

METHODS OF CELL SELECTION

FIELD OF THE INVENTION

5 The present disclosure relates to methods and compositions for identifying, selecting, or culturing cells comprising a subject nucleic acid sequence.

BACKGROUND

Cell expression systems are commonly used for the production of recombinant biological products, such as therapeutic biologics. The development of production line cells involves
10 introducing nucleic acid constructs encoding recombinant products of interest into host cells and selecting for cells that contain these nucleic acid constructs. This generally involves subjecting the cells to a selection pressure to favour cells that have taken up the foreign nucleic acids. Early selectable marker systems used antibiotic resistance markers but there has been a trend away from the use of such systems. Some alternative systems are based on complementing metabolic
15 deficiencies e.g. dihydrofolate reductase (DHFR) and glutamine synthetase (GS). There remains however a need for new selection systems that can be used to select cells used to produce recombinant biological products. Further, since production host cell engineering strategies are increasingly being used, those strategies that involve the introduction of new sequences that modify host cell characteristics would benefit from a selection system that is
20 separate from existing or future selection systems used for the introduction of sequences encoding biological products.

SUMMARY

The present disclosure relates to a tyrosine auxotrophy-based selection system and the
25 manufacture of recombinant products without the need to include tyrosine in the media. We have found that a system based on phenylalanine hydroxylase (PAH - which catalyzes the conversion of phenylalanine to tyrosine) alone is not effective and that it is necessary to include a second enzyme related to tyrosine biosynthesis, namely GTP cyclohydrolase 1 (GCH1). Further we have found that use of PAH with a truncation that removes the N-terminal regulatory domain provides
30 a significant advantage compared with the full length enzyme. Use of full length CHO PAH resulted in either no recovery in tyrosine-free medium after transfection or a much slower recovery time, whereas a truncated (tPAH) version of the molecule allowed for good recovery. The combination of PAH and GCH1 allows a cell to grow at a lower level of (e.g., in the absence of) tyrosine than a similar cell not expressing these enzymes.

Accordingly, in a first aspect, the present disclosure refers to a vector system comprising one or more nucleic acid vectors comprising:

a) a first nucleic acid sequence comprising a sequence encoding a phenylalanine hydroxylase (PAH) which lacks a functional N-terminal regulatory domain such that the PAH enzyme molecule is constitutively active, operably linked to a first control sequence which enables expression of the PAH in a host cell;

b) a second nucleic acid sequence comprising a sequence encoding a GTP cyclohydrolase 1 (GCH1) operably linked to a second control sequence which enables expression of the GCH1 in a host cell; and

c) a multiple cloning site for inserting one or more sequences encoding a product of interest operably linked to a third control sequence which enables expression of the product in a host cell, wherein the multiple cloning site and third control sequence are present in the same vector as a) and/or b).

In a related aspect the present disclosure also refers to a vector system comprising one or more nucleic acid vectors comprising:

a) a first nucleic acid sequence comprising a sequence encoding a phenylalanine hydroxylase (PAH) which lacks a functional N-terminal regulatory domain such that the PAH enzyme molecule is constitutively active, operably linked to a first control sequence which enables expression of the PAH in a host cell;

b) a second nucleic acid sequence comprising a sequence encoding a GTP cyclohydrolase 1 (GCH1) operably linked to a second control sequence which enables expression of the GCH1 in a host cell; and

c) a third nucleic acid sequence comprising a sequence encoding a product of interest operably linked to a third control sequence which enables expression of the product in a host cell, wherein the third nucleic acid sequence is present in the same vector as a) and/or b).

Such vectors may be introduced into host cells, and those cells containing the vectors selected under tyrosine-limiting conditions which do not allow for efficient growth of non-transformed cells. Accordingly in a one aspect, the present disclosure refers to a eukaryotic host cell comprising:

a) a first exogenous nucleic acid comprising a sequence which encodes a phenylalanine hydroxylase (PAH) which lacks a functional N-terminal regulatory domain such that the PAH

enzyme molecule is constitutively active, operably linked to a first control sequence which enables expression of the PAH in the host cell; and

b) a second exogenous nucleic acid which encodes a GTP cyclohydrolase 1 (GCH1), operably linked to a second control sequence which enables expression of the GCH1 in the host

5 cell; and

c) a third exogenous nucleic acid which encodes a product of interest, operably linked to a third control sequence which enables expression of the product in the host cell, wherein the third exogenous nucleic acid is present in the same exogenous nucleic acid sequence as the first and/or second exogenous nucleic acid.

