Description

INTRODUCTION

Technical field

This invention is in the field of recombinant DNA technology. More particularly it relates to the use of Kluyveromyces α-factor leader sequences to direct the secretion of heterologous polypeptides in yeast.

Background

U.S. Patent No. 4,546,082 describes the α-factor precursor gene of Saccharomyces cerevisiae and purports to show DNA constructs of the S. cerevisiae leader sequence and heterologous genes that are useful for the secretory expression of the heterologous genes.

EP-A-0 116 201 describes the actual use of the S. cerevisiae α-factor leader sequence to obtain secretory expression of human epidermal growth factor in yeast. Co-pending U.S. Patent Application Serial No. 522,909, filed August 12, 1983, describes the use of "spacer-less" S. cerevisiae α-factor leader sequences to obtain more efficient secretory expression of heterologous genes.

Science (1985) 229:1219-1224 reports the use of S. cerevisiae α-factor sequences to direct the secretion of prochymosin. It was reported that most of the prochymosin produced was present in an intracellular insoluble form rather than being secreted. Although the invertase leader was more effective at directing secretion of activatable prochymosin, an extensive mutant screen was necessary to yield reasonable amounts of prochymosin secretion.

There has been no previous demonstration of the production of mating pheromones (α-factor and a-factor) by K. lactis cells or other Kluyveromyces species. This failure to identify such peptides in K. lactis may, in part, be due to the fact that mating in K. lactis is inducible; whereas in S. cerevisiae the mating pheromones are produced constitutively.

Notwithstanding the availability of the mentioned leader sequences for directing secretion of heterologous polypeptides in yeast, there continues to be a need for alternative sequences that may afford more efficient or more practical production of particular polypeptides. Accordingly, applicants sought to determine whether a K. lactis α-factor exists and, if so, whether it might be useful for directing secretion of heterologous polypeptides, such as prochymosin, in yeast.

SUMMARY OF THE INVENTION

This invention provides DNA constructs based on the Kluyveromyces α-factor leader sequence for directing the secretion of heterologous polypeptides in yeast. Kluyveromyces transformants containing these constructs provide efficient secretion of polypeptides, such as prochymosin.

Accordingly, one aspect of the invention is a DNA construct encoding a protein whose amino acid sequence comprises a secretion-directing Kluyveromyces α-factor leader sequence linked to a heterologous polypeptide sequence via yeast processing signals for processing said protein into the heterologous polypeptide.

Another aspect of the invention is an expression vector comprising a replicative or integrative system functional in a yeast host and a DNA construct encoding a protein whose amino acid sequence comprises a secretion-directing Kluyveromyces α-factor leader sequence linked to a heterologous polypeptide sequence via a yeast processing signal for processing said protein into said heterologous polypeptide.

Still another aspect of the invention is a method for producing a heterologus polypeptide in yeast comprising growing yeast containing the expression vector as above in culture medium under conditions whereby said heterologous polypeptide is expressed in and secreted by said yeast as a "propolypeptide" and the propolypeptide is at least partially processed into said heterologous polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the strategy used to design oligonucleotide probes used to identify K. lactis α-factor DNA.

Figure 2 is the complete sequence of a DNA fragment encoding the K. lactis α-factor.

Figure 3 is a comparison of the protein sequence of K. lactis and S. cerevisiae α-factors.

Figure 4 is a schematic representation of K. lactis and S. cerevisiae α-factors.

Figure 5 is a comparison of the DNA sequences of genes encoding the K. lactis and S. cerevisiae α-factors.

Figure 6 is a schematic representation of three plasmids whose constructions are described in detail in the examples.

Figure 7 is a reaction scheme showing linkage of DNA for K. lactis α-factor leader to DNA for prochymosin.

Figure 8 represents the sequence of pAB309 BamHI/SalI insert in pUC18.

Figure 9 is a diagram of the plasmid pGB901.

Figure 10 is a diagram of the plasmid pGBTeG418.

Figure 11 represents the sequences of the primers used for mutagenesis of K. lactis α-factor leader DNA.

Figure 12 represents the DNA sequences around the junctions in K. lactis α-factor leader/prochymosin DNA fusions.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, yeast cells are employed for the production of mature heterologous polypeptides, where such polypeptides may be harvested directly from a nutrient medium. The polypeptides are produced by employing a DNA construct coding for a Kluyveromyces α-factor leader and processing signal sequence joined to the heterologous polypeptide of interest, which may be a single heterologous polypeptide or a plurality of heterologous polypeptides separated by processing signals. The resulting construct encodes a prepropolypeptide which will contain the signals for secretion of the prepropolypeptide and processing of the propolypeptide, either intracellularly or extracellularly, to the mature polypeptide.

The constructs of the subject invention will comprise a DNA sequence having the following formula encoding a propolypeptide:

$$(XY\text{-}(ZW)_n\text{-}Gene^*)_y$$

wherein:

X is a codon for lysine or arginine;

Y is a codon for arginine;

W is a codon for alanine or proline;

Z is a codon for an amino acid, preferably a codon for serine, lysine or asparagine when W encodes proline and a codon for glutamic acid, asparagine, aspartic acid, or serine when W encodes alanine;

y is an integer of at least one and usually not more than 10, more usually not more than four, providing for monomers and multimers;

Gene* is a gene other than the wild type Kluyveromyces α-factor associated with the leader sequence that is foreign to a yeast host, usually a plant or mammalian gene;

n is 0 or an integer which will generally vary from 1 to 8, usually 3 to 7. Preferably n is 0.

In this formula, the amino acids encoded by X, Y, Z and W define the α-factor processing signal in which the amino acids encoded by X and Y define a dipeptide cleavage site and those encoded by Z and W define an oligopeptide spacer. As indicated by the fact that n can be zero, the spacer is optional. If a spacer is present, the construct will typically include additional sequences that allow the removal of N-terminal residues defined by the spacer to provide the mature protein encoded by Gene*.

For the most part, the constructs of the subject invention will have at least the following formula:

$$L\text{-}(XY\text{-}(ZW)_n\text{-}Gene^*)_y$$

encoding a prepropolypeptide, wherein L is a Kluyveromyces leader sequence providing for secretion of the prepropolypeptide and the other symbols are as defined above. Any Kluyveromyces leader sequence may be employed which provides for secretion, leader sequences generally being of about 20 to 120 amino acids, usually about 30 to 100 amino acids having a hydrophobic region and having a methionine at its N-terminus.

The leader sequence, L, may be a full length α-factor leader sequence of any species for Kluyveromyces, such as K. lactis and K. fragilis, or a functional (i.e. capable of directing secretion) fragment, mutation, either conservative or non-conservative, usually not more than 5, more usually not more than 3 different amino acids, or analogs thereof. Functional fragments may be identified by preparing

constructs having various lengths of the wild type full length sequence and screening them for their ability to direct secretion. The gene sequence for the wild type K. lactis α-factor is set forth in Figure 2 with the leader sequence being the DNA encoding amino acids 1-83. The DNA sequence of Figure 2 is not essential. Any sequence which codes for a functional Kluyveromyces leader oligopeptide is sufficient. Different sequences will be more or less efficiently translated. Desirably, at least a majority of yeast preferred codons are employed in the sequence.

