Use of an amino acid auxotrophy cured prokaryotic strain for the recombinant production of a polypeptide

The current invention is in the field of recombinant polypeptide production. More precisely herein is reported a method for the recombinant production of a non-glycosylated polypeptide using an auxotrophy cured prokaryotic strain in a chemically defined minimal growth medium.

Background of the Invention

In recent years the production of therapeutic polypeptides has steadily increased and it is likely that therapeutic polypeptides will become the biggest group of therapeutics available for the treatment of various diseases in the near future. The impact of therapeutic polypeptides emerges from their specificity, such as specific target recognition and/or binding function.

Cell cultures are used in fermentative processes to produce substances and in particular polypeptides. A distinction is made between processes in which the cell cultures are genetically unmodified and form their own metabolic products and processes in which the organisms are genetically modified in such a manner that they either produce a larger amount of their own substances such as polypeptides or produce foreign substances. The organisms producing the substances are supplied with a nutrient medium which guarantees the survival of the organisms and enables the production of the desired target compound. Numerous culture media are known for these purposes which enable an optimal cultivation of the specific host.

The use of a chemically defined minimal growth medium in the cultivation of a recombinant cell for the recombinant production of therapeutic polypeptides is advantageous. It enables easy development of downstream processing and purification of the produced therapeutic polypeptide, provides for a robust productions process due to minimized raw material differences and reduces costs of goods.

A chemically defined minimal growth medium does not comprise free amino acids and it is required to use prototrophic cell lines which have intact metabolic pathways to produce the required amino acids from the available components of the chemically defined minimal growth medium.

When, for example, E.coli is used as host cell line generally wild-type strains, such as MG1655, W3110 or BL21, are employed. These strains show good growth characteristics but inferior product titer.

Mutant prokaryotic strains, which have been obtained by non-directed mutagenesis and selection, show profound differences in their genomic DNA when compared to wild-type strains. The mutant strains have been selected based on the maximum product titer that can be obtained. As the mutant strains harbor a number of auxotrophies they cannot be used for cultivation in a chemically defined minimal growth medium. The mutant strains required the feeding of amino acids to complement their auxotrophies resulting in increased cultivation costs.

US 5,932,439 reports Escherichia coli K-12 strains for production of recombinant proteins.

Summary

By curing an auxotrophic prokaryotic strain of one or more of its auxotrophies the auxotrophy cured strain can grow on a chemically defined minimal growth medium to which the substance corresponding to the auxotrophy that has been cured no further needs to be added. In case of expensive substances a reduction of the costs of goods can be achieved and a recombinant polypeptide can be produced more economically.

In addition to the restoration of the ability to grow on a chemically defined minimal growth medium in the absence of the substance corresponding to the auxotrophy that has been cured it has been found that the obtainable product titer can be maintained or even increased compared to the non-cured strain. It has furthermore been found that the obtainable product titer using an auxotrophy cured prokaryotic strain is higher than the product titer that can be obtained with a corresponding prototrophic wild-type strain.

Without being bound by this theory it is assumed that an amino acid auxotrophy cured prokaryotic strain as reported herein has a different metabolism and metabolic fluxes when compared to a corresponding prototrophic wild-type strain in which such a deletion and cure has not been performed. Thus, the inactivation and re-activation of certain enzymes within a prokaryotic strain has a detectable/pronounced effect on the entire metabolism of the strain, which can be

seen, e.g. in the increased productivity of such a strain compared to a corresponding prototrophic wild-type strain.

The present disclosure relates to an amino acid auxotrophy cured prokaryotic strain, characterized in that

- at least one deficiency in an essential amino acid metabolic pathway has been cured, and
- the strain can grow in chemically defined minimal growth medium that is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic strain.

In one embodiment the auxotrophy cured prokaryotic strain is an auxotrophy cured E.coli strain.

The present disclosure relates to an amino acid auxotrophy cured prokaryotic cell, characterized in that

- at least one deficiency in an essential amino acid metabolic pathway has been cured, and
- the cell can grow in chemically defined minimal growth medium that is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell.

In one embodiment the auxotrophy cured prokaryotic cell is an auxotrophy cured E.coli cell.

The auxotrophy cured prokaryotic strain/cell as reported herein can be used in a method for the recombinant production of a therapeutic polypeptide wherein compared to the non-auxotrophy cured strain/cell the supplementation of at least one amino acid residue is not required.

