It is desirable to provide improved recombinant eukaryotic cell lines to produce fermentation products that can be isolated with high yields. Therefore, it is the object of the present invention to provide for alternative regulatory elements suitable for recombinant production methods, which are simple and efficient.

SUMMARY ~~OF THE INVENTION~~

The object is solved by the subject matter as claimed.

According to the ~~invention~~ disclosure there is provided an isolated nucleic acid sequence comprising a promoter, which is a native sequence of *Pichia pastoris* comprising or consisting of the pCS1 nucleic acid sequence of SEQ ID 1, or a functionally active variant thereof which is a size variant, a mutant or hybrid of SEQ ID 1, or a combination thereof.

In one aspect, there is provided an isolated pCS1 promoter nucleic acid sequence, which is a native sequence of *Pichia pastoris* comprising the pCS1 nucleic acid sequence (SEQ ID 1), or a functionally active variant thereof which is a size variant, a mutant or hybrid of SEQ ID 1, or a combination thereof, which has a length of 200-1500 bp and at least 60% sequence homology to the sequence of SEQ ID 1.

According to a specific ~~aspect~~embodiment, there is provided an isolated nucleic acid sequence comprising a promoter, which is a native sequence of Pichia pastoris comprising or consisting of the pCS1 nucleic acid sequence of SEQ ID 1, or a functionally active variant thereof which is a size variant, a mutant or hybrid of SEQ ID 1, or a combination thereof, wherein said functionally active variant exhibits substantially the same activity as pCS1, specifically the pCS1 nucleic acid sequence of SEQ ID 1.

Specifically, the pCS1 nucleotide sequence is identical with the sequence of SEQ ID 1.

Preferably, the nucleic acid sequence of the ~~invention~~disclosure is not identical to the nucleic acid of SEQ ID 87 as listed in WO2007/015178 A2 (i.e. SEQ ID 24 of this application).

Specifically, the functionally active variant is

a) a size variant of pCS1 of SEQ ID 1, preferably consisting of or comprising the nucleic acid sequence selected from the group consisting of SEQ ID 2, 3, 4, 5, 6, 7 and 8;

b) a mutant of the pCS1 of SEQ ID 1, or a mutant of the size variant of a), which mutant has at least 60% homology to the sequence SEQ ID 1 or to the size variant;

c) a hybrid comprising

- a sequence selected from the group consisting of pCS1 of SEQ ID 1, a size variant of a), and a mutant of b); and

- at least one further sequence selected from the group consisting of pCS1 of SEQ ID 1, a size variant of a), a mutant of b), and a heterologous sequence; or

d) a sequence which hybridizes under stringent conditions to any of the size variant, or the mutant nucleic acid sequences of a), or b).

Preferably, the size variant of pCS1 of SEQ ID 1 consists of any of SEQ ID 2, SEQ ID 3, SEQ ID 4, SEQ ID 5 or SEQ ID 6

According to a specific embodiment, the functionally active variant is selected from the group consisting of homologs with

i) at least about 60% nucleotide sequence identity;

ii) homologs obtainable by modifying the nucleotide sequence of pCS1 of SEQ ID 1 or size variants thereof, by insertion, deletion or substitution of one or more nucleotides within the sequence or at either or both of the distal ends of the sequence, preferably with a nucleotide sequence of 80 bp to 1500 bp, or of 200 bp to 1500 bp, more preferably at least 200 bp;and

iii) analogs derived from species other than *Pichia pastoris*.

Specifically the functionally active variant of the ~~invention~~disclosure has substantially the same promoter activity as pCS1.

According to a specific embodiment, the nucleic acid sequence is operably linked to a nucleotide sequence encoding a protein of interest (POI), which nucleic acid is not natively associated with the nucleotide sequence encoding the POI.

According to a specific ~~aspect~~embodiment, the nucleic acid sequence further comprises a signal peptide gene enabling the secretion of the POI, preferably wherein the signal peptide gene is located adjacent to the 5' end of the nucleotide sequence encoding the POI.