In providing for useful DNA sequences which can be used as cassettes for expression, the following sequence can be conveniently employed:

$$Tr\text{-}L\text{-}(XY\text{-}(ZW)_n\text{-}(Gene^*)_d)_y$$

wherein:

Tr intends a DNA sequence encoding for the transcriptional regulatory signals, particularly the promoter and such other regulatory signals as operators, activators, cap signal, or other sequence involved with transcriptional or translational control;

d is 0 or 1, being 1 when y is greater than 1.

The Tr sequence will generally be at least about 100 bp and not more than about 2000 bp. Particularly useful is employing the Tr sequence associated with the leader sequence L, so that a DNA fragment can be employed which includes the transcriptional and translational signal sequences associated with the signal sequence endogenous to the host. Alternatively, one may employ other transcriptional and translational signals to provide for enhanced production of the expression product.

The remaining symbols have been defined previously.

The 3'-terminus of Gene* can be manipulated much more easily in the form of a construct which allows for insertion of Gene* into a unique restriction site in the construct. Such a preferred construct would provide for a restriction site with insertion of the Gene* into the restriction site to be in frame with the initiation codon. Such a construction can be symbolized as follows:

$$(Tr)_a\text{-}L\text{-}XY\text{-}(ZW)_n\text{-}(SC)_b\text{-}Te$$

wherein:

those symbols previously defined have the same definition;

a is 0 or 1 intending that the construct may or may not have the transcriptional and translational signals;

SC represents a stop codon;

Te is a transcriptional termination sequence and may include other signals, e.g. polyadenylation; and

b is an integer which will generally vary from about 0 to 4, more usually from 0 to 3, it being understood, that Gene* may include its own stop codon.

The sequence upstream from Gene* may be designed to provide convenient restriction sites to permit a given species of Gene* to be replaced with another species of Gene*. Linkers and adapters may be used, as required, to achieve such replacement.

The construct provides a portable sequence for insertion into vectors, which provide the desired replication system. As already indicated, in some instances it may be desirable to replace the wild-type promoter associated with the signal sequence with a different promoter. In yeast, promoters involved with enzymes in the glycolytic pathway can provide for high rates of transcription. These promoters are associated with such enzymes as phosphoglucoisomerase, phosphofructokinase, phosphotriose isomerase, phosphoglucomutase, enolase, pyruvic kinase, glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase. These promoters may be inserted upstream from the signal sequence. The 5'-flanking region to the leader sequence may be retained or replaced with the 3' sequence of the alternative promoter. Vectors can be prepared and have been reported which include promoters having convenient restriction sites downstream from the promoter for insertion of such constructs as described above.

The final expression vector will comprise the expression cassette and a replicative or integrative system that is functional in and provides stable maintenance in the yeast host. The vector may optionally contain a replication system that allows for cloning in a prokaryote. In addition, one or more markers for selection will be included, which will allow for selective pressure for maintenance in the host. Furthermore, the vector may be a high or low copy number, the copy number generally ranging from about 1 to 200. With high-copy-number vectors, there will generally be at least 10, preferably at least 20, and usually not exceeding about 150, more usually not exceeding about 100, copy number. Depending upon the Gene*, eiter high or low copy numbers may be desirable, depending upon the effect of the vector on the host. Where the presence of the expression product of the vector may have a deleterious effect on the viability of the host, a

low copy number may be indicated.

Various hosts may be employed, particularly mutants having desired properties. It should be appreciated that depending upon the rate of production of the expression product of the construct, the processing enzyme may or may not be adequate for processing at that level of production. Therefore, a mutant having enhanced production of the processing enzyme (Dipeptidyl Amino Peptidase A and/or the lys-arg endopeptidase) may be indicated or enhanced production of the enzyme may be provided by means of a vector. Generally, the production of the enzyme should be of a lower order than the production of the desired expression product.

Alternatively, there may be situations where intracellular processing is not desired. In this situation, it would be useful to have a mutant, where secretion occurs, but the product is not processed. In this manner, the product may be subsequently processed in vitro.

Host mutants which provide for controlled regulation of expression may be employed to advantage. For example, with the constructions of the subject invention where a fused protein is expressed, the transformants have slow growth which appears to be a result of toxicity of the fused protein. Thus, by inhibiting expression during growth; the host may be grown to high density before changing the conditions to permissive conditions for expression.

Furthermore, as already indicated, Gene* may have a plurality of sequences in tandem, either the same or different sequences, with intervening processing signals. In this manner, the product may be processed in whole or in part, with the result that one will obtain the various sequences either by themselves or in tandem for subsequent processing. In many situations it may be desirable to provide for different sequences, where each of the sequences is a subunit of a particular protein product.

Gene* may encode any type of polypeptide of interest. The polypeptide may be as small as an oligopeptide of 8 amino acids or may be 100,000 daltons or higher. Usually, single chains will be less than about 300,000 daltons, more usually less than about 150,000 daltons. Of particular interest are polypeptides of about 5,000 to 150,000 daltons, more particularly of about 5,000 to 100,000 daltons. Illustrative polypeptides of interest include hormones and factors, such as growth hormone, somatomedins, and epidermal growth factor; the endocrine secretions, such as luteinizing hormone, thyroid stimulating hormone, oxytocin, insulin, vasopressin, renin, calcitonin, follicle stimulating hormone, prolactin, etc.; hematopoietic factors, e.g. erythropoietin, colony stimulating factor, etc.; lymphokines; globins; globulins, e.g. immunoglobulins; albumins, e.g. serum albumins such as human serum albumin; interferons, such as α, β and δ; repressors; enzymes, e.g. chymosin or its zymogens, α- and β-amylase, glucoamylase, pullulanase, xylanase, glucose isomerase, cellulase, lipase, phospholipase, etc.; endorphins, e.g. β-endorphin, encephalin, dynorphin, etc.

Having prepared the vectors containing the constructs of this invention, one may then introduce them into the yeast host. The genus and species of yeast is not critical. Examples of yeast hosts are Kluyveromyces and Saccharomyces. The manner of introduction is conventional, there being a wide variety of ways to introduce DNA into the yeast host. See, for example, Hinnen et al., Proc. Acad. Natl. Sci. USA (1978) 75:1919-1933 or Das et al., J. Bacteriol. (1984) 158:1165-1167 or Stinchcomb et al., EP-A 0 045 573. The resulting transformants may then be grown in an appropriate nutrient medium, where appropriate maintaining selective pressure on the transformants. Where expression is inducible, one can allow for growth of the yeast to high density and then induce expression. In those situations, where a substantial proportion of the product may be retained in the periplasmic space, one can release the product by treating the yeast cells with an enzyme such as zymolyase or lyticase.

The product may be harvested by any convenient means, including purifying the protein by chromatography, electrophoresis, dialysis, solvent-solvent extraction, etc.

The following procedure can be carried out in order to obtain genes coding for α-factors from Kluyveromyces species strains other than the K. lactis strain exemplified below. A radioactively labelled oligonucleotide probe having either of the structures set forth in Figure 1 is prepared and used to probe digests of genomic DNA from the other strain. Alternatively a nick translated fragment of the S. cerevisiae α-factor gene could be used as a probe. One useful technique is to insert segments from a limited endonuclease digest of genomic DNA into plasmids and to use these plasmids to transform E. coli or another bacterial strain so as to obtain a plasmid library. DNA from the colonies can then be conveniently hybridized to the probe on the nitrocellulose filters.