The present disclosure relates to is a method for producing a polypeptide in a (recombinant) prokaryotic cell, comprising the following step:

- cultivating a (recombinant) prokaryotic cell, which has been obtained by introducing into the genome of a parent prokaryotic cell a nucleic acid curing an amino acid auxotrophy of the parent prokaryotic cell, comprising one or more nucleic acids encoding the polypeptide in a

chemically defined minimal growth medium and recovering the polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium.

One aspect as reported herein is a method for producing a polypeptide in a prokaryotic cell, comprising the following step:

- curing a prokaryotic cell that is auxotrophic for at least one amino acid from at least one amino acid auxotrophy by introducing into the genome of a parent prokaryotic cell a nucleic acid curing an amino acid auxotrophy of the parent prokaryotic cell,
- cultivating the the amino acid auxotrophy cured prokaryotic cell comprising one or more nucleic acids encoding the polypeptide in a chemically defined minimal growth medium and recovering the polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium,

wherein the product titer when using the auxotrophy cured prokaryotic strain is higher than the product titer that can be obtained with a corresponding prototrophic wild-type strain.

The present disclosure relates to a method for producing a polypeptide in a (recombinant) prokaryotic cell, comprising the following steps:

- curing an prokaryotic cell that is auxotrophic for at least one amino acid from at least one amino acid auxotrophy,
- cultivating the amino acid auxotrophy cured (recombinant) prokaryotic cell comprising one or more nucleic acids encoding the polypeptide in a chemically defined minimal growth medium and recovering the polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium.

The present disclosure relates to a method for producing a polypeptide in a (recombinant) prokaryotic cell, comprising the following step:

- cultivating a (recombinant) prokaryotic cell comprising one or more nucleic acids encoding the polypeptide in a chemically defined minimal growth medium and recovering the polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium,

wherein the (recombinant) prokaryotic cell is an amino acid auxotrophy cured prokaryotic cell.

In one embodiment of all methods for producing a polypeptide the auxotrophy cured prokaryotic cell has at least one further (non-cured) amino acid auxotrophy. This auxotrophy can be used for the selection of recombinants/transformants after the introduction of one or more nucleic acids encoding a polypeptide of interest (the polypeptide).

In one embodiment of all methods for producing a polypeptide the growth of the amino acid auxotrophy cured prokaryotic cell compared to the non-cured prokaryotic cell (parent cell) under the same cultivation conditions and in the same growth medium requires the supplementation of fewer amino acids to the growth medium.

In one embodiment of all methods for producing a polypeptide the chemically defined minimal growth medium is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell.

In one embodiment of all methods for producing a polypeptide the cultivation of the auxotrophy cured prokaryotic cell requires supplementation of one or two or three or four amino acids less to the growth medium than required for the cultivation of the non-auxotrophy cured prokaryotic cell (parent/parent cell).

In one embodiment of all methods for producing a polypeptide the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell is not added during the cultivation.

In one embodiment of all methods for producing a polypeptide the cultivation is in the absence of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell.

In one embodiment of all methods for producing a polypeptide the prokaryotic cell is an E.coli cell.

In one embodiment of all methods for producing a polypeptide the polypeptide is a full length antibody chain, a single chain antibody, a single domain antibody, a scFv, a scFab, or a conjugate of one of the before with a non-antibody polypeptide.

In one embodiment of all methods for producing a polypeptide the polypeptide is a conjugate of a scFv or scFab with a cytotoxic agent.

The present disclosure relates to the use of an amino acid auxotrophy cured prokaryotic strain for the (recombinant) production of a polypeptide.

In one embodiment

- in the amino acid auxotrophy cured prokaryotic strain at least one deficiency in an essential amino acid metabolic pathway has been cured, and
- the amino acid auxotrophy cured strain can grow in chemically defined minimal growth medium that is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic strain.

In one embodiment the auxotrophy cured prokaryotic strain is an auxotrophy cured E.coli strain.

In one embodiment the polypeptide is a full length antibody chain, a single chain antibody, a single domain antibody, a scFv, a scFab, or a conjugate of one of the before with a non-antibody polypeptide.