Accordingly, the ~~invention~~ disclosure specifically refers to the nucleic acid sequence which further comprises a nucleic acid sequence encoding a signal peptide enabling the secretion of the POI, preferably wherein nucleic acid sequence encoding the signal peptide is located adjacent to the 5' end of the nucleotide sequence encoding the POI.

~~The invention further provides for~~In one aspect, there is provided an expression construct comprising ~~a~~ the nucleic acid sequence ~~of the invention~~as defined herein, preferably an autonomously replicating vector or plasmid, or one which integrates into the chromosomal DNA of a host cell.

~~The invention further provides for~~In one aspect, there is provided a recombinant host cell which comprises ~~the~~a nucleic acid sequence ~~of the invention~~as defined herein or ~~the~~an expression construct ~~of the invention~~as defined herein,

preferably a eukaryotic cell, more preferably a yeast or filamentous fungal cell, more preferably a yeast cell of the Saccharomyces or Pichia genus.

According to a specific ~~aspect~~embodiment, the recombinant host cell comprises multiple copies of the nucleic acid sequence, and/or multiple copies of the expression construct. For example, the recombinant cell comprises 2, 3, 4, or more copies (gene copy number, GCN).

Specifically, the recombinant host cell is selected from the group consisting of mammalian, insect, yeast, filamentous fungi and plant cells, preferably a yeast, preferably any of the *P. pastoris* strains CBS 704, CBS 2612, CBS 7435, CBS 9173-9189, DSMZ 70877, X-33, GS115, KM71 and SMD1168. In some embodiments, the recombinant host cell is a *P. pastoris* strain other than X-33.

~~The invention further provides for~~In one aspect, there is provided a stable culture of a plurality of the cell ~~of the invention~~as defined herein.

~~The invention further provides for~~In one aspect, there is provided a method of producing a POI by culturing a recombinant host cell line comprising ~~the nucleic acid sequence or~~ the promoter ~~of the invention~~as defined herein, or the expression construct ~~of the invention~~as defined herein, and a nucleic acid encoding the POI under the transcriptional control of said promoter, or the recombinant host cell as defined herein, comprising the steps of

a) cultivating the cell line under conditions to express said POI, and

b) recovering the POI.

Specifically, the POI is expressed under growth-limiting conditions, e.g. by cultivating the cell line at a growth rate of less than the maximal growth rate, typically less than 90%, preferably less than 80%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, less than 5%, less than 3%, less than 2%, less than 1%, less than 0,5%, less than 0,4%, less than 0,3%, or less than 0,2% of the maximum growth rate of the cells. Typically the maximum growth rate is individually determined for a specific host cell.

According to a specific embodiment, the cell line is cultivated under batch, fed-batch or continuous cultivation conditions, and/or in media containing limited carbon substrate.

Specifically, the cultivation is performed in a bioreactor starting with a batch phase followed by a fed-batch phase or a continuous cultivation phase.

Specifically, the host cells are grown in a carbon source rich medium during the phase of high growth rate (e.g. at least 50%, or at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or up to the maximum

growth rate) and producing the POI during a phase of low growth rate (e.g. less than 90%, preferably less than 80%, less than 70%, less than 60%, less than 50%, or less than 40%, less than 30%, less than 20%, less than 10%, less than 5%, less than 3%, less than 2%, less than 1%, less than 0,5%, less than 0,4%, less than 0,3%, or less than 0,2% of the maximum growth rate) e.g. while limiting the carbon source, preferably by feeding a defined minimal medium. Specifically the defined minimal medium does not comprise a transcription-inducing carbon source.

The POI is specifically a heterologous protein, preferably selected from therapeutic proteins, including antibodies or fragments thereof, enzymes and peptides, protein antibiotics, toxin fusion proteins, carbohydrate - protein conjugates, structural proteins, regulatory proteins, vaccines and vaccine like proteins or particles, process enzymes, growth factors, hormones and cytokines, or a metabolite of a POI, specifically including a cell metabolite of the recombinant cell culture that expresses a gene of interest under the transcriptional control of a promoter of the ~~invention~~disclosure.