DNA segments identified by hybridization to the probes identified above are then digested with a variety of restriction enzymes and the resulting fragments analyzed by Southern blot or similar analysis method using the same hybridization probes in order to identify restriction fragments of a size suitable for DNA sequence analysis. Identified fragments are then purified and cloned using appropriate vectors in order that sufficient DNA can be prepared for a DNA sequence analysis.

Using these techniques, Kluyveromyces α-factor genes can be obtained from strains other than those specifically identified herein.

In accordance with the subject invention, one can provide for secretion of a wide variety of polypeptides, so as to greatly enhance product yield, simplify purification, minimize degradation of the desired product and simplify processing, equipment, and engineering requirements. Furthermore, utilization of nutrients based on productivity can be greatly enhanced, so that more economical and more efficient production of polypeptides may be achieved. Also, the use of yeast has many advantages in avoiding endotoxins, which may be present with prokaryotes and employing known techniques, which have been developed for yeast over long periods of time, which techniques include isolation of yeast products.

The following examples further illustrate the invention. These examples are not intended to limit the scope of the invention in any manner.

EXAMPLES

Identification and isolation of K. lactis α-factor

Biological assays of culture supernatants were carried out as described in Julius et al., Cell (1983) 32: 839, using as a tester strain the S. cerevisiae Mat a sst2-3 strain RC687. K. lactis strain CBS 141(α) was grown in a medium consisting of 0.5% glucose, 0.17% yeast nitrogen base without ammonium sulfate (Difco), 0.002% ammonium sulfate. After removal of cells by centrifugation, acetic acid was added to the culture supernatant to a concentration of 0.1 M and the culture supernatant was passed over a column of Bio-Rex 70 (Biorad). The column was washed with 0.1 M acetic acid, and then the α-factor was eluted with 80% EtOH/10 mM HCl. The eluate was evaporated to dryness and then dissolved in 0.1% TFA/20% acetonitrile and applied to a reverse-phase HPLC guard column. The column was washed stepwise with solutions containing 0.1% TFA and 20%, 40%, 60% and 80% acetonitrile. The 60% fraction, containing the α-factor activity, was then applied to an analytical C-18 HPLC column and eluted with a gradient of 20% to 80% acetonitrile in 0.1% TFA. Fractions were collected and assayed for α-factor activity. The fractions containing α-factor were dried and subjected to amino acid sequence analysis using Applied Biosystems gas phase protein sequencer Model 470A coupled to a Model 120A PTH analyzer.

Hybridization screening of plasmid libraries

Pools of oligonucleotides were labelled using γ-[^{32}P]-ATP and T4 polynucleotide kinase. These oligonucleotide probes were used to probe Southern blots or bacterial colonies at 42°C in the following hybridization solution:

4xSSC, 50 mM KH_2PO_4 pH 7, 1% sarkosyl, 10% dextran sulfate, 200 µg/ml of sonicated, denatured salmon sperm DNA. Filters were washed in 2xSSC, 0.1% SDS at 42°C.

A plasmid library in the cloning vector pJS109 (other standard cloning vectors such as pUC18 and pBR322 may be used in the place of pJS109), containing inserts resulting from a limited Sau3AI digest of genomic DNA from K. lactis strain SD11 (a trp1 lac4), size-fractionated to purify fragments >5000 bp, was screened with these probes by plating transformants of E. coli strain HB101 at density of 500-2000 colonies per 80 mm plate of L-agar containing 100 µg/ml ampicillin. DNA was transferred from the colonies to nitrocellulose filters and these filters hybridized as described above. Areas on the original plates corresponding to regions of hybridization signals on the filters were picked and replated and then retested by hybridization to isolate single colonies with plasmids containing hybridizing sequences. Positive colonies were further tested by Southern blot analysis of DNA purified from small cultures.

Plasmids purified from hybridization-positive colonies were digested with a variety of restriction enzymes and the resulting fragments analyzed by Southern blot analysis using the same hybridization probes in order to identify restriction fragments of size suitable for DNA sequence analysis. Fragments thus identified were purified by agarose gel electrophoresis and cloned into appropriate MP18 and MP19 vectors and DNA sequence analysis was performed.

Chymosin assays of culture supernatants

Cells were removed from cultures by centrifugation and the resulting supernatants were acidified to pH 2 by the addition of 1 M H_2SO_4 and incubated for 2 hours at room temperature. The solutions were then neutralized to pH 6 by the addition of 2 M Tris base. A 50 µl volume of an appropriate dilution was added to 200 µl of a suspension of 12% non-fat dry milk in 10 mM $CaCl_2$ and incubated at 37°C until a clot

formed. A unit of chymosin activity is defined as the amount of active chymosin required to produce a clot in 10 min under these conditions.

Results

The first 10 amino acids of the K. lactis α-factor showed a definite homology to that from S. cerevisiae, with 6 identical residues. This sequence is shown below:
Trp-Ser-Trp-Ile-Thr-Leu-Arg-Pro-Gly-Gln.

This protein sequence was used to design a set of oligonucleotides deduced to be complementary to the structural gene as shown in Figure 1. Oligonucleotides including all of the possible codons for a segment of the α-factor peptide were synthesized as two pools of 96 and 48 different molecules.

These two pools were radioactively labelled using γ-[^{32}P]-ATP and T4 polynucleotide kinase and were each used to probe a Southern blot of a restriction digest of K. lactis DNA. Pool 2 was found to give strong hybridization to a single fragment in several different digests. Thus, pool 2 was chosen to screen plasmid libraries of K. lactis genomic DNA.

Use of these probes to screen plasmid libraries resulted in the isolation of a number of hybridizing clones. DNA sequence analysis of one of these clones, αfkl8b, showed it to encode an α-factor-related peptide which bore a strong similarity to the precursor of the S. cerevisiae α-factor peptide. The hybridizing segment was found to be on a PstI-EcoRI fragment of about 1000 bp. The sequence of this fragment is shown in Figure 2.

Comparison of the deduced α-factor precursors of this fragment is shown in Figures 3 and 4. As can be seen they have leader sequences of (amino acids 1-83 in Figure 3) identical length with considerable sequence homology. The K. lactis precursor, however, contains only two sites for the addition of N-linked carbohydrate chains (Figure 4). In addition, the spacers of the K. lactis repeats are longer than those of the S. cerevisiae repeats and show a more diverse sequence with the pattern X-Ala/Pro rather than the Glu/Asp-Ala sequences found in S. cerevisiae. A comparison of the DNA sequences (Figure 5) also shows a strong degree of homology throughout the coding region.

A series of plasmids shown in Figure 6 were constructed in order to provide a fusion of the K. lactis α-factor leader to prochymosin expressed under the transcriptional control of a strong promoter. First, a 673 bp SspI-EcoRI fragment from αfkl8b was modified by filling the EcoRI overhang by Klenow enzyme and addition of BglII linkers to the blunt ends. This fragment was then inserted into a BglII site joining the promoter and terminator regions of the S. cerevisiae glyceraldehyde-3-phosphate dehydrogenase gene (GAP). This cassette was cloned as a BamHI fragment in pUC18, resulting in pAB307.

