding the helper proteins identified herein have so far not been associated with a beneficial effect on protein yield.

[00165] The helper proteins may be native to the species or the genus of the host cell or taken or derived from other prokaryotic or eukaryotic organisms. The foreign DNA sequences encoding the helper proteins may be obtained from a variety of sources, preferably from a

plant, insect, fungal or mammalian species, preferably from the class of *Saccharomycetes*, preferably from the order of *Saccharomycetales*, preferably from the family of *Saccharomycetaceae*, and preferably from the genus of *Komagataella*. Homologous nucleotide sequences from other organisms such as vertebrates may be used.

[00166] The amino acid sequences of each helper proteins and their corresponding designations in the Examples are listed in Table 2 below:

Table 2

Helper protein (HP) and alternative designation ('xx')	Designation (in analogy to S. cerevisiae)	Amino acid sequences (SEQ ID NO:)	Polynucleotide (SEQ ID NO:)	Designation in the Examples (see Table 7)
HP1 ('56')		SEQ ID NO: 4	SEQ ID NO: 16	PP7435_Chr3-0607
HP2 ('2')		SEQ ID NO: 1	SEQ ID NO: 13	PP7435_Chr3-0933
HP3 ('3')	SBH1	SEQ ID NO: 2	SEQ ID NO: 14	PP7435_Chr2-0220
HP4 ('27')	CPR6	SEQ ID NO: 3	SEQ ID NO: 15	PP7435_Chr3-0639
HP5 ('4')	MXR2	SEQ ID NO: 5	SEQ ID NO: 17	PP7435_Chr4-0108
HP6 ('54')	MDR1	SEQ ID NO: 7	SEQ ID NO: 19	PP7435_Chr1-1225
HP7 ('55')		SEQ ID NO: 8	SEQ ID NO: 20	PP7435_Chr1-0667
HP8 ('40')	-	SEQ ID NO: 6	SEQ ID NO: 18	PP7435_Chr1-1232
HP9 ('60')	-	SEQ ID NO: 9	SEQ ID NO: 21	PP7435_Chr4-0448
HP10 ('34')	SEC61	SEQ ID NO: 162	SEQ ID NO: 163	PP7435_Chr1-0204

[00167] In particular, out of a screen for secretion helper factors in *Pichia pastoris* 55 candidate genes were identified which were validated by wet-lab expression experiments in order to select prime candidate genes. A prime candidate gene is one which enhances yield of the two model proteins SDZ-Fab#9 and/or HyHEL-Fab#8 as described herein to an extent of 20% or more in comparison to a *P. pastoris* strain not over-expressing the prime candidate gene, i.e., a host cell prior to engineering as described herein. To this end, out of the 55 genes, 9 prime candidates were identified. 4 prime candidates, i.e. PP7435_Chr3-0607, PP7435_Chr3-0933, PP7435_Chr2-0220 (SBH1), PP7435_Chr3-0639 (CPR6) showed more than 20% secretion and/or expression enhancement of both model proteins SDZ-Fab and HyHEL Fab and 5 further candidates (PP7435_Chr4-0108 (MXR2), PP7435_Chr1-1232, PP7435_Chr1-1225 (MDR1), PP7435_Chr1-0667, PP7435_Chr4-0448) showed >20-30% secretion yield enhancement for one of the two model proteins SDZ-Fab and HyHEL Fab, respectively, when being overexpressed.

[00168] Basis for the screen was a direct comparison between the transcriptional profile of a *P. pastoris* producer strain and a *P. pastoris* non-producer strain which resulted in the identification of 55 genes. These genes belong to various metabolic pathways and have different functions or may even have an unknown function. Thus, no guidance as to the

nature of the thus-obtained helper factors is apparent from the genes or proteins encoded thereby. Accordingly, tests have to be performed in order to validate whether or not a potential secretion helper factor is indeed a helper factor under real conditions. However, it is *prima facie* not apparent that a potential helper factor is indeed a helper factor, merely because it was identified by its enhanced transcript appearance in a producer strain. In fact, it may be that a potential helper factor has no positive effect on protein secretion or may even have a negative effect. This was indeed observed by the inventors, since two genes encoding chaperones (SCJ1, HCH1) identified among the 55 genes, from which it could be reasonably expected to enhance secretion, did the opposite. Thus, tests for each gene are required, but no guidance exists as to which protein encoded by one of the approx. 60 genes is indeed a secretion helper factor.

[00169] Hence, the finding of a "true" secretion helper factor is not a straightforward matter, but an inventive choice for which no guidance is available or apparent from the mere gene/protein sequence or the function of a protein identified in a screen as done by the present inventors.

[00170] What is also unusual about the prime candidate genes that have to be overexpressed as is described herein – they are not typical secretion helper factors, i.e., genes which encode a protein having a function that is not deemed to play a role in protein secretion or even have an unknown function.

[00171] In a preferred embodiment, the host cell is further engineered to express or overexpress at least one, such as at least 2, 3, 4, 5, 6, 7, 8, or at least 9 polynucleotide encoding a helper protein having an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162 or a functional homologue thereof, wherein the functional homologue has having at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162.

[00172] Preferably, the present invention provides a recombinant host cell for manufacturing a protein of interest, wherein the host cell is engineered to overexpress a polynucleotide encoding a helper protein having an amino acid having at least 40% sequence identity to SEQ ID NO: 1, 45% sequence identity to SEQ ID NO: 2, 50% sequence identity to SEQ ID NO: 3, 45% sequence identity to SEQ ID NO: 4, 50% sequence identity to SEQ ID NO: 5, 45% sequence identity to SEQ ID NO: 6, 40% sequence identity to SEQ ID NO: 7, 40% sequence identity to SEQ ID NO: 8, 40% sequence identity to SEQ ID NO: 9, or 45% sequence identity to SEQ ID NO: 162. Such a host cell is applied in the methods and uses described herein.

[00173] Preferably, the host cell is engineered to express or overexpress at least one polynucleotide encoding a helper protein having an amino acid sequence having at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162. If the polynucleotide(s) already exist in the host cell, the host cell can manipulated in a way such that they are overexpressed, as will be described later.

[00174] In a preferred embodiment, 2, 3, 4, 5, 6, 7, 8 9 or more types of helper proteins disclosed by present invention are overexpressed. For example, the host cell can be engineered to overexpress 2, 3, 4, 5, 6, 7, 8 or more of helper proteins selected from SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162 or functional homologues thereof, where a functional homologue thereof has an amino acid having at least 30% sequence identity to SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162.

[00175] The term "overexpress" generally refers to any amount greater than or equal to an expression level exhibited by a reference standard. The terms "overexpress," "overexpressing," "overexpressed" and "overexpression" in the present invention refer an expression of a gene product or a polypeptide at a level greater than the expression of the same gene product or polypeptide prior to a genetic alteration of the host cell or in a comparable host which has not been genetically altered at defined conditions. If a host cell does not comprise a given gene product, it is possible to introduce the gene product into the host cell for expression; in this case, any detectable expression is encompassed by the term "overexpression." However, it is not required, although preferred, that the host cell overexpresses the helper protein HP1-HP10 or functional homologues thereof.

Overexpression of Helper Proteins by the Host Cell

[00176] Overexpression can be achieved in any ways known to a skilled person in the art. In general, it can be achieved by increasing transcription/translation of the gene, e.g. by increasing the copy number of the gene or altering or modifying regulatory sequences or sites associated with expression of a gene. For example, the gene can be operably linked to a strong constitutive promoter and/or strong ubiquitous promoter in order to reach high expression levels. Such promoters can be endogenous promoters or recombinant promoters. Alternatively, it is possible to remove regulatory sequences such that expression becomes constitutive. One can substitute a promoter with a heterologous promoter which increases expression of the gene or leads to constitutive expression of the gene. Using inducible promoters additionally makes it possible to increase the expression in the course of cultivation. Furthermore, overexpression can also be achieved by, for example, modifying the chromosomal location of a particular gene, altering nucleic acid sequences adjacent to a

particular gene such as a ribosome binding site or transcription terminator, introducing a frame-shift in the open reading frame, modifying proteins (e.g., regulatory proteins, suppressors, enhancers, transcriptional activators and the like) involved in transcription of the gene and/or translation of the gene product, or any other conventional means of deregulating expression of a particular gene routine in the art (including but not limited to use of antisense nucleic acid molecules, for example, to block expression of repressor proteins or deleting or mutating the gene for a transcriptional factor which normally represses expression of the gene desired to be overexpressed. Prolonging the life of the mRNA may also improve the level of expression. For example, certain terminator regions may be used to extend the half-lives of mRNA (Yamanishi et al., Biosci. Biotechnol. Biochem. (2011) 75:2234 and US 2013/0244243). If multiple copies of genes are included, the genes can either be located in plasmids of variable copy number or integrated and amplified in the chromosome. If the host cell does not comprise the gene product encoding the helper protein, it is possible to introduce the gene product into the host cell for expression. In this case, "overexpression" means expressing the gene product using any methods known to a skilled person in the art.

[00177] The overexpression of the polypeptide encoding the helper proteins, can be achieved by other methods known in the art, for example by genetically modifying their endogenous regulatory regions, as described by Marx et al., 2008 (Marx, H., Mattanovich, D. and Sauer, M. *Microb Cell Fact* 7 (2008): 23), and Pan et al., 2011 (Pan et al., *FEMS Yeast Res.* (2011) May; (3):292-8.), such methods include, for example, integration of a recombinant promoter that increases expression of the helper proteins. Transformation is described in Cregg et al. (1985) Mol. Cell. Biol. 5:3376-3385.

[00178] Those skilled in the art will find relevant instructions in Martin et al. (Bio/Technology 5, 137-146 (1987)), Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), Eikmanns et al. (Gene 102, 93-98 (1991)), EP 0 472 869, US 4,601,893, Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), LaBarre et al. (Journal of Bacteriology 175, 1001- 1007 (1993)), WO 96/15246, Malumbres et al. (Gene 134, 15- 24 (1993)), JP-A-10-229891, Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)) and Makrides (Microbiological Reviews 60, 512-538 (1996)), inter alia, and in well-known textbooks on genetics and molecular biology.

[00179] According to a preferred embodiment, the polynucleotide encoding the helper protein can be presented in a single copy or in multiple copies per cell. The copies may be adjacent to or distant from each other. According to another preferred embodiment, the method of the invention employs recombinant nucleotide sequences encoding the helper proteins provided on one or more plasmids suitable for integration into the genome of the

host cell, in a single copy or in multiple copies per cell. The copies may be adjacent to or distant from each other. Overexpression can be achieved by expressing multiple copies of the polynucleotide, such as 2, 3, 4, 5, 6 or more copies of said polynucleotide per host cell.

[00180] The recombinant nucleotide sequence encoding the POI(s), as well as those encoding the helper proteins, may also be provided on one or more autonomously replicating plasmids in a single copy or in multiple copies per cell.

[00181] Alternatively, the recombinant nucleotide sequence encoding the POI and the recombinant nucleotide sequence encoding a protein that increases protein secretion are present on the same plasmid in single copy or multiple copies per cell.

[00182] The polynucleotide encoding the helper protein is preferably integrated into the genome of the host cell. The term "genome" generally refers to the whole hereditary information of an organism that is encoded in the DNA (or RNA for certain viral species). It may be present in the chromosome, on a plasmid or vector, or both. Preferably, the polynucleotide encoding the helper protein is integrated into the chromosome of said cell.