5 In one example the first, second and third nucleic acid molecules are integrated into the genome of the host cell.

 In one example, the host cell is a mammalian cell, such as a Chinese Hamster Ovary (CHO) cell.

10 In the various examples of the disclosure, the lack of a functional N-terminal regulatory domain in the PAH, may for example be due to a deletion to form a truncated PAH. Based on the human and CHO PAH amino acid sequences this is typically a deletion of about the first 116 amino acids.

 In one example, the PAH is CHO PAH or human PAH.

 In one example the first and/or second control sequence comprises an SV40 promoter.

15 The vector system of the present disclosure is typically used to select cells that have been successfully transformed with a nucleic acid encoding a product of interest, such as a recombinant polypeptide. Accordingly in another aspect, the present disclosure refers to a method of selecting a eukaryotic cell comprising a nucleic acid sequence encoding a product of interest, the method comprising:

20 a) contacting a population of cells that are unable to survive or grow in the absence of tyrosine, with the vector system comprising: i) a first nucleic acid sequence comprising a sequence encoding a phenylalanine hydroxylase (PAH) which lacks a functional N-terminal regulatory domain such that the PAH enzyme molecule is constitutively active, operably linked to a first control sequence which enables expression of the PAH in a host cell; ii) a second nucleic acid sequence comprising a sequence encoding a GTP cyclohydrolase 1 (GCH1) operably linked to a second control sequence which enables expression of the GCH1 in a host cell; and iii) a third
25 nucleic acid sequence comprising a sequence encoding a product of interest operably linked to a third control sequence which enables expression of the product in a host cell, wherein the third sequence is present in the same vector as i) and/or ii), under conditions that permit uptake of the
30 vector system by the cells;

b) culturing the cells in the presence of phenylalanine and under conditions where the level of tyrosine is lower than the level required for survival or growth of cells that do not express the PAH and GCH1 enzymes encoded by the vector system; and

5 c) selecting one or more cells that are able to grow under such conditions to obtain one or more cells which contain the nucleic acid sequence encoding the product.

The level of tyrosine is selected to ensure a stringent selection and is optionally supplemented with phenylalanine. In one example the culture media includes no added tyrosine.

10 In a related example, the present disclosure refers to the use of a vector system of the disclosure for selecting from a population of cells, one or more cells comprising a nucleic acid sequence that has been introduced into the cells.

The selected host cells obtained by the selection method of the present disclosure form another example of the disclosure. Accordingly, in another example, the present disclosure refers to a host cell comprising:

a) a first exogenous nucleic acid comprising a sequence which encodes a phenylalanine hydroxylase (PAH) which lacks a functional N-terminal regulatory domain, operably linked to a first control sequence which enables expression of the PAH in the host cell; and

b) a second exogenous nucleic acid which encodes a GTP cyclohydrolase 1 (GCH1), operably linked to a second control sequence which enables expression of the GCH1 in the host cell; and

c) a third exogenous nucleic acid which encodes a product of interest, operably linked to a third control sequence which enables expression of the product in the host cell.

A host cell of the present disclosure may be genetically modified to inhibit or abolish any endogenous PAH and/or GCH1 activity. In one example this can be achieved by mutations
5 (insertions, deletions and/or substitutions) in the genomic sequences encoding and/or regulating expression of endogenous PAH and/or GCH1.

The selected host cells of the present disclosure comprising a nucleic acid sequence encoding the product of interest will typically be used in the manufacturing of that product. Accordingly in a further aspect, the present disclosure refers to a method of making a product,
10 the method comprising culturing a host cell of the disclosure in the presence of phenylalanine and under conditions suitable for expressing the product, and recovering the product, and optionally subjecting the recovered product to one or more treatment or purification steps.