Fusion of sequences encoding the α-leader and bovine prochymosin was then performed. First pAB307 was digested with NcoI and the cohesive ends made blunt by treatment with mung bean nuclease. The resulting product was then digested with SalI. To this was ligated a 2000 bp EcoRV-SalI fragment (sequences shown in Figure 7 and 8) containing sequences encoding prochymosin and the S. cerevisiae GAP transcriptional termination region. This fragment was derived from plasmid pJS111 in which a XbaI-BamHI adaptor had been added to the 5' end of a fragment containing prochymosin cDNA fused to the S. cerevisiae GAP transcriptional termination region. This ligation mixture was used to transform E. coli strain HB101 and a transformant carrying the plasmid pAB309 was isolated. The sequences around the junction of this fusion are shown in Figure 7, and the sequence of the entire BamHI-SalI insert of pAB309 is shown in Figure 8.

In order to allow for transformation of K. lactis strains, a 3560 bp HindIII fragment containing the E. coli kanamycin resistance gene under the transcriptional control of the S. cerevisiae ADH1 promoter inserted within the 3' region of the K. lactis LAC4 gene was inserted into pAB309 to result in the plasmid pAB312. Screening for the insert was effected by restriction digest analysis and checked for the correct orientation.

The 3560 bp HindIII fragment was derived from the plasmid pGB901, a diagram of which is shown in Figure 9. Plasmid pGB901 was constructed by ligating (1) 3.6 kb XbaI-HaeII fragment containing the lactase promoter to about position -90 from the lactase ATG start codon isolated from pUCla56 (described below), (2) an HaeII-SalI fragment having the following nucleotide sequence:

```
5' TTAAC ACTTGAAATT TAGGAAAGAG CAGAATTTGG CAAAAAAAAT AAAAAAAAAA TAAACACG 3'
3' CGCGAATTG TGAACTTTAA ATCCTTTCTC GTCTTAAACC GTTTTTTTTA TTTTTTTTTT ATTTGTGCAG CT 5'
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(3) a 5.1 kb SalI-XbaI fragment encoding prochymosin and a gene conferring resistance to the antibiotic G418 from pGB900 (described below), and (4) pUC19 cleaved with XbaI.

During the construction of the plasmid the CG sequence from the HaeII site was inadvertently removed, thereby creating a HindIII site at this position.

Plasmid pUCla56 was constructed as follows. Chromosomal DNA was isolated from K. lactis strain CBS 2360, cleaved with XhoI, and separated according to size on a sucrose gradient. Fractions containing the lactase gene were detected with a LAC4 probe after spotting the DNA on a nitrocellulose filter. DNA containing the LAC4 gene was cloned into the XhoI site of plasmid pPA153-215 (P.M. Andreoli, Mol. Gen. Genet. (1985) 199:372-380) giving rise to plasmid pPA31. An XbaI fragment of pPA31 containing the lactase gene was subcloned in the XbaI site of pUC19 (Yanisch-Perron et al., Gene (1985) 33:103-119) which yields plasmid pUCla56.

Plasmid pGB900 was constructed by ligating (1) a 3.6 kb HindIII-XbaI fragment from plasmid pGBTeG418 (shown in Figure 10 and described below) containing the G418 resistance gene and (2) a SalI-HindIII fragment from plasmid pGB123 containing the prochymosin gene in pUC19 cleaved with SalI and XbaI. Plasmid pGB123 is described in European Patent Application EP-A 0 096 430.

Plasmid pGBTeG418 (Figure 10) consists of the plasmid pPA215 as described by Andreoli, Mol. Gen. Genet. (1985) 199:372-380, and a 5.6 kb fragment consisting of the 3.7 kb BamHI K. lactis lactase terminator fragment (Breunig et al., Nucleic Acids Res. (1984) 12:2327-2341) and the Tn5 gene (Reiss et al., EMBO J. (1984) 3:3317-3322) conferring resistance to G418 under the direction of the promoter alcohol dehydrogenase I (ADHI) from yeast, similar to that as described by Bennetzen and Hall, J. Biol. Chem. (1982) 257:3018-3025. Plasmid pGBTeG418 was deposited with CBS on February 26, 1987 under number CBS 184.87.

Plasmid pAB312 was digested with EcoRV (to target integration to the LAC4 region of the K. lactis genome) and was then used to transform K. lactis strain 2UV21 (a ura3 trp1 lac4 [kil$^-$] to G418 resistance using the LiCl technique described by Das et al., J. Bacteriol. (1984) 158: 1165-1167).

A number of these transformants, as well as an untransformed control strain, were grown for 36 hr in 1 ml of medium composed of 1% yeast extract, 2% peptone, 2% glucose, 0.17% yeast nitrogen base, 50 µg/ml tryptophan and 50% µg/ml uracil. Culture supernatants were then assayed for chymosin activity after acid activation. All of the transformants were found to secrete between 100 and 120 units/ml of activatable chymosin.

Removal of spacer codons by in vitro mutagenesis

A 1900 bp SacI-HindIII fragment was isolated from pAB309 and cloned into MP19 (Yanisch-Perron et al., Gene (1985) 33:103). Single-stranded phage DNA was prepared and used as template for in vitro mutagenesis with the oligonucleotide primers shown in Figure 11. The M13 phages MP19/αkl1.5 and MP19/αkl2.2 were prepared using Primer #1 and Primer #2, respectively.

Double-stranded RF DNA was prepared from these phages, and 1100 bp SacI-StuI fragments isolated from each. These fragments were ligated to a 7100 bp SacI-StuI fragment from pAB312. The resulting plasmids pAB313 and pAB314 were isolated with the sequence alterations illustrated in Figure 12.

The plasmids pAB313 and pAB314 were used to transform strain 2UV21 to G418 resistance. Cultures of transformants 2UV21::pAB312, 2UV21::pAB313 and 2UV21::pAB314 were grown and culture supernatants assayed for chymosin activity as above. The results of these assays are reported in Table 1, below.

Table 1

Strain	Host	Plasmid	Chymosin Activity (units/ml culture)
2UV21	2UV21	-	<2
KRN303-1	2UV21	pAB312	256
KRN304-4	2UV21	pAB313	175
KRN305-2	2UV21	pAB314	206

Each of the transformants was found to secrete a single prochymosin-related species as judged by SDS polyacrylamide gel electrophoresis of trichloroacetic acid-precipitated culture supernatants. The prochymosin-related protein secreted by pAB312 transformants appeared to be of slightly higher molecular

weight than those secreted by pAB313 and pAB314 transformants as determined by electrophoretic mobility.

The cell pellets from the above strains were analyzed for chymosin activity and found to contain less than 2% of the activity measured in the supernatants. Thus the Kluyveromyces α-factor is approximately 98% efficient in directing the secretion of prochymosin from Kluyveromyces lactis.

The major species secreted by KRN303-1 and KRN304-4 were purified by preparative SDS polyacrylamide gel electrophoresis and subjected to gas phase amino acid sequence analysis. The N-terminal sequences of these species are given below:

KRN303-1

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 1               5                  10                  15
Glu-Ala-Asp-Ala-Ser-His-His-Met-Ala-Glu-Ile-Thr-Arg-Ile-Pro
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KRN304-4

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 1          5
Ala-Glu-Ile-Thr-Arg-Ile
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These results indicate that the prochymosin-related species secreted by KRN303-1 has undergone no processing of the amino-terminal spacer sequence, while the species secreted from KRN304-4 has the authentic mature prochymosin amino terminus.