[00183] The polynucleotide encoding the helper protein may be integrated in its natural locus. “Natural locus” means the location on a specific chromosome, where the polynucleotide encoding the helper protein is located, for example at the natural locus of HP1 to 9 as shown in Table 1. However, in another embodiment, the polynucleotide encoding the helper protein is present in the genome of the host cell not at their natural locus, but integrated ectopically. The term "ectopic integration" means the insertion of a nucleic acid into the genome of a microorganism at a site other than its usual chromosomal locus, i.e., predetermined or random integration. In another embodiment, the polynucleotide encoding the helper protein is integrated into the natural locus and ectopically. Heterologous recombination can be used to achieve random or non-targeted integration. Heterologous recombination refers to recombination between DNA molecules with significantly different sequences. Methods of recombinations are known in the art and for example described in Boer et al., Appl Microbiol Biotechnol (2007) 77:513–523. One may also refer to Principles of Gene Manipulation and Genomics by Primrose and Twyman (7th edition, Blackwell Publishing 2006) for genetic manipulation of yeast cells.

[00184] For yeast cells, the polynucleotide encoding the helper protein and/or the polynucleotide encoding the POI may be inserted into a desired locus, such as AOX1, GAP, ENO1, TEF, HIS4 (Zamir et al., Proc. NatL Acad. Sci. USA (1981) 78(6):3496-3500), HO (Voth et al. Nucleic Acids Res. 2001 June 15; 29(12): e59), TYR1 (Mirisola et al., Yeast 2007;

24: 761–766), His3, Leu2, Ura3 (Taxis et al., BioTechniques (2006) 40:73-78), Lys2, ADE2, TRP1, GAL1, ADH1 or on the integration of 5S ribosomal RNA gene.

[00185] In other embodiments, the polynucleotide encoding the helper protein and/or the polynucleotide encoding the POI can be integrated in a plasmid or vector. The terms "plasmid" and "vector" include autonomously replicating nucleotide sequences as well as genome integrating nucleotide sequences. A skilled person is able to employ suitable plasmids or vectors depending on the host cell used.

[00186] Preferably, the plasmid is a eukaryotic expression vector, preferably a yeast expression vector.

[00187] Preferably, the plasmid is a eukaryotic expression vector, preferably a yeast expression vector. Examples of plasmids using yeast as a host include YIp type vector, YEp type vector, YRp type vector, YCp type vector, pGPD-2, pAO815, pGAPZ, pGAPZα, pHIL-D2, pHIL-S1, pPIC3.5K, pPIC9K, pPICZ, pPICZα, pPIC3K, pHWO10, pPUZZLE and 2 μm plasmids. Such vectors are known and are for example described in Cregg et al., Mol Biotechnol. (2000) 16(1):23-52.

[00188] Plasmids can be used for the transcription of cloned recombinant nucleotide sequences, i.e. of recombinant genes and the translation of their mRNA in a suitable host organism. Plasmids can also be used to integrate a target polynucleotide into the host cell genome by methods known in the art, such as described by J. Sambrook et al., Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York (2001). A "plasmid" usually comprises an origin for autonomous replication in the host cells, selectable markers, a number of restriction enzyme cleavage sites, a suitable promoter sequence and a transcription terminator, which components are operably linked together. The polypeptide coding sequence of interest is operably linked to transcriptional and translational regulatory sequences that provide for expression of the polypeptide in the host cells.

[00189] Most plasmids exist in only one copy per bacterial cell. Some plasmids, however, exist in higher copy numbers. For example, the plasmid ColE1 typically exists in 10 to 20 plasmid copies per chromosome in *E. coli*. If the nucleotide sequences of the present invention are contained in a plasmid, the plasmid may have a copy number of 20-30, 30-100 or more per host cell. With a high copy number of plasmids, it is possible to overexpress helper proteins by the cell.

[00190] Large numbers of suitable plasmids or vectors are known to those of skill in the art and many are commercially available. Examples of suitable vectors are provided in

Sambrook et al, eds., Molecular Cloning: A Laboratory Manual (2nd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory (1989), and Ausubel et al, eds., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York (1997).

[00191] A vector or plasmid of the present invention encompass yeast artificial chromosome, which refers to a DNA construct that can be genetically modified to contain a heterologous DNA sequence (e.g., a DNA sequence as large as 3000 kb), that contains telomeric, centromeric, and origin of replication (replication origin) sequences.

[00192] A vector or plasmid of the present invention also encompasses bacterial artificial chromosome (BAC), which refers to a DNA construct that can be genetically modified to contain a heterologous DNA sequence (e.g., a DNA sequence as large as 300 kb), that contains an origin of replication sequence (Ori), and may contain one or more helicases (e.g., parA, parB, and parC).

[00193] Examples of plasmids using Escherichia coli as their host include pBR322, pUC18, pUC19, pUC118, pVC119, pSP64, pSP65, pTZ-18R/-18U, pTZ-19R/-19U, pGEM-3, pGEM-4, pGEM-3Z, pGEM-4Z, pGEM-5Zf(-), and pBluescript KSTM (Stratagene). Examples of plasmids suitable for expression in Escherichia coli include pAS, pKK223 (Pharmacia), pMC1403, pMC931, and pKC30.

[00194] Polynucleotides encoding the helper proteins and the POI may be recombined in to the host cell by ligating the relevant genes each into one vector. It is possible to construct single vectors carrying the genes, or two separate vectors, one to carry the helper protein genes and the other one the POI genes. These genes can be integrated into the host cell genome by transforming the host cell using such vector or vectors. In some embodiments, the genes encoding the POI is integrated in the genome and the gene encoding the helper protein is integrated in a plasmid or vector. In some embodiments, the genes encoding the helper protein is integrated in the genome and the gene encoding the POI is integrated in a plasmid or vector. In some embodiments, the genes encoding the POI and the helper protein are integrated in the genome. In some embodiments, the gene encoding the POI and the helper protein is integrated in a plasmid or vector. If multiple genes encoding the POI are used, some genes encoding the POI are integrated in the genome while others are integrated in the same or different plasmids or vectors. If multiple genes encoding the helper proteins are used, some of the genes encoding the helper protein are integrated in the genome while others are integrated in the same or different plasmids or vectors.

[00195] Overexpression of the endogenous polypeptide in the recombinant microbial cell can be achieved by modifying expression control sequences. Expression control sequences

are known in the art and include, for example, promoters, enhancers, polyadenylation signals, transcription terminators, internal ribosome entry sites (IRES), and the like, that provide for the expression of the polynucleotide sequence in a host cell. Expression control sequences interact specifically with cellular proteins involved in transcription (Maniatis et al., Science, 236: 1237-1245 (1987)). Exemplary expression control sequences are described in, for example, Goeddel, Gene Expression Technology: Methods in Enzymology, Vol. 185, Academic Press, San Diego, Calif. (1990).

[00196] In a preferred embodiment, the overexpression is achieved by using an enhancer to express the helper protein. Transcriptional enhancers are relatively orientation and position independent, having been found 5' and 3' to the transcription unit, within an intron, as well as within the coding sequence itself. The enhancer may be spliced into the expression vector at a position 5' or 3' to the coding sequence, but is preferably located at a site 5' from the promoter. Most yeast genes contain only one UAS, which generally lies within a few hundred base pairs of the cap site and most yeast enhancers (UASs) cannot function when located 3´ of the promoter, but enhancers in higher eukaryotes can function both 5´ and 3´ of the promoter.

[00197] Many enhancer sequences are now known from mammalian genes (globin, RSV, SV40, EMC, elastase, albumin, a-fetoprotein and insulin). One may also use an enhancer from a eukaryotic cell virus, such as the SV40 late enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. Yeast enhancers, also called upstream activating sequences (UASs), such as the UAS/Gal system from *Saccharomyces cerevisiae*, can be advantageously used with yeast promoters (described in European Patent No. 0317254 and Rudoni et al., The International Journal of Biochemistry and Cell Biology, (2000), 32(2):215-224).

[00198] Overexpression of the endogenous polypeptide in the recombinant cell can be achieved by modifying transcriptional and translational regulatory sequences, including, for example, promoters, enhancers, polyadenylation signals, transcription terminators, internal ribosome entry sites (IRES), and the like, that provide for the expression of the polynucleotide sequence in a host cell. Such sequences interact specifically with cellular proteins involved in transcription (Maniatis et al., Science, 236: 1237-1245 (1987)). Exemplary sequences are described in, for example, Goeddel, Gene Expression Technology: Methods in Enzymology, Vol. 185, Academic Press, San Diego, Calif. (1990). . The polynucleotides encoding the POI and/or helper proteins are preferably operably linked to transcriptional and translational regulatory sequences that provide for expression in the host cells. The term "transcriptional regulatory sequences" as used herein refers to nucleotide sequences that are associated with a gene nucleic acid sequence and which regulate the

transcription of the gene. The term "translational regulatory sequences" as used herein refers to nucleotide sequences that are associated with a gene nucleic acid sequence and which regulate the translation of the gene. Transcriptional and/or translational regulatory sequences can either be located in plasmids or vectors or integrated in the chromosome of the host cell. Transcriptional and/or translational regulatory sequences are located in the same nucleic acid molecule of the gene which it regulates.

[00199] For example, overexpression of the endogenous helper protein in the recombinant cell can be achieved by modifying the promoters, for example, by replacing the endogenous promoter which is operably linked to the helper protein with another stronger promoter in order to reach high expression levels. Such promoter may be inductive or constitutive. Modification of endogenous promoter may be performed by mutation or homologous recombination using methods known in the art.

[00200] The overexpression of the polynucleotide encoding the helper proteins, can be achieved by other methods known in the art, for example by genetically modifying their endogenous regulatory regions, as described by Marx et al., 2008 (Marx, H., Mattanovich, D. and Sauer, M. *Microb Cell Fact* 7 (2008): 23), and Pan et al., 2011 (Pan et al., *FEMS Yeast Res.* (2011) May; (3):292-8.), such methods include, for example, integration of a recombinant promoter that increases expression of the helper proteins. Transformation is described in Cregg et al. (1985) Mol. Cell. Biol. 5:3376-3385. A "recombinant" promoter is referred to with respect to the sequence whose expression it drives. As used herein, a recombinant promoter means when the promoter is not a native promoter to the given sequence, i.e., when the promoter is different from a naturally occurring promoter (the "native promoter"). The term "promoter" as used herein refers to a region that facilitates the transcription of a particular gene. A promoter typically increases the amount of recombinant product expressed from a nucleotide sequence as compared to the amount of the expressed recombinant product when no promoter exists. A promoter from one organism can be utilized to enhance recombinant product expression from a sequence that originates from another organism. The promoter can be integrated into a host cell chromosome by homologous recombination using methods known in the art (e.g. Datsenko et al, Proc. Natl. Acad. Sci. U.S.A., 97(12): 6640-6645 (2000)). In addition, one promoter element can increase the amount of products expressed for multiple sequences attached in tandem. Hence, one promoter element can enhance the expression of one or more recombinant products.

[00201] Promoter activity may be assessed by its transcriptional efficiency. This may be determined directly by measurement of the amount of mRNA transcription from the promoter, e.g. by Northern Blotting, quantitative PCR or indirectly by measurement of the amount of gene product expressed from the promoter.

[00202] The promoter could be an "inducible promoter" or "constitutive promoter." "Inducible promoter" refers to a promoter which can be induced by the presence or absence of certain factors, and "constitutive promoter" refers to an unregulated promoter that allows for continuous transcription of its associated gene or genes.

[00203] In a preferred embodiment, both the nucleotide sequences encoding the helper protein and the POI are driven by an inducible promoter. In another preferred embodiment, both the nucleotide sequences encoding the helper protein and POI is driven by a constitutive promoter. In yet another preferred embodiment, the nucleotide sequences encoding the helper protein is driven by a constitutive promoter and the POI is driven by an inducible promoter. In yet another preferred embodiment, the nucleotide sequences encoding the helper protein is driven by an inducible promoter and the POI is driven by a constitutive promoter. As an example, the HP may be driven by a constitutive GAP promoter and the POI may be driven by an inducible AOX1 promoter. In one embodiment, the nucleotide sequences encoding the helper protein and POI is driven by the same promoter or similar promoters in terms of promoter activity and/or expression behaviour.