When a cell line developed using the host cells and selection processes of the present disclosure is used in large scale manufacturing, it may no longer be essential to exert a selection
15 pressure by omitting tyrosine during culturing steps. However, tyrosine, which is considered an essential amino acid, has after cysteine the second lowest solubility in water of any of the amino acids. The low solubility of tyrosine can be a challenge for generating feed solutions of sufficient concentration to support culture of cells under biomanufacturing conditions, e.g., in fed-batch bioprocesses, e.g., in a bioreactor.

The host cells of the present disclosure can be efficiently grown in lower levels of (including
20 in the absence of) tyrosine and reducing the need for high concentration tyrosine feed solutions. Since phenylalanine is consumed by the cells to produce tyrosine, in one example, the culture medium is supplemented with phenylalanine.

The present disclosure also refers to a culture medium, such as a feed, comprising a plurality of amino acids, such as at least 3 or 4 amino acids, wherein there is less than 0.01 g/L tyrosine, such as less than 50, 20 or 10 μ M tyrosine (e.g., no tyrosine) and at least 2, preferably at least 3, 4, 5, 6, 7, 8, 9, mM phenylalanine in aqueous solution. Typically the culture medium comprises less than 10 mM phenylalanine. The present disclosure also refers to a culture medium mixture in substantially dry form (e.g., comprising less than 5, 4, 3, 2, or 1% water, e.g., does not appreciably comprise water) comprising a plurality of amino acids, such as at least 3 or 4 amino acids, with levels of tyrosine and phenylalanine such that by the addition of an appropriate volume of water the above culture medium is prepared.

The present disclosure further refers to the use of the culture medium and culture medium mixture to select and/or grow cells transformed with a vector system of the disclosure, such as in the expression of the product of interest encoded by the vector system.

The present disclosure also refers to a mixture comprising a host cell of the invention and a culture medium of the invention.

In another example, the disclosure features a bioreactor comprising a population of host cells of the invention. In another example, the disclosure features a bioreactor comprising a culture medium and a population of production cells of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows a schematic of the PAH enzyme's domain structure.

Fig. 2 shows (A) histograms obtained using flow cytometry of the mean fluorescence from the population of cells after transfection and recovery for 3 weeks of the same CHO cell pools and (B) tables of the fluorescence data.

Fig. 3 shows a graph of PAH mRNA amounts relative to control as measured by qRT-PCR.

Fig. 4 shows (A) a graph of cell growth by viable cell concentration of various cell pools, some over-expressing truncated PAH, in the absence of tyrosine or glutamine, optionally supplemented with phenylalanine, over 18 days; and (B) a graph of culture viability of the same cell pools under the same conditions.

Fig. 5 shows growth characteristics of tyrosine prototrophic cell pools with varied phenylalanine supplementation.

Fig. 6: shows graphs of growth characteristics of tyrosine prototrophic cell pools in CD CHO no tyrosine with 6 mM phenylalanine. (A) viable cell concentration of various cell pools

CLAIMS

1. A method of selecting a eukaryotic cell comprising a nucleic acid sequence encoding a product of interest, the method comprising:

i) contacting a population of cells that are unable to survive or grow in the absence of tyrosine, with a vector system comprising

a) a first nucleic acid sequence comprising a sequence encoding a phenylalanine hydroxylase (PAH) which lacks a functional N-terminal regulatory domain such that the PAH enzyme molecule is constitutively active, operably linked to a first control sequence which enables expression of the PAH in a host cell;

(b) a second nucleic acid sequence comprising a sequence encoding a GTP cyclohydrolase 1 (GCH1) operably linked to a second control sequence which enables expression of the GCH1 in a host cell; and

(c) a third nucleic acid sequence comprising a sequence encoding a product of interest operably linked to a third control sequence which enables expression of the product in a host cell, wherein the third sequence is present in the same vector as (a) and/or (b), under conditions that permit uptake of the vector system by the cells;

ii) culturing the cells in the presence of phenylalanine and under conditions where the level of tyrosine is lower than the level required for survival or growth of cells that do not express the PAH and GCH1 enzymes encoded by the vector system; and

iii) selecting one or more cells that are able to grow under such conditions to obtain one or more cells which contain the nucleic acid sequence encoding the product.