The following organisms were deposited with the American Type Culture Collection on June 30, 1987: HB101 pAB307, ATCC Accession No. 67454; HB101 pAB312, ATCC Accession No. 67455.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Claims

Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. A DNA construct encoding a protein having an amino acid sequence which comprises a secretion-directing Kluyveromyces α-factor leader sequence linked to a heterologous polypeptide sequence.

2. A DNA construct according to claim 1, wherein said protein further comprises a yeast processing signal for processing said protein into said heterologous polypeptide between said leader sequence and said heterologous polypeptide sequence.

3. A DNA construct according to claim 1, further comprising a yeast promoter at the 5' end of said leader sequence.

4. The DNA sequence of claim 1, wherein said leader sequence is the full length leader sequence of wild type Kluyveromyces α-factor.

5. The DNA sequence of claim 4, wherein said leader sequence is the leader sequence shown in Figure 2.

6. The DNA sequence of claim 1, wherein said leader sequence is a functional fragment of the full length leader sequence of wild type Kluyveromyces α-factor.

7. The DNA sequence of claim 6, wherein said full length leader sequence is the leader sequence shown in Figure 2.

8. The DNA sequence of claim 1, wherein said Kluyveromyces α-factor leader sequence is a Kluyveromyces lactis α-factor leader sequence.

* C L M S *

weight than those secreted by pAB313 and pAB314 transformants as determined by electrophoretic mobility.

The cell pellets from the above strains were analyzed for chymosin activity and found to contain less than 2% of the activity measured in the supernatants. Thus the Kluyveromyces α-factor is approximately 98% efficient in directing the secretion of prochymosin from Kluyveromyces lactis.

The major species secreted by KRN303-1 and KRN304-4 were purified by preparative SDS polyacrylamide gel electrophoresis and subjected to gas phase amino acid sequence analysis. The N-terminal sequences of these species are given below:

KRN303-1

1				5				10				15
Glu	Ala	Asp	Ala	Ser	His	His	Met	Ala	Glu	Ile	Thr	Arg	Ile	Pro

KRN304-4

1				5		
Ala	Glu	Ile	Thr	Arg	Ile	

These results indicate that the prochymosin-related species secreted by KRN303-1 has undergone no processing of the amino-terminal spacer sequence, while the species secreted from KRN304-4 has the authentic mature prochymosin amino terminus.

The following organisms were deposited with the American Type Culture Collection on June 30, 1987: HB101 pAB307, ATCC Accession No. 67454; HB101 pAB312, ATCC Accession No. 67455.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Claims

Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. A DNA construct encoding a protein having an amino acid sequence which comprises a secretion-directing Kluyveromyces α-factor leader sequence linked to a heterologous polypeptide sequence.

2. A DNA construct according to claim 1, wherein said protein further comprises a yeast processing signal for processing said protein into said heterologous polypeptide between said leader sequence and said heterologous polypeptide sequence.

3. A DNA construct according to claim 1, further comprising a yeast promoter at the 5' end of said leader sequence.

4. The DNA sequence of claim 1, wherein said leader sequence is the full length leader sequence of wild type Kluyveromyces α-factor.

5. The DNA sequence of claim 4, wherein said leader sequence is the leader sequence shown in Figure 2.

6. The DNA sequence of claim 1, wherein said leader sequence is a functional fragment of the full length leader sequence of wild type Kluyveromyces α-factor.

7. The DNA sequence of claim 6, wherein said full length leader sequence is the leader sequence shown in Figure 2.

8. The DNA sequence of claim 1, wherein said Kluyveromyces α-factor leader sequence is a Kluyveromyces lactis α-factor leader sequence.

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9. The DNA sequence of claim 2, wherein said yeast processing signal comprises a basic dipeptide cleavage site and a spacer.

10. The DNA sequence of claim 2, wherein said yeast processing signal comprises a basic dipeptide cleavage site directly fused to the N-terminus of said heterologous polypeptide and lacks a spacer.

11. The DNA sequence of claim 9 or 10, wherein said basic dipeptide cleavage site comprises at least one arginine.

12. The DNA sequence of claim 11, wherein said basic dipeptide cleavage site is lysine-arginine or arginine-arginine.

13. The DNA construct of claim 3, wherein said yeast promoter is an α-factor promoter or a GAP promoter, said Kluyveromyces α-factor leader sequence is a Kluyveromyces lactis α-factor leader sequence, and said Kluyveromyces lactis α-factor leader sequence is linked to said heterologous polypeptide sequence via a yeast processing signal which comprises a lysine-arginine cleavage site and a spacer.

14. The DNA construct of claim 13, wherein said heterologous polypeptide is prochymosin.

15. The DNA construct of claim 3, wherein said yeast promoter is an α-factor promoter or a GAP promoter, said Kluyveromyces α-factor leader sequence is a Kluyveromyces lactis α-factor leader sequence, and said Kluyveromyces lactis α-factor leader sequence is linked to said heterologous polypeptide sequence via a yeast processing signal which includes a lysine-arginine cleavage site fused directly to said heterologous polypeptide and lacks a spacer.

16. The DNA construct of claim 15, wherein said heterologous polypeptide is prochymosin.

17. An expression vector comprising a replicative or integrative system for providing stable maintenance in yeast and a DNA construct according to any one of claims 1 to 16.

18. A method for producing a heterologous polypeptide in yeast comprising:
growing yeast containing an expression vector according to claim 17 in culture medium under conditions whereby said heterologous polypeptide is expressed in and secreted by said yeast as a propolypeptide which is at least partially processed into said heterologous polypeptide.

19. A method for producing a heterologous polypeptide in yeast wherein said heterologous polypeptide is secreted under the direction of a yeast α-factor leader sequence, characterised in that said yeast α-factor leader sequence is a Kluyveromyces α-factor leader sequence.

20. The method of claim 19, wherein said Kluyveromyces α-factor leader sequence is a Kluyveromyces lactis α-factor leader sequence.

Claims for the following Contracting State : ES

1. Method for preparing a DNA construct which comprises linking a DNA fragment encoding a secretion-directing Kluyveromyces α-factor leader sequence to a DNA fragment encoding a heterologous polypeptide sequence.

2. Method according to claim 1, which further comprises linking a DNA fragment encoding a yeast processing signal between said DNA fragment encoding said leader sequence and said DNA fragment encoding said heterologous polypeptide sequence.

3. Method according to claim 1, which further comprises linking a yeast promoter at the 5' end of said leader sequence.

4. Method according to claim 1, wherein said leader sequence is the full length leader sequence of wild type Kluyveromyces α-factor.