[00204] Many inducible promoters are known in the art. Many are described in a review by Gatz, Curr. Op. Biotech., 7: 168 (1996) (see also Gatz, Ann. Rev. Plant. Physiol. Plant Mol. Biol., 48:89 (1997)). Examples include tetracycline repressor system, Lac repressor system, copper-inducible systems, salicylate-inducible systems (such as the PR1 a system), glucocorticoid-inducible (Aoyama et al., 1997), alcohol-inducible systems, e.g., AOX promoters, and ecdysome-inducible systems. Also included are the benzene sulphonamide-inducible (U.S. Patent No. 5,364,780) and alcohol-inducible systems (WO 97/06269 and WO 97/06268) and glutathione S-transferase promoters.

[00205] Suitable promoter sequences for use with yeast host cells are described in Mattanovich et al., Methods Mol. Biol. (2012) 824:329-58 and include glycolytic enzymes like triosephosphate isomerase (TPI), phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH or GAP) and variants thereof, lactase (LAC) and galactosidase (GAL), *P. pastoris* glucose-6-phosphate isomerase promoter (PPGI), the 3-phosphoglycerate kinase promoter (PPGK), the glycerol aldehyde phosphate dehydrogenase promoter (PGAP), translation elongation factor promoter (PTEF), and the promoters of *P. pastoris* enolase 1 (PENO1), triose phosphate isomerase (PTPI), ribosomal subunit proteins (PRPS2, PRPS7, PRPS31, PRPL1), alcohol oxidase promoter (PAOX) or variants thereof with modified characteristics, the formaldehyde dehydrogenase promoter (PFLD), isocitrate lyase promoter (PICL), alpha-ketoisocaproate decarboxylase promoter (PTHI), the promoters of heat shock protein family members (PSSA1, PHSP90, PKAR2), 6-Phosphogluconate dehydrogenase (PGND1), phosphoglycerate mutase (PGPM1), transketolase (PTKL1),

phosphatidylinositol synthase (PPIS1), ferro-O2-oxidoreductase (PFET3), high affinity iron permease (PFTR1), repressible alkaline phosphatase (PPHO8), N-myristoyl transferase (PNMT1), pheromone response transcription factor (PMCM1), ubiquitin (PUBI4), single-stranded DNA endonuclease (PRAD2), the promoter of the major ADP/ATP carrier of the mitochondrial inner membrane (PPET9) (WO2008/128701) and the formate dehydrogenase (FMD) promoter. The GAP promoter, AOX promoter or a promoter derived from GAP or AOX promoter is particularly preferred. AOX promoters can be induced by methanol and are repressed by e.g. glucose.

[00206] Further examples of suitable promoters include Saccharomyces cerevisiae enolase (ENO-1), Saccharomyces cerevisiae galactokinase (GAL1), Saccharomyces cerevisiae alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), Saccharomyces cerevisiae triose phosphate isomerase (TPI), Saccharomyces cerevisiae metallothionein (CUP1), and Saccharomyces cerevisiae 3-phosphoglycerate kinase (PGK), and the maltase gene promoter (MAL).

[00207] Other useful promoters for yeast host cells are described by Romanos et al, 1992, Yeast 8:423-488.

[00208] Suitable promoter sequences for use with *E. coli* include T7 promoter, T5 promoter, tryptophan (trp) promoter, lactose (lac) promoter, tryptophan/lactose (tac) promoter, lipoprotein (lpp) promoter, and λ phage PL promoter in plasmids.

[00209] The promoter which drives the expression of the polynucleotide encoding the helper protein is preferably not the endogenous to the promoter of the helper gene. Preferably, a recombinant promoter is used instead of the endogenous promoter of the helper protein gene. A "recombinant" promoter is referred to with respect to the sequence whose expression it drives. As used herein, a recombinant promoter means when the promoter is not a native promoter to the given sequence, i.e., when the promoter is different from a naturally occurring promoter (the "native promoter"). Such a promoter is sometimes also referred to herein as heterologous promoter.

[00210] In a preferred embodiment, the overexpression is achieved by using an enhancer to enhance the promoter activity which drives the expression of the helper protein. Transcriptional enhancers are relatively orientation and position independent, having been found 5' and 3' to the transcription unit, within an intron, as well as within the coding sequence itself. The enhancer may be spliced into the expression vector at a position 5' or 3' to the coding sequence, but is preferably located at a site 5' from the promoter. Most yeast genes contain only one UAS, which generally lies within a few hundred base pairs of the cap

site and most yeast enhancers (UASs) cannot function when located 3′ of the promoter, but enhancers in higher eukaryotes can function both 5′ and 3′ of the promoter.

[00211] Many enhancer sequences are now known from mammalian genes (globin, RSV, SV40, EMC, elastase, albumin, a-fetoprotein and insulin). One may also use an enhancer from a eukaryotic cell virus, such as the SV40 late enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. Yeast enhancers, also called upstream activating sequences (UASs), such as the UAS/Gal system from *Saccharomyces cerevisiae*, can be advantageously used with yeast promoters (described in European Patent No. 0317254 and Rudoni et al., The International Journal of Biochemistry and Cell Biology, (2000), 32(2):215-224).

[00212] It is preferred that the present host cell underexpressing the KO protein(s) would coexpress the protein of interest and a helper protein having an amino acid sequence having at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162.

[00213] Preferably, the host cell can be engineered to overexpress 2, 3, 4, 5, 6, 7, 8 or more of helper proteins selected from SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162 or functional homologues thereof. In a preferred embodiment, the host cell can be engineered to (1) underexpress 1, 2, 3 or more of KO proteins KO1, KO2, KO3 or functional homologues thereof and (2) overexpress 1, 2, 3, 4, 5, 6, 7, 8, 9 or more of helper proteins HP1, HP2, HP3, HP4, HP5, HP6, HP7, HP8, HP9, HP10 or functional homologues thereof.

[00214] However, a specific combination of helper proteins HP3 (SEQ ID NO: 2) or a functional homologue and HP1 (SEQ ID NO. 4) or a functional homologue, or helper proteins HP10 (SEQ ID NO: 162) or a functional homologue and HP3 (SEQ ID NO: 2) or a functional homologue, is a preferred embodiment of the host cells of the present invention that are preferably applied in the methods and uses of the present invention.

[00215] Also, a specific combination of helper protein HP2 (SEQ ID NO: 1) or a functional homologue and HP3 (SEQ ID NO: 2) or a functional homologue and KO protein KO1 (SEQ ID NO: 10), or helper protein HP2 (SEQ ID NO: 1) or a functional homologue and HP1 (SEQ ID NO: 4) or a functional homologue and KO protein KO1 (SEQ ID NO: 10), is a preferred embodiment of the host cells of the present invention that are preferably applied in the methods and uses of the present invention.

[00216] Host cells being engineered to reflect such a combination are envisaged in a preferred embodiment. These host cells are preferably applied in the methods and uses described herein. “Reflected” means that the skilled person knows in accordance with the

teaching of the present invention that a helper protein is overexpressed, while a KO protein is underexpressed.

[00217] It is preferred that the present host cell for manufacturing a protein of interest is engineered to underexpress at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof and further overexpresses at least one polynucleotide encoding a helper protein having an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162 or a functional homologue thereof, wherein the functional homologue of the helper protein has at least 30%, such as at least 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, sequence identity to an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162.

[00218] In a preferred embodiment, the underexpression of at least one polynucleotide encoding a KO protein, in concert with the overexpression of at least one polynucleotide encoding a helper protein, may increase the yield of the protein of interest, for example SDZ-Fab (SEQ ID NO: 25 and 26) or HyHEL-Fab (SEQ ID NO: 29 and 20) in the host cell by at least 1%, such as at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or at least 200% or more, compared to the host cell prior to the engineering to underexpress the polynucleotide encoding the KO protein.

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[00219] It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, constructs, and reagents described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be limited only by the appended claims.

[00220] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

[00221] All publications mentioned herein are for the purpose of describing and disclosing, for example, the cell lines, constructs, and methodologies that are described in the

publications, which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

[00222] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject invention, and are not intended to limit the scope of what is regarded as the invention and defined in the claims. Efforts have been made to ensure accuracy with respect to the numbers used (e.g. amounts, temperature, concentrations, etc.) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees centigrade; and pressure is at or near atmospheric.

Examples

[00223] The below examples will demonstrate that the newly identified gene(s) increases the titer (product per volume in mg/L) and the yield (product per biomass in mg/g biomass measured as dry cell weight or wet cell weight), respectively, of recombinant proteins upon its/their underexpression. As an example, the yield of recombinant antibody Fab fragments and recombinant enzymes in the yeast *Pichia pastoris* are increased. The positive effect was shown in shaking cultures (conducted in shake flasks or deep well plates) and in lab scale fed-batch cultivations.

Example 1 Generation of *P. pastoris* production strains

a) Construction of *P. pastoris* strains secreting antibody Fab fragment HyHEL

[00224] *P. pastoris* CBS7435 (CBS, genome sequenced by Küberl et al. 2011) mut^S variant was used as host strain. The pPM2d_pGAP and pPM2d_pAOX expression vectors are derivatives of the pPuzzle_ZeoR vector backbone described in WO2008/128701A2, consisting of the pUC19 bacterial origin of replication and the Zeocin antibiotic resistance cassette. Expression of the heterologous gene is mediated by the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter or alcohol oxidase (AOX) promoter, respectively, and the S. *cerevisiae* CYC1 transcription terminator. The light chain (LC) and the heavy chain (HC) of the antibody Fab fragment HyHEL (Fig. 2) were amplified from vector DNA template (carrying the gene of interests with N-terminal *S. cerevisiae* alpha mating factor signal leader sequence) using the primers for HyHEL-HC and HyHEL-LC in

Table 3, and each ligated into both vectors pPM2d_pGAP and pPM2d_pAOX digested with *Sbf*I and *Sfi*I. The LC fragments were ligated into variants of pPM2d_pGAP and pPM2d_pAOX, where one restriction enzyme site in the promoter region was exchanged for another to allow subsequent linearization (*Nde*I instead of *Avr*II in pPM2d_pGAP, *Bsu36*I instead of *Bpu*1102I in pPM2d_pAOX), the HC fragments were ligated into the unmodified versions of the vectors. After sequence verification of LC and HC, the expression cassettes for both chains were combined onto one vector by using the compatible restriction enzymes *Mre*I and *Age*I.

[00225] Plasmids were linearized using *Nde*I restriction enzyme (for pPM2d_pGAP) or *Bsu36*I restriction enzyme (for pPM2d_pAOX), respectively, prior to electroporation (using a standard transformation protocol described in Gasser et al. 2013. Future Microbiol. 8(2):191-208) into *P. pastoris*. Selection of positive transformants was performed on YPD plates (per liter: 10 g yeast extract, 20 g peptone, 20 g glucose, 20 g agar-agar) containing 50 µg/mL of Zeocin. Colony PCR was used to ensure the presence of the transformed plasmid. Therefore, genomic DNA was obtained by cooking and freezing of *P. pastoris* colonies for 5 minutes each and directly applied for PCR with the appropriate primers.

b) Construction of a *P. pastoris* strain secreting antibody Fab fragment SDZ

[00226] The light chain (LC) and the heavy chain (HC) of the antibody Fab fragment SDZ (Fig. 2) were amplified from vector DNA template (carrying the gene of interests with N-terminal alpha mating factor signal leader sequence) using the primers for SDZ-HC and SDZ-LC in Table 3, and each ligated into pPM2d_pAOX or the variant of pPM2d_pAOX with the *Bsu36*I restriction site, respectively, each digested with *Sbf*I and *Sfi*I. After sequence verification of LC and HC, the expression cassettes for both chains were combined onto one vector by using the compatible restriction enzymes *Mre*I and *Age*I.