2. A method according to claim 1 wherein (a), (b) and (c) are present in the same vector.

3. A method according to claim 1 or claim 2 comprising two vectors wherein (c) is present in the same vector as (a) or (b).

4. A method according to any one of claims 1 to 3 wherein the eukaryotic cell is a mammalian cell, for example a CHO cell.

5. A method according to any one of claims 1 to 4, wherein the cell culture medium lacks tyrosine.
6. A eukaryotic host cell comprising:
 - a) a first exogenous nucleic acid comprising a sequence which encodes a phenylalanine hydroxylase (PAH) which lacks a functional N-terminal regulatory domain such that the PAH enzyme molecule is constitutively active, operably linked to a first control sequence which enables expression of the PAH in the host cell; and
 - b) a second exogenous nucleic acid which encodes a GTP cyclohydrolase 1 (GCH1), operably linked to a second control sequence which enables expression of the GCH1 in the host cell; and
 - c) a third exogenous nucleic acid which encodes a product of interest, operably linked to a third control sequence which enables expression of the product in the host cell, wherein the third exogenous nucleic acid is present in the same exogenous nucleic acid sequence as the first and/or second exogenous nucleic acid.
7. A host cell according to claim 6 wherein the PAH has a deletion of the N-terminal regulatory domain.
8. A host cell according to claim 6 or claim 7 wherein the PAH is CHO or human PAH.
9. A host cell according to any one of claims 6 to 8 which is a mammalian cell, for example a CHO cell.
10. A host cell according to any one of claims 6 to 9 wherein the first, second and third nucleic acid molecules are integrated into the genome of the host cell.
11. A host cell according to any one of claims 6 to 9 wherein the activity of the cell's endogenous genes encoding PAH and/or GCH1 has been reduced or abolished.

12. A vector system comprising one or more nucleic acid vectors comprising:

a) a first nucleic acid sequence comprising a sequence encoding a phenylalanine hydroxylase (PAH) which lacks a functional N-terminal regulatory domain such that the PAH enzyme molecule is constitutively active, operably linked to a first control sequence which enables expression of the PAH in a host cell;

b) a second nucleic acid sequence comprising a sequence encoding a GTP cyclohydrolase 1 (GCH1) operably linked to a second control sequence which enables expression of the GCH1 in a host cell; and

c) a multiple cloning site for inserting a sequence encoding a product of interest operably linked to a third control sequence which enables expression of the product in a host cell, wherein the multiple cloning site and third control sequence are present in the same vector as (a) and/or (b).

13. A vector system comprising one or more nucleic acid vectors comprising:

a) a first nucleic acid sequence comprising a sequence encoding a phenylalanine hydroxylase (PAH) which lacks a functional N-terminal regulatory domain such that the PAH enzyme molecule is constitutively active, operably linked to a first control sequence which enables expression of the PAH in a host cell;

b) a second nucleic acid sequence comprising a sequence encoding a GTP cyclohydrolase 1 (GCH1) operably linked to a second control sequence which enables expression of the GCH1 in a host cell; and

c) a third nucleic acid sequence comprising a sequence encoding a product of interest operably linked to a third control sequence which enables expression of the product in a host cell, wherein the third nucleic acid sequence is present in the same vector as (a) and/or (b).

14. A method of making a product, the method comprising culturing a host cell according to any one of claims 7 to 11 in the presence of phenylalanine and under conditions suitable for expressing the product, and recovering the product, and optionally subjecting the recovered product to one or more treatment or purification steps.

15. A method according to claim 14, wherein the cells are cultured under conditions where the level of tyrosine is lower than the level required for survival or growth of cells that do not express the PAH and GCH1 enzymes encoded by the vector system defined in claim 12 or 13.