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5. Method according to claim 4, wherein said leader sequence is the leader sequence shown in Figure 2.

6. Method for preparing a DNA sequence according to claim 1, wherein said leader sequence is a functional fragment of the full length leader sequence of wild type Kluyveromyces α-factor.

7. Method according to claim 6, wherein said full length leader sequence is the leader sequence shown in Figure 2.

8. Method according to claim 1, wherein said Kluyveromyces α-factor leader sequence is a Kluyveromyces lactis α-factor leader sequence.

9. Method according to claim 2, wherein said yeast processing signal comprises a basic dipeptide cleavage site and a spacer.

10. Method according to claim 2, wherein said yeast processing signal comprises a basic dipeptide cleavage site directly fused to the N-terminus of said heterologous polypeptide and lacks a spacer.

11. Method according to claim 9 or 10, wherein said basic dipeptide cleavage site comprises at least one arginine.

12. Method according to claim 11, wherein said basic dipeptide cleavage site is lysine-arginine or arginine-arginine.

13. Method according to claim 3, wherein said yeast promoter is an α-factor promoter or a GAP promoter, said Kluyveromyces α-factor leader sequence is a Kluyveromyces lactis α-factor leader sequence, and said Kluyveromyces lactis α-factor leader sequence is linked to said heterologous polypeptide sequence via a yeast processing signal which comprises a lysine-arginine cleavage site and a spacer.

14. Method according to claim 13, wherein said heterologous polypeptide is prochymosin.

15. Method according to claim 3, wherein said yeast promoter is an α-factor promoter or a GAP promoter, said Kluyveromyces α-factor leader sequence is a Kluyveromyces lactis α-factor leader sequence, and said Kluyveromyces lactis α-factor leader sequence is linked to said heterologous polypeptide sequence via a yeast processing signal which includes a lysine-arginine cleavage site fused directly to said heterologous polypeptide and lacks a spacer.

16. Method according to claim 15, wherein said heterologous polypeptide is prochymosin.

17. Method for preparing an expression vector which comprises combining a replicative or integrative system for providing stable maintenance in yeast and a DNA construct obtained to the method according to any one of claims 1 to 16.

18. A method for producing a heterologous polypeptide in yeast comprising:
growing yeast containing an expression vector obtained according by the method of claim 17 in culture medium under conditions whereby said heterologous polypeptide is expressed in and secreted by said yeast as a propolypeptide which is at least partially processed into said heterologous polypeptide.

19. A method for producing a heterologous polypeptide in yeast wherein said heterologous polypeptide is secreted under the direction of a yeast α-factor leader sequence, characterised in that said yeast α-factor leader sequence is a Kluyveromyces α-factor leader sequence.

20. The method of claim 19, wherein said Kluyveromyces α-factor leader sequence is a Kluyveromyces lactis α-factor leader sequence.

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Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. DNA-Konstruktion, die kodiert für ein Protein mit einer Aminosäuresequenz, die eine die Sekretion lenkende Kluyveromyces-α-Faktor-Signalsequenz aufweist, die an eine heterologe Polypeptidsequenz gebunden ist.

2. DNA-Konstruktion nach Anspruch 1, dadurch gekennzeichnet, dass das genannte Protein weiter ein Hefeprozessierungssignal für die Prozessierung des genannten Proteins zu dem genannten heterologen Polypeptid zwischen der genannten Signalsequenz und der genannten heterologen Polypeptidsequenz aufweist.

3. DNA-Konstruktion nach Anspruch 1, dadurch gekennzeichnet, dass sie ferner einen Hefepromotor am 5'-Ende der genannten Signalsequenz aufweist.

4. DNA-Sequenz nach Anspruch 1, dadurch gekennzeichnet, dass die genannte Signalsequenz die Signalsequenz mit voller Länge von Wildtyp-Kluyveromyces-α-Faktor ist.

5. DNA-Sequenz nach Anspruch 4, dadurch gekennzeichnet, dass die genannte Signalsequenz die in Fig. 2 dargestellte Signalsequenz ist.

6. DNA-Sequenz nach Anspruch 1, dadurch gekennzeichnet, dass die genannte Signalsequenz ein funktionstüchtiges Fragment der Signalsequenz mit voller Länge von Wildtyp-Kluyveromyces-α-Faktor ist.

7. DNA-Sequenz nach Anspruch 6, dadurch gekennzeichnet, dass die genannte Signalsequenz mit voller Länge die in Fig. 2 dargestellte Signalsequenz ist.

8. DNA-Sequenz nach Anspruch 1, dadurch gekennzeichnet, dass die genannte Kluyveromyces-α-Faktor-Signalsequenz eine Kluyveromyces lactis-α-Faktor-Signalsequenz ist.

9. DNA-Sequenz nach Anspruch 2, dadurch gekennzeichnet, dass das genannte Hefeprozessierungssignal eine basisches Dipeptid-Spaltstelle und ein Zwischenstück aufweist.

10. DNA-Sequenz nach Anspruch 2, dadurch gekennzeichnet, dass das genannte Hefeprozessierungssignal eine basisches Dipeptid-Spaltstelle aufweist, die direkt an den N-Terminus des genannten heterologen Polypeptids fusioniert ist, und dass ihm ein Zwischenstück fehlt.

11. DNA-Sequenz nach Anspruch 9 oder 10, dadurch gekennzeichnet, dass die genannte basisches Dipeptid-Spaltstelle mindestens ein Arginin aufweist.

12. DNA-Sequenz nach Anspruch 11, dadurch gekennzeichnet, dass die genannte basisches Dipeptid-Spaltstelle Lysin-Arginin oder Arginin-Arginin ist.

13. DNA-Konstruktion nach Anspruch 3, dadurch gekennzeichnet, dass der genannte Hefepromotor ein α-Faktorpromotor oder ein GAP-Promotor ist, die genannte Kluyveromyces-α-Faktor-Signalsequenz eine Kluyveromyces lactis-α-Faktor-Signalsequenz ist und die genannte Kluyveromyces lactis-α-Faktor-Signalsequenz an die genannte heterologe Polypeptidsequenz über ein Hefeprozessierungssignal gebunden ist, das eine Lysin-Arginin-Spaltstelle und ein Zwischenstück aufweist.

14. DNA-Konstruktion nach Anspruch 13, dadurch gekennzeichnet, dass das genannte heterologe Polypeptid Prochymosin ist.

15. DNA-Konstruktion nach Anspruch 13, dadurch gekennzeichnet, dass der genannte Hefepromotor ein α-Faktorpromotor oder ein GAP-Promotor ist, die genannte Kluyveromyces-α-Faktor-Signalsequenz eine Kluyveromyces lactis-α-Faktor-Signalsequenz ist und die genannte Kluyveromyces lactis-α-Faktor-Signalsequenz an die genannte heterologe Polypeptidsequenz über ein Hefeprozessierungssignal gebunden ist, das eine Lysin-Arginin-Spaltstelle aufweist, die direkt an das genannte heterologe

Polypeptid fusioniert ist, und dem ein Zwischenstück fehlt.

16. DNA-Konstruktion nach Anspruch 15, dadurch gekennzeichnet, dass das genannte heterologe Polypeptid Prochymosin ist.

17. Expressionsvektor, dadurch gekennzeichnet, dass er ein replikatives oder integratives System zur Erzielung von stabiler Manifestierung und Weitergabe an die Nachkommen in Hefe und eine DNA-Konstruktion nach einem der Ansprüche 1 bis 16 aufweist.

18. Verfahren zur Herstellung eines heterologen Polypeptids in Hefe, dadurch gekennzeichnet, dass man Hefe, die einen Expressionsvektor nach Anspruch 17 enthält, in Kulturmedium unter Bedingungen kultiviert, unter denen das genannte heterologe Polypeptid in der genannten Hefe exprimiert und von ihr sekretiert wird als Propolypeptid, das mindestens teilweise zu dem genannten heterologen Polypeptid prozessiert wird.