[00227] Plasmids were linearized using *Bsu36*I restriction enzyme prior to electroporation (using a standard transformation protocol described in Gasser et al. 2013. Future Microbiol. 8(2):191-208) into *P. pastoris*. Selection of positive transformants was performed on YPD plates (per liter: 10 g yeast extract, 20 g peptone, 20 g glucose, 20 g agar-agar) containing 50 µg/mL of Zeocin. Colony PCR was used to ensure the presence of the transformed plasmid. Therefore, genomic DNA was obtained by cooking and freezing of *P. pastoris* colonies for 5 minutes each and directly applied for PCR with the appropriate primers.

[00228] Table 3 shows oligonucleotide primers for PCR amplification of HyHEL LC and HC as well as SDZ LC and HC (Alpha-mating factor_ forward is the forward primer for amplification of all Fab chains).

Table 3

Primer	Restriction site attached	sequence
Alpha-mating factor_ forward*	*Sbf*I	ACTACCTGCAGGCGAAACGATGAGATTCCCATC SEQ ID NO: 33
HyHEL-HC backward	*Sfi*I	TCATGGCCGAGGCGGCCCTATTACTTGTCACAGG ACTTTGGCTC SEQ ID NO: 34
HyHEL-LC backward	*Sfi*I	CTATGGCCGAGGCGGCCCTATTAACACTCACCTCT GTTG SEQ ID NO: 35
SDZ-HC back	*Sfi*I	TATCGGCCGAGGCGGCCCTATTACTTACCTGGGG ACAAG SEQ ID NO: 36
SDZ-LC back	*Sfi*I	CTATGGCCGAGGCGGCCCTATTAACACTCACCTCT GTTG SEQ ID NO: 37

Example 2 Chemostat cultivation

[00229] Cultivations were performed with 1.4 L DASGIP reactors (Eppendorf, Germany) with a maximum working volume of 1.0 L.

[00230] Following media were used:

[00231] *PTM_1 trace salts stock solution (per litre) contains:* 6.0 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.36 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.82 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4 \cdot 7H_2O$, and 5.0 mL H_2SO_4 (95 %-98 %).

[00232] *Glycerol Batch medium (per litre) contains:* 2 g Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$), 39.2 g Glycerol, 20.8 g $NH_4H_2PO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.6 g KCl, 0.022 g $CaCl_2 \cdot 2H_2O$, 0.8 mg biotin and 4.6 mL PTM1 trace salts stock solution. HCl was added to set the pH to 5.

[00233] *Glucose Chemostat medium (per litre) contains:* 2.5 g Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$), 55.0 g glucose monohydrate, 21.8 g $NH_4H_2PO_4$, 1.0 g $MgSO_4 \cdot 7H_2O$, 2.5 g KCl, 0.04 g $CaCl_2 \cdot 2H_2O$, 4.0 mg biotin and 2.43 mL PTM1 trace salts stock solution. HCl was added to set the pH to 5.

[00234] *Methanol/Glycerol Chemostat medium (per litre) contains:* 2.5 g Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$), 8.5 g methanol, 50.0 g glycerol, 21.8 g $NH_4H_2PO_4$, 1.0 g $MgSO_4 \cdot 7H_2O$, 2.5 g KCl, 0.04 g $CaCl_2 \cdot 2H_2O$, 4.0 mg biotin and 2.43 mL PTM1 trace salts stock solution. HCl was added to set the pH to 5.

[00235] The dissolved oxygen was controlled at DO = 20 % with stirrer speed (400 – 1200 rpm) and aeration rate (12 – 72 standard Liter/hour (sL/h)) air, the temperature was controlled at 25°C and the pH setpoint of 5 was controlled with addition of NH_4OH (25 %). Foaming was controlled by addition of antifoam agent (5% Glanapon 2000) on demand.

[00236] To start the cultivation, 0.4 L batch medium was sterile filtered and transferred into the fermenter under a sterile work bench and was inoculated (from a *P. pastoris* overnight pre-culture in YPG containing 50 µg/mL Zeocin, 180 rpm, 28°C) with a starting optical density (OD_{600}) of 1. The batch phase of approximately 24 h reached a dry biomass concentration of approximately 20 g/L, it was followed by a constant feed with chemostat medium at 40 mL/h (for µ=D=0.1/h) with simultaneous constant removal of culture to keep the total volume constant. A dry biomass concentration of approximately 25 g/L was reached after 7 residence times (70 hours) when samples for microarray experiments were taken and the cultivation was terminated. This chemostat cultivation was performed three times with each production strain (CBS7435 pGAP HyHEL-Fab using glucose chemostat medium and CBS7435mut^S pAOX HyHEL-Fab using methanol/glycerol chemostat medium) and non-producing wildtype control strain (CBS7435 pGAP control and CBS7435mut^S pAOX control, respectively) to obtain the biological replicates necessary for reliable microarray analysis.

[00237] Samples were taken after approximately 70 hours in steady state conditions of the chemostat. Routine sampling as determination of optical density or yeast dry mass, qualitative microscopic inspection and cell viability analysis was done alongside during each cultivation. For microarray analysis, samples were taken and treated as follows: For optimal quenching, 9 mL cell culture broth was immediately mixed with 4.5 mL of ice cold 5% phenol (Sigma) solution (in Ethanol abs.), and aliquoted. Each 2 mL were centrifuged (13,200 rpm for 1 minute) in pre-cooled collection tubes (GE healthcare, NJ), supernatant was removed completely and the tubes were stored at -80°C until RNA isolation.

Example 3 Microarrays & data evaluation for transcriptomic experiments

a) RNA isolation and sample preparation for microarray hybridization

[00238] The RNA was isolated from chemostat sample cells using TRI reagent according to the supplier's instructions (Ambion, US). The cell pellets were resuspended in TRI reagent and homogenized with glass beads using a FastPrep 24 (M.P. Biomedicals, CA) at 5 m s^{-1} for 40 seconds. After addition of chloroform, the samples were centrifuged and the total RNA was precipitated from the aqueous phase by adding isopropanol. The pellet was washed with 70% ethanol, dried and re-suspended in RNAse free water. RNA concentrations were determined by measuring OD_{260} using a Nanodrop 1000 spectrophotometer (NanoDrop

products, DE). Remaining DNA from the samples was removed using the DNA free Kit (Ambion, CA). Sample volume equal to 10 µg RNA was diluted to 50 µL in RNAse free water, then DNAse buffer I and rDNAse I were added and incubated at 37°C for 30 minutes. After addition of DNAse Inactivation Reagent, the sample was centrifuged and the supernatant was transferred into a fresh tube. RNA concentrations were determined again like described above. Additionally, RNA integrity was analyzed using RNA nano chips (Agilent). To monitor the microarray workflow from amplification and labelling to hybridisation of the samples, the Spike In Kit (Agilent, Product Nr.: 5188-5279) was used as positive control. It contains 10 different polyadenylated transcripts from an adenovirus, which are amplified, labelled and cohybridised together with the own RNA samples. The samples were labelled with Cy 3 and Cy 5 using the Quick Amp Labelling Kit (Agilent, Prod. Nr.5190-0444). Therefore 500 ng of purified sample RNA were diluted in 8.3 µL RNAse free water, 2 µL Spike A or B, and 1.2 µL T7 promoter primer were added. The mixture was denatured for 10 minutes at 65°C and kept on ice for 5 minutes. Then 8.5 µL cDNA mastermix (per sample: 4 µL 5x first strand buffer, 2 µL 0.1 M DTT, 1 µL 10 mM dNTP mix, 1 µL MMLV-RT, 0.5 µL RNAse out) were added, incubated at 40°C for 2 hours, then transferred to 65°C for 15 minutes and put on ice for 5 minutes. The transcription mastermix (per sample: 15.3 µL nuclease free water, 20 µL transcription buffer, 6 µL 0.1 M DTT, 6.4 µL 50% PEG, 0.5 µL RNAse Inhibitor, 0.6 µL inorg. phosphatase, 0.8 µL T7 RNA Polymerase, 2.4 µL Cyanin 3 or Cyanin 5) was prepared and added to each tube and incubated at 40°C for 2 hours. In order to purify the obtained labelled cRNA, the RNeasy Mini Kit (Qiagen, Cat.No. 74104) was used. Samples were stored at -80 °C. Quantification of the cRNA concentration and labelling efficiency was done at the Nanodrop spectrophotometer.

b) Microarray analysis

[00239] The Gene Expression Hybridisation Kit (Agilent, Cat. No. 5188-5242) was used for hybridisation of the labelled sample cRNAs. For the preparation of the hybridisation samples each 300 ng cRNA (Cy3 and Cy 5) and 6 µL 10-fold blocking agent were diluted with nuclease free water to a final volume of 24 µL. After addition of 1 µL 25-fold fragmentation buffer, the mixture was incubated at 60°C for 30 minutes. Then 25 µL GEx Hybridisation Buffer HI-RPM was added to stop the reaction. After centrifugation for one minute with 13,200 rpm, the sample was chilled on ice and used for hybridisation immediately. In-house designed *P. pastoris* specific oligonucleotide arrays (AMAD-ID: 034821, 8x15K custom arrays, Agilent) were used. Microarray hybridisation was done according to the Microarray Hybridisation Chamber User Guide (Agilent G2534A). First, the gasket slide was uncovered and put onto the chamber base, Agilent label facing up. The sample (40 µL per array) was loaded in the middle of each of the eight squares. Then the

microarray slide was carefully put onto the gasket slide (Agilent label facing down) and the chamber cover was placed on and fixed with the clamp. All samples were hybridized against a reference pool sample in a dye-swap manner. The pool RNA sample had been generated by combining RNA from a variety of cultivations in equal amounts. Hybridisation was done in the hybridisation oven for 17 hours at 65°C. Before scanning, the microarray chip was washed. Therefore, the chamber was dismantled, and the sandwich slides were detached from each other while submerged in wash buffer 1. The microarray was directly transferred into another dish with wash buffer 1, washed for 1 minute, transferred into wash buffer 2 (temperature at least 30°C) and washed for another minute. After drying of the microarray slide by touching the slide edge with a tissue, it was put into the slide holder (Agilent label facing up). The slide holder was put into the carousel and scanning was started.

c) Data acquisition and statistical evaluation of microarray data

[00240] Images were scanned at a resolution of 50 nm with a G2565AA Microarray scanner (Agilent) and were imported into the Agilent Feature Extraction 9.5 software. Agilent Feature Extraction 9.5 was used for the quantification of the spot intensities. The raw mean spot intensity data was then imported into the open source software R for further normalisation and data analysis.

[00241] The intensity data were subjected to normalization (no background correction, the within slide normalization method Loess and the between slide normalization method Aquantile was used), before the differential expression values were calculated. The p-values associated with the differential expression values were calculated using a linear model fit (limma R package), subsequently they were adjusted for multiple testing using the method of Benjamini and Yekutieli (BY method of limma R package). Log2 fold changes were calculated for HyHEL-Fab producing strains compared to their respective control.

[00242] The microarray data was browsed for entries with significant (adjusted p-value <0.05) difference in expression levels (fold change >1.5) between the chemostat triplicates of CBS7435 producing HyHEL-Fab and its non-producing host control.

[00243] Table 4 shows up-regulated genes from microarray analysis of HyHEL-Fab producing *P. pastoris*.