The ~~invention~~ disclosure further provides for a method to identify a constitutive promoter from eukaryotic cells, comprising the steps of

a) cultivating eukaryotic cells at a high growth rate;

b) further cultivating the cells at a low growth rate;

c) providing samples of the cell culture of step a) and b),

d) performing transcription analysis in said samples and comparing the transcript levels with the transcript levels of the native pGAP promoter of the cells; and

f) selecting the constitutive promoter that has a higher transcription strength as compared to the native pGAP promoter at high and low growth rates, preferably by determining a transcript level of the identified constitutive promoter which is at least 1.1-fold higher as compared to the native pGAP promoter, preferably at least 1.2-fold, preferably at least 1.3-fold, preferably at least 1.4-fold, preferably at least 1.5-fold, preferably at least 1.6-fold, preferably at least 1.7-fold, preferably at least 1.8-fold, preferably at least 1.9-fold, preferably at least 2-fold, preferably at least 3-fold, preferably at least 4-fold, preferably at least 5-fold, preferably at least 10-fold, or at least 15-fold higher.

Specifically, the transcript level is determined at a high growth rate and a low growth rate within the range of 0,01 to 0,2 h^{-1}, preferably within the range of 0,015 to 0,15 h^{-1}, e.g. by examining at least two samples of a cell culture, a first one representing a high growth rate, such as at least 0,05 h^{-1}, preferably at least 0,06 h^{-1},

or at least 0,07 h^{-1}, at least 0,08 h^{-1}, at least 0,09 h^{-1}, at least 0,1 h^{-1}, e.g. at a growth rate of 0,15 h^{-1}, and a second one representing a low growth rate, such as less than the first one, e.g. less than 0,05 h^{-1}, preferably less than 0,04 h^{-1}, less than 0,03 h^{-1}, or less than 0,02 h^{-1}, e.g. at a growth rate of 0,015 h^{-1}. The transcription level is specifically determined by analysis of the gene expression patterns using DNA microarrays, e.g. according to Example 11 below.

The ~~invention~~disclosure further provides for the use of an isolated nucleic acid sequence comprising or consisting of a promoter which when operatively linked to a nucleotide sequence encoding a POI directs the expression thereof in a host cell at an expression level that is higher than under control of the native pGAP promoter at high and low growth rates, preferably in a method of producing a POI by cultivating a host cell transformed with the nucleic acid sequence, wherein the cultivation is performed in a bioreactor starting with a batch phase followed by a fed-batch phase or a continuous cultivation phase.

Specifically, the expression level is determined at a high growth rate and a low growth rate within the range of 0,01 to 0,2 h^{-1}, preferably within the range of 0,015 to 0,15 h^{-1}, e.g. by examining at least two samples of a cell culture, a first one representing a high growth rate, such as at least 0,05 h^{-1}, preferably at least 0,06 h^{-1}, or at least 0,07 h^{-1}, at least 0,08 h^{-1}, at least 0,09 h^{-1}, at least 0,1 h^{-1}, e.g. at a growth rate of 0,15 h^{-1}, and a second one representing a low growth rate, such as less than the first one, e.g. less than 0,05 h^{-1}, preferably less than 0,04 h^{-1}, less than 0,03 h^{-1}, or less than 0,02 h^{-1}, e.g. at a growth rate of 0,015 h^{-1}. The expression level is specifically determined under growth-limited conditions. An example of determining the expression strength in growth-limited conditions e.g. in a glucose-limited chemostat cultivation, at high and low growth rates is provided in Example 10 below.