In one embodiment the polypeptide is a conjugate of a scFv or scFab with a cytotoxic agent.

In one embodiment of all aspects the auxotrophy cured prokaryotic strain is a non-lysogenic strain. A lysogenic strain is infected with a temporal bacteriophage, i.e. a non-lysogenic strain is free of bacteriophages.

In one embodiment of all aspects the cure is a directed cure targeting only the amino acid auxotrophy.

In one embodiment of all aspects the cure is the introduction of one or more enzymes required for the synthesis of the auxotrophic amino acid.

In one embodiment of all aspects the prokaryotic strain has the genotype thi-1, ΔompT, ΔpyrF.

Detailed Description of the Invention

One aim in the development of large scale recombinant polypeptide production processes is the reduction of the required amount, i.e. the consumption, of medium components during the cultivation. Especially the reduction of the consumption of expensive medium components, such as amino acids, is advantageous in view of costs of goods.

This aim has been achieved by curing an amino acid auxotrophic strain from at least one, some, or all of its amino acids auxotrophies in order to avoid feeding of expensive amino acids and therewith to reduce the costs of the recombinant production of a polypeptide of interest. Additionally a chemical defined medium can be used which results in an improved process stability and facilitates downstream processing (DSP).

Thus, herein is reported a method for the recombinant production of a non-glycosylated polypeptide using an auxotrophy cured prokaryotic strain in a chemically defined minimal growth medium.

It has been found that by curing an auxotrophic prokaryotic strain of one or more of its auxotrophies the auxotrophy cured strain can grow on a chemically defined minimal growth medium to which the substance corresponding to the auxotrophy that has been cured no further needs to be added. In case of expensive substances, such as e.g. amino acids, a reduction of the costs of goods can be achieved and a recombinant polypeptide can be produced more economically.

In addition to the restoration of the ability to grow on a chemically defined minimal growth medium in the absence of the substance corresponding to the auxotrophy that has been cured it has been found that the obtainable product titer can be maintained or even increased. It has furthermore been found that the obtainable product titer using an auxotrophy cured prokaryotic strain is higher than the product titer that can be obtained with a corresponding prototrophic wild-type strain.

One aspect as reported herein is a method for producing a polypeptide in a prokaryotic cell, comprising the following step:

- cultivating a prokaryotic cell, which has been obtained by introducing into the genome of a parent prokaryotic cell a nucleic acid curing an amino acid auxotrophy of the parent prokaryotic cell, comprising one or more nucleic acids encoding the polypeptide in a chemically defined minimal growth medium and recovering the polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium.

One aspect as reported herein is a method for producing a polypeptide in a prokaryotic cell, comprising the following steps:

- curing an prokaryotic cell that is auxotrophic for at least one amino acid from at least one amino acid auxotrophy,
- cultivating the amino acid auxotrophy cured prokaryotic cell comprising one or more nucleic acids encoding the polypeptide in a chemically defined minimal growth medium and recovering the

polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium.

One aspect as reported herein is a method for producing a polypeptide in a (recombinant) prokaryotic cell, comprising the following step:

- cultivating a (recombinant) prokaryotic cell comprising one or more nucleic acids encoding the polypeptide in a chemically defined minimal growth medium and recovering the polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium,

wherein the (recombinant) prokaryotic cell is an amino acid auxotrophy cured prokaryotic cell,

wherein growth of the amino acid auxotrophy cured prokaryotic cell compared to the non-cured prokaryotic cell (parent cell) under the same cultivation conditions and in the same growth medium requires the supplementation of fewer amino acids to the growth medium, and

wherein the chemically defined minimal growth medium is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell.

An "amino acid auxotrophic prokaryotic cell" is a prokaryotic cell that cannot synthesize an essential amino acid e.g. due to a mutation or deletion within a gene locus comprising the structural gene encoding the proteins of the corresponding biosynthetic pathway. Without the addition of the respective amino acid to the cultivation medium the cell cannot proliferate. The auxotrophy can be for any amino acid. The prokaryotic cell can also be auxotrophic for more than one amino acid. Thus, in one embodiment the amino acid auxotrophic prokaryotic cell is auxotrophic for at least two amino acids. In another embodiment the amino acid auxotrophic prokaryotic cell is auxotrophic for at least two, at least three, at least four, at least five amino acids. In a further embodiment the amino acid auxotrophic prokaryotic cell is auxotrophic for at least 2 and up to 5, or up to 10, or up to 15 amino acids. In another embodiment the amino acid auxotrophic prokaryotic cell is auxotrophic for two to five amino acids, or two to three amino acids, or for two amino acids, or for three amino acids, or for four amino acids. In one embodiment the (amino acid) auxotrophy cured cell has at least one amino acid auxotrophy, or

at least two amino acid auxotrophies, or at least three amino acid auxotrophies, or from one to three amino acid auxotrophies, or two amino acid auxotrophies.