Table 4

Gene identifier *P. pastoris* CBS7435	Microarray probe name	Transcript level fold change in CBS7435 pPM2d_pGAP HyHEL vs. control	Transcript level fold change in CBS7435 pPM2d_pAOX

			HyHEL vs. control
PAS_chr4_0822	Pipas_chr4_0822	1.20	8.27
PP7435_Chr4-1007	Pipas_chr4_0009	2.68	5.56
PP7435_Chr3-0183	Pipas_chr3_0987	2.17	5.47
PP7435_Chr1-1225	Pipas_chr1-4_0431	2.48	4.76
PP7435_Chr4-0976	PIPA00444	2.27	4.22
PP7435_Chr2-0351	Pipas_chr2-2_0331	1.74	4.10
PP7435_Chr1-0941	Pipas_chr1-4_0167	0.93	3.12
PP7435_Chr3-0933	Pipas_chr3_0288	2.06	3.11
PAS_chr3_0401	Pipas_chr3_0401	2.59	2.92
PP7435_Chr1-1232	Pipas_chr1-4_0681	1.57	2.79
PP7435_Chr4-0294	Pipas_chr4_0673	2.11	2.70
PP7435_Chr1-0667	Pipas_chr1-1_0348	2.02	2.69
PP7435_Chr3-0607	Pipas_chr3_0598	2.02	2.59
PP7435_Chr2-0722	Pipas_chr2-1_0566	1.13	2.50
PP7435_Chr2-0842	Pipas_chr2-1_0454	4.16	2.43
PAS_chr3_0821	Pipas_chr3_0821	1.40	2.18
PP7435_Chr2-0501	Pipas_chr2-1_0887	2.69	2.18
PP7435_Chr2-0220	Pipas_chr2-2_0210	1.27	2.17
PP7435_Chr3-0278	Pipas_chr3_0895	1.06	2.09
PP7435_Chr4-0108	Pipas_chr4_0843	1.80	2.04
PP7435_Chr2-1019	Pipas_chr2-1_0287	1.52	2.04
PP7435_Chr3-1062	Pipas_chr3_0170	1.75	1.96
PP7435_Chr4-0448	Pipas_chr4_0972	2.31	1.95
PP7435_Chr3-0837	Pipas_chr3_0377	1.38	1.87
PP7435_Chr1-0176	Pipas_chr1-3_0174	1.70	1.84
PP7435_Chr3-0156	Pipas_chr3_1014	1.30	1.84
PP7435_Chr4-0582	Pipas_chr4_0403	1.34	1.82
PP7435_Chr2-0638	Pipas_chr2-1_0642	2.35	1.78
PP7435_Chr3-0639	Pipas_chr3_0567	1.70	1.73
PP7435_Chr2-0866	Pipas_chr2-1_0433	1.36	1.71
PP7435_Chr2-0028	Pipas_chr2-2_0031	1.70	1.71
PP7435_Chr1-1009	Pipas_chr1-4_0229	4.09	1.70
PP7435_Chr2-0848	Pipas_chr2-1_0448	1.39	1.70
PP7435_Chr1-0470	Pipas_chr1-1_0160	1.39	1.69
PP7435_Chr1-0077	Pipas_chr1-3_0080	1.31	1.68
PP7435_Chr1-1220	Pipas_chr1-4_0428	1.49	1.68
PP7435_Chr1-0571	Pipas_chr1-1_0259	2.18	1.68
PP7435_Chr1-0204	Pipas_chr1-3_0202	0.71	1.67
PP7435_Chr4-0182	Pipas_chr4_0775	1.22	1.65
PP7435_Chr4-0320	Pipas_chr4_0650	1.26	1.65
PP7435_Chr3-0548	Pipas_chr3_0652	2.29	1.62
PP7435_Chr2-1300	Pipas_chr2-1_0002	3.71	1.62
PP7435_Chr1-1077	Pipas_chr1-4_0294	1.46	1.61
PP7435_Chr4-0699	Pipas_chr4_0299	1.43	1.61
PP7435_Chr2-0729	Pipas_chr2-1_0560	1.12	1.56
PP7435_Chr4-0923	Pipas_chr4_0093	0.94	1.53
PP7435_Chr1-0592	Pipas_chr1-1_0276	1.59	1.52

Example 4 Generation of strains overexpressing identified genes

[00244] For the investigation of positive effects on Fab secretion, the identified genes were overexpressed in two different Fab producing strains: CBS7435 pPM2d_pAOX HyHEL,

which was the source of the microarray data (see Example 3) and CBS7435 pPM2d_pAOX SDZ (generation see Example 1).

a) Amplification and cloning of the identified potential secretion helper genes into pPM2aK21 expression vectors

[00245] The genes identified in Example 3 were amplified by PCR (Phusion Polymerase, New England Biolabs) from start to stop codon using the primers shown in Table 5. The sequences were cloned into the MCS of the pPM2a expression vector with the two restriction enzymes *Sbf*I and *Sfi*I. pPMKaK21 is a derivative of pPM2d (described in Example 1a), consisting of an AOX terminator sequence (for integration into the native AOX terminator locus), an origin of replication for *E. coli* (pUC19), an antibiotic resistance cassette (kanMX conferring resistance to Kanamycin and G418) for selection in *E. coli* and yeast, an expression cassette for the gene of interest (GOI) consisting of a *GAP* promoter, a multiple cloning site (MCS) and the *S. cerevisiae CYC1* transcription terminator. Gene sequences were verified by Sanger sequencing.

Table 5

Gene identifier (ORF name CBS7435)	Forward primer (*Sbf*I attached)	Backward primer (*Sfi*I attached)
PAS_chr4_0822	CTTGCCTGCAGGATGCTAACGGCCAGTTGGTC SEQ ID NO: 38	GATCGGCCGAGGCGGCCTCAGCAGTATTCCCACCAGAATC SEQ ID NO: 39
PP7435_Chr4-1007	CTTGCCTGCAGGATGTCAGTTCATTTCGTTATAGCAGC SEQ ID NO: 40	GATCGGCCGAGGCGGCCTCATATAAAAGGTTTATCATAATTCTCATCCTCAG SEQ ID NO: 41
PP7435_Chr3-0183	GAAACCTGCAGGATGTCTGAATTTGTTGCTAAAATTAACATTC SEQ ID NO: 42	GATCGGCCGAGGCGGCCTTAGGCGGTTGGAACGTTC SEQ ID NO: 43
PP7435_Chr1-1225	GAAACCTGCAGGATGTCTCATCTATTACTGCGTGACAGC SEQ ID NO: 44	GATCGGCCGAGGCGGCCTCATGGCCCGGCATATCTAG SEQ ID NO: 45
PP7435_Chr4-0976	GACACCTGCAGGATGTCTGAATCCTCCAGTATCTCTCTAGTTG SEQ ID NO: 46	GATCGGCCGAGGCGGCCCTAGATACATCCCAAAAGTGCACCG SEQ ID NO: 47
PP7435_Chr2-0351	GATACCTGCAGGATGATCCTGGGTTCAGTTTGGG SEQ ID NO: 48	GATCGGCCGAGGCGGCCCTAAAAGTTTGCTGCAGCATTTGAAG SEQ ID NO: 49
PP7435_Chr1-0941	CTTGCCTGCAGGATGGGTTGCTTTAGATTTTGTCTGG SEQ ID NO: 50	GATCGGCCGAGGCGGCCCTATTTGTATACGTGCTGTGGAGCC SEQ ID NO: 51
PP7435_Chr3-0933	CTTGCCTGCAGGATGTTAAACAAGCTGTTCATTGCAATACTC SEQ ID NO: 52	GATCGGCCGAGGCGGCCTTAGCTGGCAAGGGTAATTGTCTC SEQ ID NO: 53
PAS_chr3_0401	GACACCTGCAGGATGGCTCCTCAAACACCAAGG SEQ ID NO: 54	GATCGGCCGAGGCGGCCTCAAAAAAACAATCTCAAAATCTCCAG SEQ ID NO: 55

PP7435_Chr1-1232	GTACCCTGCAGGATGACCAAGGAAAATGAAGCC SEQ ID NO: 56	GATCGGCCGAGGCGGCCTTATTTTTTCTCCAATTCAGCCAG SEQ ID NO: 57
PP7435_Chr4-0294	GAAACCTGCAGGATGCTGTTGTCACATACCATGATACTTC SEQ ID NO: 58	GATCGGCCGAGGCGGCCTTAAGATTGCTTCTTTTTGAGATTGG SEQ ID NO: 59
PP7435_Chr1-0667	GAAACCTGCAGGATGACGGACTATGTCACTTCTAAGCG SEQ ID NO: 60	GATCGGCCGAGGCGGCCTCATAATCTCCCTCCAGGGG SEQ ID NO: 61
PP7435_Chr3-0607	GAAACCTGCAGGATGTCGTCTGATGCTGTGGAG SEQ ID NO: 62	GATCGGCCGAGGCGGCCTCAAATAATGCTACATTTGCGTTTCTTTC SEQ ID NO: 63
PP7435_Chr2-0722	CTTGCCTGCAGGATGTCTTATACGTCGGACAACAAAGAG SEQ ID NO: 64	GATCGGCCGAGGCGGCCTTACGTGTATCCGCTTCCTCTGTAC SEQ ID NO: 65
PP7435_Chr2-0842	GATACCTGCAGGATGAACTTGTACCTAATTACATTACTATTCGC SEQ ID NO: 66	GATCGGCCGAGGCGGCCTTAGAACCCACATTGATTTGGATACTG SEQ ID NO: 67
PAS_chr3_0821	GAAACCTGCAGGATGTCGTTATCAACCTTTCTAGGCG SEQ ID NO: 68	GATCGGCCGAGGCGGCCTCAGACTCTACTCATCATTTTGTCTTCCTC SEQ ID NO: 69
PP7435_Chr2-0501	GAAACCTGCAGGATGATGTACAGGAACTTAATAATTGCTACTGC SEQ ID NO: 70	GATCGGCCGAGGCGGCCCTAACACTCTATGAGGTCTACAATGTCCAAC SEQ ID NO: 71
PP7435_Chr2-0220	CATGCCTGCAGGATGTCTACAGCAATTCCAGGAGGAC SEQ ID NO: 72	GATCGGCCGAGGCGGCCTTAGTTGATCAACTTTCCTGTCAGCTTAG SEQ ID NO: 73
PP7435_Chr3-0278	GACACCTGCAGGATGAGTGGTGACCATAAGAGCTTTACG SEQ ID NO: 74	GATCGGCCGAGGCGGCCTTACTGTGTACCATACCGATCCAATCC SEQ ID NO: 75
PP7435_Chr4-0108	CTTGCCTGCAGGATGACTAACTGGAAAGCGATATTGACTCC SEQ ID NO: 76	GATCGGCCGAGGCGGCCTTAGTTCTCTTCTTCACCTTGAAATTTTAGGC SEQ ID NO: 77
PP7435_Chr2-1019	GCAACCTGCAGGATGTCTTATCGCCCTCAGTTTCAAC SEQ ID NO: 78	GATCGGCCGAGGCGGCCTCAATAGATCTTTTTCTTTTCATCAAAACTCAAC SEQ ID NO: 79
PP7435_Chr3-1062	GATACCTGCAGGATGGTGGTGCACAACCCTAATAAC SEQ ID NO: 80	GATCGGCCGAGGCGGCCCTAGGTACTATGCTGAACATCCTGAGTATGAG SEQ ID NO: 81
PP7435_Chr4-0448	GAAACCTGCAGGATGAGATTTTCTAACGTCGTTTTAACTGC SEQ ID NO: 82	GATCGGCCGAGGCGGCCTTACAAGGCAAAGACTCCGAAAGTG SEQ ID NO: 83
PP7435_Chr3-0837	GACACCTGCAGGATGACTGTGCCTGATCTGAAAGAAAC SEQ ID NO: 84	GATCGGCCGAGGCGGCCTCAGGCCAGCGCAACG SEQ ID NO: 85
PP7435_Chr1-0176	CTTGCCTGCAGGATGAAGATATGGCTGGTACTTCTTTTAGTTTTTG SEQ ID NO: 86	GATCGGCCGAGGCGGCCTCACAATTCGTCTCTAATTTGTTGCG SEQ ID NO: 87
PP7435_Chr3-0156	CTTGCCTGCAGGATGGAGCAGGTTCCAGTCG SEQ ID NO: 88	GATCGGCCGAGGCGGCCTTATTCATCATAAACTTCTTCTATGGTGGC SEQ ID NO: 89
PP7435_Chr4-0582	CTTGCCTGCAGGATGGATCCTTTTTCAATTCTTCTCAC SEQ ID NO: 90	GATCGGCCGAGGCGGCCCTACTTTGGAGACAGATCTTCCACCTTAAC SEQ ID NO: 91
PP7435_Chr2-0638	GAAACCTGCAGGATGACCAGTCAAGGATTTTTGGATC SEQ ID NO: 92	GATCGGCCGAGGCGGCCCTATATGCTATCAACCATCTCCATCAAATAAC SEQ ID NO: 93
PP7435_Chr3-0639	GACACCTGCAGGATGACTCCCCGTTCTCATATTTTC SEQ ID NO: 94	GATCGGCCGAGGCGGCCCTACTCAAAGAACTTAGACAAAGCAGCTTTCTC SEQ ID NO: 95
PP7435_Chr2-	GATCCCTGCAGGATGGCAGAAGAAGA	GATCGGCCGAGGCGGCCCTAATTAG