19. Verfahren zur Herstellung eines heterologen Polypeptids in Hefe, wobei das genannte heterologe Polypeptid unter der Kontrolle einer Hefe-α-Faktor-Signalsequenz sekretiert wird, dadurch gekennzeichnet, dass die genannte Hefe-α-Faktor-Signalsequenz eine Kluyveromyces-α-Faktor-Signalsequenz ist.

20. Verfahren nach Anspruch 19, dadurch gekennzeichnet, dass die genannte Kluyveromyces-α-Faktor-Signalsequenz eine Kluyveromyces lactis-α-Faktor-Signalsequenz ist.

Patentansprüche für folgenden Vertragsstaat : ES

1. Verfahren zur Herstellung einer DNA-Konstruktion, dadurch gekennzeichnet, dass man ein DNA-Fragment, das für eine die Sekretion lenkende Kluyveromyces-α-Faktor-Signalsequenz kodiert, mit einem DNA-Fragment, das für eine heterologe Polypeptidsequenz kodiert, verbindet.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass man ferner ein DNA-Fragment, das für ein Hefeprozessierungssignal kodiert, zwischen das genannte DNA-Fragment, das für die genannte Signalsequenz kodiert, und das genannte DNA-Fragment, das für die genannte heterologe Polypeptidsequenz kodiert, verbindet.

3. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass man ferner einen Hefepromotor an das 5'-Ende der genannten Signalsequenz bindet.

4. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die genannte Signalsequenz die Signalsequenz mit voller Länge von Wildtyp-Kluyveromyces-α-Faktor ist.

5. Verfahren nach Anspruch 4, dadurch gekennzeichnet, dass die genannte Signalsequenz die in Fig. 2 dargestellte Signalsequenz ist.

6. Verfahren zur Herstellung einer DNA-Sequenz nach Anspruch 1, dadurch gekennzeichnet, dass die genannte Signalsequenz ein funktionsfähiges Fragment der Signalsequenz mit voller Länge von Wildtyp-Kluyveromyces-α-Faktor ist.

7. Verfahren nach Anspruch 6, dadurch gekennzeichnet, dass die genannte Signalsequenz mit voller Länge die in Fig. 2 dargestellte Signalsequenz ist.

8. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die genannte Kluyveromyces-α-Faktor-Signalsequenz eine Kluyveromyces lactis-α-Faktor-Signalsequenz ist.

9. Verfahren nach Anspruch 2, dadurch gekennzeichnet, dass das genannte Hefeprozessierungssignal eine basisches Dipeptid-Spaltstelle und ein Zwischenstück aufweist.

10. Verfahren nach Anspruch 2, dadurch gekennzeichnet, dass das genannte Hefeprozessierungssignal eine basisches Dipeptid-Spaltstelle aufweist, die direkt an den N-Terminus des genannten heterologen Polypeptids fusioniert ist, und dass ihm ein Zwischenstück fehlt.

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11. Verfahren nach Anspruch 9 oder 10, dadurch gekennzeichnet, dass die genannte basisches Dipeptid-Spaltstelle mindestens ein Arginin aufweist.

12. Verfahren nach Anspruch 11, dadurch gekennzeichnet, dass die genannte basisches Dipeptid-Spaltstelle Lysin-Arginin oder Arginin-Arginin ist.

13. Verfahren nach Anspruch 3, dadurch gekennzeichnet, dass der genannte Hefepromotor ein α-Faktorpromotor oder ein GAP-Promotor ist, die genannte Kluyveromyces-α-Faktor-Signalsequenz eine Kluyveromyces lactis-α-Faktor-Signalsequenz ist und die genannte Kluyveromyces lactis-α-Faktor-Signalsequenz an die genannte heterologe Polypeptidsequenz über ein Hefeprozessierungssignal gebunden ist, das eine Lysin-Arginin-Spaltstelle und ein Zwischenstück aufweist.

14. Verfahren nach Anspruch 13, dadurch gekennzeichnet, dass das genannte heterologe Polypeptid Prochymosin ist.

15. Verfahren nach Anspruch 13, dadurch gekennzeichnet, dass der genannte Hefepromotor ein α-Faktorpromotor oder ein GAP-Promotor ist, die genannte Kluyveromyces-α-Faktor-Signalsequenz eine Kluyveromyces lactis-α-Faktor-Signalsequenz ist und die genannte Kluyveromyces lactis-α-Faktor-Signalsequenz an die genannte heterologe Polypeptidsequenz über ein Hefeprozessierungssignal gebunden ist, das eine Lysin-Arginin-Spaltstelle aufweist, die direkt an das genannte heterologe Polypeptid fusioniert ist, und dem ein Zwischenstück fehlt.

16. Verfahren nach Anspruch 15, dadurch gekennzeichnet, dass das genannte heterologe Polypeptid Prochymosin ist.

17. Verfahren zur Herstellung eines Expressionsvektors, dadurch gekennzeichnet, dass man ein replikatives oder integratives System zur Erzielung von stabiler Manifestierung und Weitergabe an die Nachkommen in Hefe und eine DNA-Konstruktion, die gemäss dem Verfahren nach einem der Ansprüche 1 bis 16 erhalten wurde, kombiniert.

18. Verfahren zur Herstellung eines heterologen Polypeptids in Hefe, dadurch gekennzeichnet, dass man Hefe, die einen gemäss dem Verfahren nach Anspruch 17 erhaltenen Expressionsvektor enthält, in Kulturmedium unter Bedingungen kultiviert, unter denen das genannte heterologe Polypeptid in der genannten Hefe exprimiert und von ihr sekretiert wird als Propolypeptid, das mindestens teilweise zu dem genannten heterologen Polypeptid prozessiert wird.

19. Verfahren zur Herstellung eines heterologen Polypeptids in Hefe, wobei das genannte heterologe Polypeptid unter der Kontrolle einer Hefe-α-Faktor-Signalsequenz sekretiert wird, dadurch gekennzeichnet, dass die genannte Hefe-α-Faktor-Signalsequenz eine Kluyveromyces-α-Faktor-Signalsequenz ist.

20. Verfahren nach Anspruch 19, dadurch gekennzeichnet, dass die genannte Kluyveromyces-α-Faktor-Signalsequenz eine Kluyveromyces lactis-α-Faktor-Signalsequenz ist.

Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. Construction d'ADN codant une protéine ayant une séquence d'acides aminés qui comprend une séquence de tête du facteur α de Kluyveromyces dirigeant la sécrétion liée à une séquence polypeptidique hétérologue.

2. Construction d'ADN suivant la revendication 1, dans laquelle la protéine comprend aussi un signal de maturation moléculaire de levure pour opérer la maturation moléculaire de la protéine en le polypeptide hétérologue entre la séquence de tête et la séquence polypeptidique hétérologue.

3. Construction d'ADN suivant la revendication 1, comprenant aussi un promoteur de levure à l'extrémité 5' de la séquence de tête.

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4. Séquence d'ADN suivant la revendication 1, dans laquelle la séquence de tête est la séquence de tête en longueur complète du facteur α de Kluyveromyces de type sauvage.

5. Séquence d'ADN suivant la revendication 4, dans laquelle la séquence de tête est la séquence de tête représentée à la Fig. 2.

6. Séquence d'ADN suivant la revendication 1, dans laquelle la séquence de tête est un fragment fonctionnel de la séquence de tête en longueur complète du facteur α de Kluyveromyces de type sauvage.

7. Séquence d'ADN suivant la revendication 6, dans laquelle la séquence de tête en longueur complète est la séquence de tête représentée à la Fig. 2.

8. Séquence d'ADN suivant la revendication 1, dans laquelle la séquence de tête du facteur α de Kluyveromyces est une séquence de tête du facteur α de Kluyveromyces lactis.

9. Séquence d'ADN suivant la revendication 2, dans laquelle le signal de maturation moléculaire de levure comprend un site de coupure dipeptidique basique et un espaceur.

10. Séquence d'ADN suivant la revendication 2, dans laquelle le signal de maturation moléculaire de levure comprend un site de coupure dipeptidique basique fusionné directement à l'extrémité N du polypeptide hètérologue et est exempt d'un espaceur.