Specifically, the expression level is at least 1.1-fold higher compared to the pGAP promoter, preferably at least 1.2-fold, preferably at least 1.3-fold, preferably at least 1.4-fold, preferably at least 1.5-fold, preferably at least 1.6-fold, preferably at least 1.7-fold, preferably at least 1.8-fold, preferably at least 1.9-fold, preferably at least 2-fold, preferably at least 3-fold, preferably at least 4-fold, preferably at least 5-fold, preferably at least 10-fold, or at least 15-fold higher.

In one aspect, there is provided the use of the isolated nucleic acid sequence as defined herein or the expression construct as defined herein in a method of producing a POI by cultivating a host cell transformed with the nucleic acid sequence and/or the

expression construct, preferably wherein the cultivation is performed in a bioreactor starting with a batch phase followed by a fed-batch phase or a continuous cultivation phase.

According to a specific ~~aspect~~embodiment, the isolated nucleic acid sequence of the ~~invention~~disclosure or the expression construct of the ~~invention~~disclosure is used in a method of producing a POI by cultivating a host cell transformed with the nucleic acid sequence and/or the

13. A method of producing a POI by culturing a recombinant host cell line comprising the promoter according to any of claims 1 to 7 or the expression construct according to claim 8, and a nucleic acid encoding the POI under the transcriptional control of said promoter, or the recombinant host cell according to any of claims 9 to 11, comprising the steps of

a) cultivating the cell line under conditions to express said POI, and

b) recovering the POI.

14. The method according to claim 13, wherein the POI is expressed under growth-limiting conditions.

15. The method according to claim 13 or 14, wherein the cell line is cultivated under batch, fed-batch or continuous cultivation conditions, and/or in media containing limited carbon substrate.

16. The method according to claim 15, wherein the cultivation is performed in a bioreactor starting with a batch phase followed by a fed-batch phase or a continuous cultivation phase.

17. Method according to any of claims 13 to 16, wherein the POI is a heterologous protein, preferably selected from therapeutic proteins, including antibodies or fragments thereof, enzymes and peptides, protein antibiotics, toxin fusion proteins, carbohydrate - protein conjugates, structural proteins, regulatory proteins, vaccines and vaccine like proteins or particles, process enzymes, growth factors, hormones and cytokines, or a metabolite of a POI.

~~18. A method to identify a constitutive promoter from eukaryotic cells, comprising the steps of~~

~~a) cultivating eukaryotic cells at a high growth rate;~~

~~b) further cultivating the cells at a low growth rate;~~

~~c) providing samples of the cell culture of step a) and b),~~

~~d) performing transcription analysis in said samples and comparing the transcript levels with the transcript levels of the native pGAP promoter of the cells; and~~

~~f) selecting the constitutive promoter that has a higher transcription strength as compared to the native pGAP promoter at high and low growth rates, preferably by determining a transcript level of the identified constitutive promoter which is at least 1.1-fold higher as compared to the native pGAP promoter.~~

~~19. Use of an isolated nucleic acid sequence comprising a promoter which when operatively linked to a nucleotide sequence encoding a protein of interest (POI) directs the expression thereof in a host cell at an expression level that is higher than under control of the native pGAP promoter at high and low growth rates, preferably in a method of producing the POI by cultivating a host cell transformed with the nucleic acid sequence, wherein the cultivation is performed in a bioreactor starting with a batch phase followed by a fed-batch phase or a continuous cultivation phase.~~

~~20. Use according to claim 19, wherein the expression level is determined at a high growth rate and a low growth rate within the range of 0,015 to 0,15 h^{-1}.~~

~~21. Use according to claim 19 or 20, wherein the expression level is at least 1.1-fold higher compared to the pGAP promoter.~~

~~22~~18. Use of the isolated nucleic acid sequence of any of claims 1 to 7 or the expression construct of claim 8 in a method of producing a POI by cultivating a host cell transformed with the nucleic acid sequence and/or the expression construct, preferably wherein the cultivation is performed in a bioreactor starting with a batch phase followed by a fed-batch phase or a continuous cultivation phase.