0866	ACC SEQ ID NO: 96	TAATACTTGCTTCTATTTCCTGGTACA AC SEQ ID NO: 97
PP7435_Chr2-0028	GAACCCTGCAGGATGATTTTGAGCAA GCTGTCGTTTAGAC SEQ ID NO: 98	GATCGGCCGAGGCGGCCTTATTTAT TAACAATGACATCATCTTCAAACTCG SEQ ID NO: 99
PP7435_Chr1-1009	CTTGCCTGCAGGATGGGTGCCATTGG AATG SEQ ID NO: 100	GATCGGCCGAGGCGGCCCTATTGCA GAACATTCGATATCCAATC SEQ ID NO: 101
PP7435_Chr2-0848	GATACCTGCAGGATGCTACCATTTTCG TACGACGTG SEQ ID NO: 102	GATCGGCCGAGGCGGCCCTATAACT CTCCATTCTCCTCGTCGATC SEQ ID NO: 103
PP7435_Chr1-0470	GATCCCTGCAGGATGAAAATATTAAGT GCATTGCTTCTTCTTTTTAC SEQ ID NO: 104	GATCGGCCGAGGCGGCCTTATAGCT CTTGGTGTAATAACTGGGG SEQ ID NO: 105
PP7435_Chr1-0077	CTTGCCTGCAGGATGTCTAAACCCTAC AAGCTGATAGGTGAG SEQ ID NO: 106	GATCGGCCGAGGCGGCCTTAATCTT CTCCAGCAGGTATCTCATCC SEQ ID NO: 107
PP7435_Chr1-1220	CTTGCCTGCAGGATGAATCAATTTTCT CTAGCTTCACAAGTAAAC SEQ ID NO: 108	GATCGGCCGAGGCGGCCCTACTCG GTTAATGGTCCGAGTGC SEQ ID NO: 109
PP7435_Chr1-0571	GACACCTGCAGGATGAGTTATAGGAA AGACAACAAACAAAAG SEQ ID NO: 110	GATCGGCCGAGGCGGCCTTAGAAG GCAGCTTCATCATCG SEQ ID NO: 111
PP7435_Chr1-0204	CTTGCCTGCAGGATGAGCAGCTTCAG AGTTCTAGACTTGG SEQ ID NO: 112	GATCGGCCGAGGCGGCCTTACAGAT CAACGAATCC SEQ ID NO: 113
PP7435_Chr4-0182	GATACCTGCAGGATGAACATCTTTAGA ATCCTAGGTAAGTTTCC SEQ ID NO: 114	GATCGGCCGAGGCGGCCTCATTCTG GCAGCTTGAATTTC SEQ ID NO: 115
PP7435_Chr4-0320	CTTGCCTGCAGGATGTCCACAACTACT AAGAAAAACAAGAACAGG SEQ ID NO: 116	GATCGGCCGAGGCGGCCTTACCATG CACCCTTTCCTCTC SEQ ID NO: 117
PP7435_Chr3-0548	CTTGCCTGCAGGATGTCAGAGGAGTA AGAACCACAAACAG SEQ ID NO: 118	GATCGGCCGAGGCGGCCTCAATTTA TTCTAGGTTTTTTGGTTCGG SEQ ID NO: 119
PP7435_Chr2-1300	GTACCCTGCAGGATGATGGCAAGTCC AACCG SEQ ID NO: 120	GATCGGCCGAGGCGGCCCGCAACA ACGCTGGTTG SEQ ID NO: 121
PP7435_Chr1-1077	CTTGCCTGCAGGATGAGTAACCAGTAT AATCCGTATGAGCAG SEQ ID NO: 122	GATCGGCCGAGGCGGCCCTATCTTC CCCAGTTTCCGACAC SEQ ID NO: 123
PP7435_Chr4-0699	GTTACCTGCAGGATGTCTACAGAGAA CAAAGCAGAGACAAAAC SEQ ID NO: 124	GATCGGCCGAGGCGGCCCTATTTCT TTGCTTCAGCGTTTGC SEQ ID NO: 125
PP7435_Chr2-0729	CTTGCCTGCAGGATGTTAAACTTAATA TCCACAATAAGTGGGTG SEQ ID NO: 126	GATCGGCCGAGGCGGCCTTAAGCAG GAGCAGATAACCAAGC SEQ ID NO: 127
PP7435_Chr4-0923	CTTGCCTGCAGGATGGGTAGAAGGAA AATAGAGATAAATCCG SEQ ID NO: 128	GATCGGCCGAGGCGGCCTCAGCTCT TCTTAGTCACACTGCTTG SEQ ID NO: 129
PP7435_Chr1-0592	CTTGCCTGCAGGATGTCACTTCAACTG TCCATTATCTTCG SEQ ID NO: 130	GATCGGCCGAGGCGGCCCTACTCGT CCTTCTTGTTGCTCTTCTC SEQ ID NO: 131

b) Co-overexpression of identified genes in *P. pastoris* Fab producing strains

[00246] The *P. pastoris* Fab overproducing strains CBS7435mut^S pAOX HyHEL-Fab and CBS7435mut^S pAOX SDZ-Fab were used as host strains for co-overexpression of the genes

identified in Example 3 and cloned in Example 4a. Before transformation into the Fab producing strains, the pPM2aK21 vectors containing the secretion helper genes identified in Example 3 are linearized in the AOX terminator sequence with the restriction enzyme *Asc*I. Positive transformants were selected on YPD agar plates containing G418.

Example 5 Screening for Fab expression

[00247] In small-scale screenings, 8 to 12 transformants of each secretion helper gene were tested in comparison to the non-engineered parental host and ranked based on their impact on cell growth, Fab titer and Fab yield. Of all the tested clones, PP7435_Chr3-0933, PP7435_Chr2-0220, PP7435_Chr3-0639, PP7435_Chr1-1232, PP7435_Chr1-1225, PP7435_Chr3-0607, PP7435_Chr4-0448, PP7435_Chr4-0108, PP7435_Chr1-0667 were found to show the best results (increasing Fab titer or yield at least 1.2-fold).

[00248] In Example 7, all of these clones (except PP7435_Chr1-0667) were then cultivated in bioreactors for verification of strain improvement in controlled production processes.

a) Small scale cultivation of *Pichia pastoris* Fab production strains

[00249] 2 mL YP-medium (10 g/L yeast extract, 20 g/L peptone) containing 10 g/L glycerol and 50 µg/mL Zeocin were inoculated with a single colony of *P. pastoris* strains and grown overnight at 25 °C. Aliquots of these cultures (corresponding to a final OD_{600} of 2.0) were transferred to 2 mL of Synthetic screening medium M2 (media composition is given below) supplemented with 20 g/L glucose and a glucose feed tablet (Kuhner, Switzerland; CAT# SMFB63319) and incubated for 25 h at 25 °C at 170 rpm in 24 deep well plates. The cultures were washed once by centrifugation, then the pellets were resuspended in Synthetic screening medium M2 and aliquots (corresponding to a final OD_{600} of 4.0) were transferred into 2 mL of Synthetic screening medium M2 in fresh 24 deep well plates. Methanol (5 g/L) was added repeatedly every 12 h for 48 hours, before cells were harvested by centrifugation at 2500xg for 10 min at room temperature and prepared for analysis. Biomass was determined by measuring the cell weight of 1 mL cell suspension, while determination of the recombinant secreted protein in the supernatant is described in the following Examples 5b-6c.

[00250] *Synthetic screening medium M2 contained per litre:* 22.0 g Citric acid monohydrate 3.15 g $(NH_4)_2PO_4$, 0.49 g $MgSO_4*7H_2O$, 0.80 g KCl, 0.0268 g $CaCl_2*2H_2O$, 1.47 mL PTM1 trace metals, 4 mg Biotin; pH was set to 5 with KOH (solid)

b) SDS-PAGE & Western Blot analysis

[00251] For protein gel analysis the NuPAGE® Novex® Bis-Tris system was used, using 12 % Bis-Tris or 4-12 % Bis-Tris gels and MOPS running buffer (all from Invitrogen). After electrophoresis, the proteins were either visualized by silver staining or transferred to a nitrocellulose membrane for Western blot analysis. Therefore, the proteins were electroblotted onto a nitrocellulose membrane using the XCell II™ *Blot* Module for wet (*tank*) transfer (Invitrogen) according to the manufacturer´s instructions. After blocking, the Western Blots were probed with the following antibodies: For Fab light chain: anti-human kappa light chains (bound and free) - alkaline phosphatase (AP) conjugated antibody, Sigma A3813 (1:5,000); For Fab heavy chain: Mouse Anti-Human IgG antibody (Ab7497, Abcam) diluted 1:1,000 and Anti-Mouse IgG (Fc specific)–Alkaline Phosphatase antibody produced in goat (A1418, Sigma) as secondary antibody diluted 1:5,000.

[00252] Detection was performed with the colorimetric AP detection kit (BioRad) based on the NBT/BCIP system for AP-conjugates, or the chemoluminescent Super Signal West Chemiluminescent Substrate (Thermo Scientific) for HRP-conjugates.

c) Quantification of Fab by ELISA

[00253] Quantification of intact Fab by ELISA was done using anti-human IgG antibody (ab7497, Abcam) as coating antibody and a goat anti-human IgG (Fab specific) - alkaline phosphatase conjugated antibody (Sigma A8542) as detection antibody. Human Fab/Kappa, IgG fragment (Bethyl P80-115) was used as standard with a starting concentration of 100 ng/mL, supernatant samples are diluted accordingly. Detection was done with pNPP (Sigma S0942). Coating-, Dilution- and Washing buffer were based on PBS (2 mM KH_2PO_4, 10 mM $Na_2HPO_4.2\ H_2O$, 2.7 mM g KCl, 8 mM NaCl, pH 7.4) and completed with BSA (1% (w/v)) and/or Tween20 (0.1% (v/v)) accordingly.