11. Séquence d'ADN suivant la revendication 9 ou 10, dans laquelle le site de coupure dipeptidique basique comprend au moins une arginine.

12. Séquence d'ADN suivant la revendication 11, dans laquelle le site de coupure dipeptidique basique est lysine-arginine ou arginine-arginine.

13. Construction d'ADN suivant la revendication 3, dans laquelle le promoteur de levure est un promoteur de facteur α ou un promoteur GAP, la séquence de tête du facteur α de Kluyveromyces est une séquence de tête du facteur α de Kluyveromyces lactis et la séquence de tête du facteur α de Kluyveromyces lactis est liée à la séquence polypeptidique hétérologue par l'intermédiaire d'un signal de maturation moléculaire de levure qui comprend un site de coupure lysine-arginine et un espaceur.

14. Construction d'ADN suivant la revendication 13, dans laquelle le polypeptide hétérologue est la prochymosine.

15. Construction d'ADN suivant la revendication 3, dans laquelle le promoteur de levure est un promoteur de facteur α ou un promoteur GAP, la séquence de tête du facteur α de Kluyveromyces est une séquence de tête du facteur α de Kluyveromyces lactis et la séquence de tête du facteur α de Kluyveromyces lactis est liée à la séquence polypeptidique hétérologue par l'intermédiaire d'un signal de maturation moléculaire de levure qui inclut un site de coupure lysine-arginine fusionné directement au polypeptide hétérologue et est exempt d'un espaceur.

16. Construction d'ADN suivant la revendication 15, dans laquelle le polypeptide hétérologue est la prochymosine.

17. Vecteur d'expression comprenant un système réplicatif ou intégratif pour assurer le maintien stable dans une levure et une construction d'ADN suivant l'une quelconque des revendications 1 à 16.

18. Procédé pour produire un polypeptide hétérologue dans une levure, qui comprend la mise en croissance d'une levure contenant un vecteur d'expression suivant la revendication 17 dans un milieu de culture dans des conditions dans lesquelles le polypeptide hétérologue est exprimé dans et est sécrété par la levure à l'état de propolypeptide qui est au moins partiellement soumis à la maturation moléculaire en le polypeptide hétérologue.

19. Procédé pour produire un polypeptide hétérologue dans une levure dans laquelle ce polypeptide hétérologue est sécrété sous la direction d'une séquence de tête du facteur α de la levure, caractérisé en ce que la séquence de tête du facteur α de la levure est une séquence de tête du facteur α de Kluyveromyces.

20. Procédé suivant la revendication 19, dans lequel la séquence de tête du facteur α de Kluyveromyces est une séquence de tête du facteur α de Kluyveromyces lactis.

Revendications pour l'Etat contractant suivant : ES

1. Procédé pour préparer une construction d'ADN, qui comprend la liaison d'un fragment d'ADN codant une séquence de tête du facteur α de Kluyveromyces dirigeant la sécrétion avec un fragment d'ADN codant une séquence polypeptidique hétérologue.

2. Procédé suivant la revendication 1, qui comprend aussi la liaison d'un fragment d'ADN codant un signal de maturation moléculaire de levure entre le fragment d'ADN codant la séquence de tête et le fragment d'ADN codant la séquence polypeptidique hétérologue.

3. Procédé suivant la revendication 1, qui comprend aussi la liaison d'un promoteur de levure à l'extrémité 5' de la séquence de tête.

4. Procédé suivant la revendication 1, dans lequel la séquence de tête est la séquence de tête en longueur complète du facteur α de Kluyveromyces de type sauvage.

5. Procédé suivant la revendication 4, dans lequel la séquence de tête est la séquence de tête représentée à la Fig. 2.

6. Procédé de préparation d'une séquence d'ADN suivant la revendication 1, dans lequel la séquence de tête est un fragment fonctionnel de la séquence de tête en longueur complète du facteur α de Kluyveromyces de type sauvage.

7. Procédé suivant la revendication 6, dans lequel la séquence de tête en longueur complète est la séquence de tête représentée à la Fig. 2.

8. Procédé suivant la revendication 1, dans lequel la séquence de tête du facteur α de Kluyveromyces est une séquence de tête du facteur α de Kluyveromyces lactis.

9. Procédé suivant la revendication 2, dans lequel le signal de maturation moléculaire de levure comprend un site de coupure dipeptidique basique et un espaceur.

10. Procédé suivant la revendication 2, dans lequel le signal de maturation moléculaire de levure comprend un site de coupure dipeptidique basique fusionné directement à l'extrémité N du polypeptide hétérologue et est exempt d'un espaceur.

11. Procédé suivant la revendication 9 ou 10, dans lequel le site de coupure dipeptidique basique comprend au moins une arginine.

12. Procédé suivant la revendication 11, dans lequel le site de coupure dipeptidique basique est lysine-arginine ou arginine-arginine.

13. Procédé suivant la revendication 3, dans lequel le promoteur de levure est un promoteur de facteur α ou un promoteur GAP, la séquence de tête du facteur α de Kluyveromyces est une séquence de tête du facteur α de Kluyveromyces lactis et la séquence de tête du facteur α de Kluyveromyces lactis est liée à la séquence polypeptidique hétérologue par l'intermédiaire d'un signal de maturation moléculaire de levure qui comprend un site de coupure lysine-arginine et un espaceur.

14. Procédé suivant la revendication 13, dans lequel le polypeptide hétérologue est la prochymosine.

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15. Procédé suivant la revendication 3, dans lequel le promoteur de levure est un promoteur de facteur α ou un promoteur GAP, la séquence de tête du facteur α de Kluyveromyces est une séquence de tête du facteur α de Kluyveromyces lactis et la séquence de tête du facteur α de Kluyveromyces lactis est liée à la séquence polypeptidique hétérologue par l'intermédiaire d'un signal de maturation moléculaire de levure qui inclut un site de coupure lysinearginine fusionné directement au polypeptide hétérologue et est exempt d'un espaceur.

16. Procédé suivant la revendication 15, dans lequel le polypeptide hétérologue est la prochymosine.

17. Procédé de préparation d'un vecteur d'expression, qui comprend la combinaison d'un système réplicatif ou intégratif pour assurer le maintien stable dans une levure avec une construction d'ADN obtenue par le procédé suivant l'une quelconque des revendications 1 à 16.

18. Procédé pour produire un polypeptide hétérologue dans une levure, qui comprend la mise en croissance d'une levure contenant un vecteur d'expression obtenu par le procédé suivant la revendication 17 dans un milieu de culture dans des conditions dans lesquelles le polypeptide hétérologue est exprimé dans et est sécrété par la levure à l'état de propolypeptide qui est au moins partiellement soumis à la maturation moléculaire en le polypeptide hétérologue.

19. Procédé pour produire un polypeptide hétérologue dans une levure dans laquelle ce polypeptide hétérologue est sécrété sous la direction d'une séquence de tête du facteur α de la levure, caractérisé en ce que la séquence de tête du facteur α de la levure est une séquence de tête du facteur α de Kluyveromyces.

20. Procédé suivant la revendication 19, dans lequel la séquence de tête du facteur α de Kluyveromyces est une séquence de tête du facteur α de Kluyveromyces lactis.