Example 6 Generation of strains underexpressing selected identified genes

[00254] Some of the genes that had been identified in Example 3, resulted in less secreted Fab when overexpressed in HyHEL-Fab producing *P. pastoris* host strains (in Example 4 and 5). Two of these genes were encoding chaperones, namely the cytosolic chaperone HCH1/PP7435_Chr3-1062 (KO2) and the ER-resident chaperone SCJ1/PP7435_Chr1-0176 (KO3). This finding is very surprising because chaperones are generally regarded as having expression/secretion enhancing effects. To the inventors' surprise, the overexpression of PP7435_Chr1-0176 was detrimental, reducing Fab titers and yields to less than 80% of the parental non-engineered HyHEL-Fab or SDZ-Fab producing

strains. Also PP7435_Chr3-1062 overexpression reduced HyHEL Fab titers and yields to less than 80% of the parental non-engineered strains. Thus, these genes (PP7435_Chr1-0176/SCJ1, PP7435_Chr3-1062/HCH1) were disrupted in both host strains. The flocculation transcription factor PP7435_Chr4-0252/FLO8 was additionally chosen as knock-out target after many flocculation-related genes were found to be strongly down-regulated (fold change <0.66) in the transcriptomics experiment (Example 3).

[00255] The *P. pastoris* Fab overproducing strains CBS7435mut^S pAOX HyHEL-Fab and CBS7435mut^S pAOX SDZ-Fab were used as host strains. A split marker cassette approach was used as described by Heiss *et al.* (2013) [Appl Microbiol Biotechnol. 97(3):1241-9. to generate transformants with a disrupted gene locus. Verification of positive knock-out strains was done by PCR, using genomic DNA of transformants which had been able to grow on G418 and primers outside of the disruption cassettes (Table 6).

[00256] Table 6 lists all primers that were used for the construction of the knock-out cassettes (2 overlapping split marker cassettes per knock-out target): The primer pairs A_forward/A_backward, B_forward/B_backward, C_forward/C_backward, D_forward/ D_backward were used to amplify the fragments A, B, C and D by PCR (Phusion Polymerase, New England Biolabs). Fragment A is amplified from genomic *P. pastoris* DNA, starting 1700 bp in 5 prime direction of the respective ATG (of the targeted gene) until 200 bp in 5 prime direction of ATG. Fragment D is amplified from genomic *P. pastoris* DNA, starting 200 bp in 3 prime direction of the respective ATG (of the targeted gene) until 1700 bp in 3 prime direction of ATG. Fragment B consists of the first two thirds of the KanMX selection marker cassette and is amplified from pPM2aK21 vector DNA template. Fragment B consists of the last two thirds of the KanMX selection marker cassette and is amplified from pPM2aK21 vector DNA template. Fragments A and B are annealed together (AB) by overlap PCR using the primers A_forward and B_backward. Fragments C and D are annealed together (CD) by overlap PCR using the primers C_forward and D_backward. To generate knock-out strains, a Fab producing host strain was transformed with total 0.5 µg DNA of fragments AB and CD, which both overlap as well. Cells were selected on YPD agar plates containing 500 µg/mL G418. Positive knock-outs clones were verified by PCR using the primer pair check_forward (binds in 5 prime region close to primer sequence A_forward) and check_backward (binds in 3 prime region close to primer sequence D_backward). Due to the replacement of a 400 bp region (around ATG) with a KanMX cassette, PCR product bands of positive knock-out strains are bigger than those of a wild type sequence.

Table 6

Gene identifier	Primer	Sequence

PP7435_Chr4-0252	A_forward	CGAACATCCATCACCAAAACAC SEQ ID NO: 132
	A_backward	GTTGTCGACCTGCAGCGTACGGTGTTGCCGCGAAATG SEQ ID NO: 133
	B_forward	CATTTCGCGGCAACACCGTACGCTGCAGGTCGACAAC SEQ ID NO: 134
	B_backward	CGGTGAGAATGGCAAAAGCTTATG SEQ ID NO: 135
	C_forward	AAGCCCGATGCGCCAGAGTTG SEQ ID NO: 136
	C_backward	CGTCTCTTGGGCAAATTGATCAGTGGATCTGATATCACC TA SEQ ID NO: 137
	D_forward	TAGGTGATATCAGATCCACTGATCAATTTGCCCAAGAGA CG SEQ ID NO: 138
	D_backward	GACTGTTGCGATTGCTGGTG SEQ ID NO: 139
	check_forward	ATCCAGGACACGCTCATCAAG SEQ ID NO: 140
	check_backward	GTGTGTGCTCTGGAATTGGATC SEQ ID NO: 141
PP7435_Chr1-0176	A_forward	AGAGGAGGTTGAATGCGAAGAAG SEQ ID NO: 142
	A_backward	GTTGTCGACCTGCAGCGTACTTCTGGTGAGCTTATATGG CAGTAGTTAC SEQ ID NO: 143
	B_forward	GTAACTACTGCCATATAAGCTCACCAGAAGTACGCTGCA GGTCGACAAC SEQ ID NO: 144
	B_backward	CGGTGAGAATGGCAAAAGCTTATG SEQ ID NO: 145
	C_forward	AAGCCCGATGCGCCAGAGTTG SEQ ID NO: 146
	C_backward	CTCGGGATCACCAAGCACAAGTGGATCTGATATCACCTA SEQ ID NO: 147
	D_forward	TAGGTGATATCAGATCCACTTGTGCTTGGTGATCCCGAG SEQ ID NO: 148
	D_backward	TCAAAGTATGCTGGGAAGAATGG SEQ ID NO: 149
	check_forward	TGGATTGTCTCGGAGGCG SEQ ID NO: 150
	check_backward	TACTATGACTATGGGAGACCTGGGTG SEQ ID NO: 151
PP7435_Chr3-1062	A_forward	TGAAGCATCCCACCCACTG SEQ ID NO: 152
	A_backward	GTTGTCGACCTGCAGCGTACCCTTCGCAGACTGTAATTA TTGGC SEQ ID NO: 153
	B_forward	GCCAATAATTACAGTCTGCGAAGGGTACGCTGCAGGTC GACAAC SEQ ID NO: 154
	B_backward	CGGTGAGAATGGCAAAAGCTTATG SEQ ID NO: 155
	C_forward	AAGCCCGATGCGCCAGAGTTG SEQ ID NO: 156
	C_backward	GTTGACTTTGACGGTTGCAGATACAGTGGATCTGATATC ACCTA SEQ ID NO: 157
	D_forward	TAGGTGATATCAGATCCACTGTATCTGCAACCGTCAAAG

		TCAAC SEQ ID NO: 158
	D_backward	TTCTCTCCTTGATTATCGGTCTCTTTC SEQ ID NO: 159
	check_forward	TGGCAGATGACTTCACAAACG SEQ ID NO: 160
	check_backward	GTGGCATCTTTCATAACGACATCTC SEQ ID NO: 161

Example 7. Fed batch cultivations

[00257] Helper factor engineered strains from Example 5 and 6 with the best performance (increased yield of model protein by at least 1.2 fold change) in the small-scale cultivation were analyzed in fed batch bioreactor cultivations for verification of production host strain improvement. Two protocols were used.

a) Fed batch protocol A

[00258] The fed batches were carried out in 1.4 L DASGIP reactors (Eppendorf, Germany) with a maximum working volume of 1.0 L. Cultivation temperature was controlled at 25°C, pH was controlled at 5.0 by addition of 25% ammonium hydroxide and the dissolved oxygen concentration was maintained above 20% saturation by controlling the stirrer speed between 400 and 1200 rpm, and the airflow between 24 and 72 sL/h.

[00259] The inoculum for the fed batch cultivation was cultivated in shaking flasks containing 100 mL of YP medium containing 20 g/L glycerol and 50 µg/mL Zeocin, and incubated at 28°C and 180 rpm for approximately 24 hours. The cultures were used to inoculate the starting volume of 0.4 L in the bioreactor to a starting optical density (600 nm) of 1.0. The batch was finished after approximately 24 h and the first (10 mL) salt shot was given.

[00260] Glycerol fed batch solution was then fed at a constant rate of 5 mL/h for 5 hours. Then, a methanol pulse (2 g) and a salt shot (10 mL) were given to the culture. After methanol pulse consumption had been indicated by an increase in dissolved oxygen concentration in the culture, a constant feed with methanol fed batch solution was started with a feed rate of 1.0 g/h. Salt shots of 10 mL are given every 10 g of newly formed biomass, that corresponds to ~43 g methanol feed medium. With increasing biomass concentrations, the methanol feed rate was increased appropriately when methanol accumulation could be ruled out due to a sudden increase in dissolved oxygen in the culture when turning off the methanol feed for a short period of time. The final methanol feed rate was 2.5 g/h.

[00261] Samples were taken frequently for the determination of biomass and the quantification of Fab (as described in Example 6). The cultivation was harvested after approximately 100 hours when cell densities had reached more than 100 g/L cell dry weight.

[00262] The media were as follows:

[00263] *Batch medium* (*per litre*) *contained:* 2.0 g citric acid, 12.4 g $(NH_4)_2HPO_4$, 0.022 g $CaCl_2 \cdot 2H_2O$, 0.9 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 40 g glycerol, 4.6 mL PTM1 trace salts stock solution. The pH is set to 5.0 with 25% HCl.

[00264] *Glycerol fed batch solution* (*per litre*) *contained:* 623 g glycerol, 12 mL PTM1 trace salts stock solution and 40 mg biotin. PTM1 composition is given in Example 1.

[00265] *Methanol fed batch solution* (*per litre*) *of pure methanol contained:* 12 mL PTM1 trace salts stock solution and 40 mg biotin.

[00266] *Salt shot solution* (*per litre*) *contained:* 20.8 g $MgSO_4 \cdot 7H_2O$, 41.6 KCl, 1.04 g $CaCl_2 \cdot 2H_2O$

b) Fed batch protocol B

[00267] Respective strains were inoculated into wide-necked, baffled, covered 300 mL shake flasks filled with 50 mL of YPhyG and shaken at 110 rpm at 28°C over-night (pre-culture 1). Pre-culture 2 (100 mL YPhyG in a 1000 mL wide-necked, baffled, covered shake flask) was inoculated from pre-culture 1 in a way that the OD_{600} (optical density measured at 600 nm) reached approximately 20 (measured against YPhyG media) in late afternoon (doubling time: approximately 2 hours). Incubation of pre-culture 2 was performed at 110 rpm at 28°C, as well.

[00268] The fed batches were carried out in 1.0 L working volume bioreactor (Minifors, Infors, Switzerland). All bioreactors (filled with 400 mL BSM-media with a pH of approximately 5.5) were individually inoculated from pre-culture 2 to an OD600 of 2.0. Generally, *P. pastoris* was grown on glycerol to produce biomass and the culture was subsequently subjected to glycerol feeding followed by methanol feeding.

[00269] In the initial batch phase, the temperature was set to 28°C. Over the period of the last hour before initiating the production phase it was decreased to 25°C and kept at this level throughout the remaining process, while the pH dropped to 5.0 and was kept at this level. Oxygen saturation was set to 30% throughout the whole process (cascade control: stirrer, flow, oxygen supplementation). Stirring was applied between 700 and 1200 rpm and a

flow range (air) of 1.0 – 2.0 L/min was chosen. Control of pH at 5.0 was achieved using 25% ammonium. Foaming was controlled by addition of antifoam agent Glanapon 2000 on demand.

[00270] During the batch phase, biomass was generated (μ ~ 0.30/h) up to a wet cell weight (WCW) of approximately 110-120 g/L. The classical batch phase (biomass generation) would last about 14 hours. A constant glycerol-feed with 6 g/(L*h) was initiated after 11 hours, and lasted 5 hours. The first sampling point was selected to be 16 hours (in the following named as "0 hours" of induction time).

[00271] A total of 290 g of methanol was supplied over a period of approximately 95 hours (with a linearly increasing feed rate defined by the equation 1 + 0.04 * t (g/L)).

[00272] Samples were taken at various time points with the following procedure: the first 3 mL of sampled cultivation broth (with a syringe) were discarded. 1 mL of the freshly taken sample (3-5 mL) was transferred into a 1.5 mL centrifugation tube and spun for 5 minutes at 13,200 rpm (16,100 g). Supernatants were diligently transferred into a separate vial.

[00273] 1 mL of cultivation broth was centrifuged in a tared Eppendorf vial at 13,200 rpm (16,100 g) for 5 minutes and the resulting supernatant was accurately removed. The vial was weighed (accuracy 0.1 mg), and the tare of the empty vial was subtracted to obtain wet cell weights.

[00274] The media were as follows:

[00275] *YPhyG preculture medium* (*per litre*) *contained:* 20 g Phytone-Peptone, 10 g Bacto-Yeast Extract, 20 g glycerol

[00276] *Batch medium: Modified Basal salt medium (BSM)* (*per litre*) *contained:* 13.5 mL H_3PO_4 (85%), 0.5 g $CaCl \cdot 2H_2O$, 7.5 g $MgSO_4 \cdot 7H_2O$, 9 g K_2SO_4, 2 g KOH, 40 g glycerol, 0.25 g NaCl, 4.35 mL PTM1, 8.7 mg biotin, 0.1 mL Glanapon 2000 (antifoam)

[00277] *Feed-solution glycerol* (*per kg*) *contained:* 600 g glycerol, 12 mL PTM1

[00278] *Feed-solution Methanol contained*: pure methanol.

c) Results

[00279] Table 7 lists the genes whose overexpression was shown to increase Fab secretion in *P. pastoris* in fed batch production processes in comparison to the not engineered Fab producing control strains. The Fab product was quantified by ELISA

(Example 5c). Biomass was determined as wet cell weight or dry cell weight. Changes in product titers and yields are represented as fold change values relative to the respective control strain. Fold change values show the improvement in titers and product yields in fed batch production processes relative to the AOX HyHEL and AOX SDZ parental hosts which were grown and sampled in parallel for direct comparison.

Table 7

gene identifier	host strain	Fab titer fold change	Fab yield fold change	cultivation time	protocol
PP7435_Chr3-0933	CBS7435 pAOX HyHEL	2.24	2.38	109 h	B
	CBS7435 pAOX SDZ	1.21	1.38	111 h	B
PP7435_Chr2-0220	CBS7435 pAOX HyHEL	2.48	1.91	111 h	B
	CBS7435 pAOX SDZ	1.64	1.55	137 h	A
PP7435_Chr3-0639	CBS7435 pAOX HyHEL	1.62	1.41	109 h	B
	CBS7435 pAOX SDZ	1.17	1.20	111 h	B
PP7435_Chr1-1232	CBS7435 pAOX SDZ	1.13	1.22	111 h	B
PP7435_Chr1-1225	CBS7435 pAOX SDZ	1.39	1.74	111 h	B
PP7435_Chr3-0607	CBS7435 pAOX HyHEL	2.28	1.67	111 h	B
	CBS7435 pAOX SDZ	1.48	1.35	141 h	A
PP7435_Chr4-0448	CBS7435 pAOX HyHEL	1.65	2.21	111 h	B
PP7435_Chr4-0108	CBS7435 pAOX HyHEL	1.82	2.41	109 h	B

[00280] As shown, all the listed genes succeeded in increasing the yield (mg/biomass) of the model protein SDZ-Fab or HyHEL-Fab by at least 20 % (fold change > 1.2) upon over-expression.

[00281] Table 8 lists the genes whose deletion was shown to increase Fab secretion in *P. pastoris* in fed batch production processes in comparison to the not engineered Fab producing control strains. The Fab product was quantified by ELISA (Example 5c). Changes in product titers and yields are represented as fold change values relative to the respective control strain.

Table 8

gene identifier	host strain	Fab titer fold change	Fab yield fold change	cultivation time	protocol
PP7435_Chr4-0252	CBS7435 pAOX HyHEL	1.45	1.65	119 h	A
	CBS7435 pAOX SDZ	1.35	1.46	113 h	A
PP7435_Chr1-0176	CBS7435 pAOX HyHEL	1.35	1.28	111 h	A
PP7435_Chr3-1062	CBS7435 pAOX HyHEL	1.29	2.09	89 h	B

[00282] As shown, all the listed genes when underexpressed were able to increase the production in Fab titer (mg/L) or Fab yield (mg/biomass) of the model protein SDZ-Fab or HyHEL-Fab by at least 28% (fold change > 1.28).

Examples 8: Combination of HPs and HPs and KO proteins

[00283] For combinations of overexpression targets, CBS7435mutS pAOX SDZ-Fab strains overexpressing either HP3 ('3') or HP10 ('34') under control of the constitutive pGAP promoter (generated as described in Examples 4a and b) were used. For combination of overexpression with underexpression targets, CBS7435mutS pAOX SDZ-Fab strain with a disrupted KO1 gene locus (described in Example 6) was used. In all those strains, the plasmid encoding for the model protein SDZ-Fab were based on Zeocin as selection marker, whereas the plasmids for co-overexpression of the HP or the cassettes used for disruption of the KO gene locus carried the KanMX resistance cassette flanked by co-directional loxP recognition sites.

Prior to transformation with a further HP or KO cassette, the marker gene expression cassette (KanMX – flanked by loxP sites) was recycled by Cre recombinase. Therefore, the background strains were transformed with the episomal pTAC_Cre_HphMX4 plasmid, which is expressing Cre recombinase under control of *S. cerevisiae* TPI promoter and is transiently kept in *P. pastoris* as long as selection pressure by hygromycin (Hyg) is present in the culture medium. Transformants were grown on YPD/Zeo/Hyg agar plates at 28 °C for 2 days, and replica-plated on selective agar plates for growth at 28 °C for further 2 days. Only clones that lost their ability to grow on G418 and on Hyg after 2-3 plating rounds were selected for 24 deep well plate (DWP) screening (described in Example 5 a). Fab titer and yield were determined as described in Example 5c. The best strain in terms of Fab yield and/or titer was then transformed with another plasmid overexpressing a HP protein (described in Examples

4a and b). Transformants with two combined HPs or KOs were selected on selective agar plates (containing Zeo and G418) and screened for Fab secretion as described in Example 5. For further combinatorial steps, the procedure described above was repeated, thus yielding a strain with three combinations and so on. In all screening experiments, the parental (=preceding) strain was used as reference.

The results are as follows – Table 9:

	VS. PARENTAL STRAIN		VS. ORIGINATING STRAIN	
	FC TITER average	FC YIELD average	FC TITER average	FC YIELD average
HP3 ('3') HP1 ('56')	2.09 (n=16)	1.26 (n=16)	1.97 (n=11)	1.79 (n=11)
HP10 ('34') HP3 ('3')	1.63 (n=16)	1.6 (n=16)	n.a.	n.a.
KO1 HP2 ('2') HP3 ('3')	1.51 (n=8)	1.33 (n=8)	n.a.	n.a.
KO1 HP2 ('2') HP1 ('56')	1.42 (n=8)	1.33 (n=8)	n.a.	n.a.

"Originating strain" means a *P. pastoris* strain pAOX-SDZ-Fab#9 without a HP or without a knock-out, respectively.

"Parental strain" means the SDZ-Fab expressing *P. pastoris* strain serving as host strain for the transformation with the next HP or KO (e.g. a strain only overexpressing HP3 for the combination of HP3 and HP1, a strain having a knock-out of KO1 overexpressing HP2 for the combinations of KO1 HP2 with HP3 or HP1), respectively. It can be seen that each of the combinations leads to an increase of the Fab titer of the model protein SDZ-Fab, both in comparison to the originating strain and the parental strain. The increase of the Fab titer in comparison to the parental strain indicates that combinations of HPs or HPs and KO proteins can even further improve the yield of a POI exemplified by the model protein SDZ-Fab.

Claims

1. A recombinant host cell for manufacturing a protein of interest, wherein the host cell is engineered to underexpress at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof compared to the host cell prior to genetic manipulation, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or12.

2. The host cell of claim 1, wherein said polynucleotide encoding the KO protein increases the yield of the model protein SDZ-Fab (SEQ ID NO: 25 and 26) and/or HyHEL-Fab (SEQ ID NO: 29 and 30) compared to the host cell prior to engineering.

3. The host cell in claim 1 or 2, wherein underexpression is achieved by knocking out the polynucleotide encoding the KO protein or a functional homologue thereof from the genome of said host cell, disrupting the polynucleotide encoding the KO protein or a functional homologue thereof in the host cell, disrupting a promoter which is operably linked with said polypeptide encoding the KO protein or a functional homologue thereof, or post-transcriptional gene silencing.

4. The host cell in any one of the preceding claims, wherein the host cell is a *Pichia pastoris, Hansenula polymorpha, Trichoderma reesei, Saccharomyces cerevisiae, Kluyveromyces lactis, Yarrowia lipolytica, Pichia methanolica, Candida boidinii, and Komagataella, and Schizosaccharomyces pombe*.

5. The host cell of any of the preceding claims, wherein the protein of interest is an enzyme, a therapeutic protein, a food additive, feed additive, or an antibody or antibody fragment.

6. The host cell in any one of the preceding claims, wherein the host cell comprises at least one polynucleotide encoding a helper protein.

7. The host cell of claim 6, wherein the helper protein has an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, or 162.

8. A host cell obtained by providing a cell which expresses KO protein 1, KO protein 2, and/or KO protein 3, or functional homologue thereof and engineering the cell to underexpress the gene which encodes KO1, KO2 and/or KO3, or functional homologue thereof.

9. The host cell of any one of the preceding claims, wherein the host cell is engineered to

(i) underexpress a polynucleotide encoding a protein having an amino acid as shown in SEQ ID NO: 10 or a functional homologue thereof and further engineered to overexpress a polynucleotide encoding a helper protein having an amino acid sequence as shown in SEQ ID NO: 1 or a functional homologue thereof and a helper protein having an amino acid sequence as shown in SEQ ID NO: 2 or a functional homologue thereof, or

(ii) underexpress a polynucleotide encoding a protein having an amino acid as shown in SEQ ID NO: 10 or a functional homologue thereof and further engineered to overexpress a polynucleotide encoding a helper protein having an amino acid sequence as shown in SEQ ID NO: 4 or a functional homologue thereof and SEQ ID NO: 1 or a functional homologue thereof.

10. Use of the host cell in any one of the preceding claims for manufacturing a protein of interest.

11. A method of increasing the yield of a protein of interest in a host cell, comprising underexpressing at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12.

12. A method of increasing the yield of a protein of interest in a host cell comprising:

- engineering the host cell to underexpress at least one polynucleotide encoding a KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12,

- recombining in said host cell a heterologous polynucleotide encoding a protein of interest,

- culturing said host cell under suitable conditions to express the protein of interest, and optionally

- isolating the protein of interest from the cell culture.

13. A method of producing a protein of interest in a host cell comprising:

- providing a host cell engineered to underexpress at least one polynucleotide encoding an KO protein having an amino acid sequence as shown in SEQ ID NO: 10, 11, 12 or a functional homologue thereof, wherein the functional homologue has at least 30% sequence identity to an amino acid sequence as shown in SEQ ID NO: 10, 11 or 12, wherein said host cell comprises a heterologous polynucleotide encoding a protein of interest;

- culturing the host cell under suitable conditions to express protein of interest, and optionally

- -isolating the protein of interest from the cell culture.

14. The method in any of the preceding claims, wherein the protein of interest is an enzyme, a therapeutic protein, a food additive or feed additive, preferably a detoxifying enzyme.

15. .The method of any of the preceding claims, wherein said polynucleotide encoding the KO protein increases the yield of the model protein SDZ-Fab and/or HyHEL-Fab compared to the host cell prior to engineering