The amino acid auxotrophic prokaryotic cell is in one embodiment a bacterial cell.

In one embodiment the cell is an Escherichia cell, or a Bacillus cell, or a Lactobacillus cell, or a Corynebacterium cell, or a Yeast cell (Saccharomyces, Candida, or Pichia). In a further embodiment the cell is an Escherichia coli cell, or a Bacillus subtilis cell, or a Lactobacillus acidophilus cell, or a Corynebacterium glutamicum cell, or a Pichia pastoris yeast cell.

Prokaryotic cells that can be used in the method as reported herein can comprise one or more amino acid auxotrophies. For example, E.coli cells deficient in the Leucine biosynthetic pathway can be selected from the LeuB6 deficient cells 13-6, χ148, χ156, χ2224, χ462, χ463, χ474, χ478, χ515, χ65, χ697, χ760, 2000k MSE248, 342-167, 342MG, 679-680, A586, A592, A593, AA100, AA7852, AA787, AB1102, AB1111, AB1115, AB1122, AB1129, AB113, AB1132, AB1133, AB114, AB1157, AB1157-D, AB1314, AB1330, AB1331, AB1881, AB1884, AB1885, AB188, CP78, CP79, CR34 Thy-, CR34 Thy- SR, CR34/308, CR34/313, CR34/399, CR34/43, CR34/454, CR34/500, CR34/7a, CS130, CS312, CS419, CS425, CS426, CS460, CS471, CS472, CS50, CS81, CS85, CSR06, CSR603, CSR603/pDR1996, CT28-3b, DA10, DA11, DB1161, DB1257, DE1878, DE1882, DE2345, DF225, DF41, JRG94, JS10 C600r-m-, T6R, P678SSR pro-, PA20SR, PA200 SR, PA201 SR, PA214SRT6R, PA265 SR, PA309, PDE70, PA340, PA340/T6, PA360, PA414, PAM161, PAM162, PAM163, PAM164, PAM660, PAT84, PB349, PB69, PC1, PC2, PC3, PC5, PC6, PC8, PJ1, PJ2, PJ3, PJ4, PJ5, PJ C600 (= CRSR), W208 SR AzR, W2660, LAM-, W945his, WA2127, WA2379, WA2548, WA2552, WA2574, WA2899, WA921, WA946, WA960, Y10, Y46, Y53, Y70, YYC100.

In one embodiment the prokaryotic cell is an E.coli K12 cell or an E.coli B cell.

The term “growth” refers to the change in viable cell density in a cultivation. This change can be a change over time. The viable cell density can be determined by determining the optical density at 578 nm.

In one embodiment the growth is the increase of viable cell density during a time period. In one embodiment the time period is the time period of 0 to 30 hours of the cultivation after inoculation.

The term "same" denotes that a second value is within +/- 20 % of a first value. With respect to cultivation conditions the term "same" refers to cultivation time, cultivation volume, cultivation temperature, seeding cell density, aeration rate, pH value, stirrer speed, feed rate (if applicable), etc. With respect to the cultivation medium the term "same" denotes that a second medium compared to a first medium differs in at most 5 components (differ = presence or absence).

In one embodiment the same cultivation conditions are the identical cultivation conditions. In one embodiment the same cultivation medium is the identical cultivation medium.

Methods for cultivating a prokaryotic cell and also for cultivating an amino acid auxotrophic prokaryotic cell are known to a person of skill in the art (see e.g. Riesenberg, D., et al., Curr. Opin. Biotechnol. 2 (1991) 380-384).

The cultivating can be with any method. In one embodiment the cultivating is a batch cultivating, a fed-batch cultivating, a perfusion cultivating, a semi-continuous cultivating, or a cultivating with full or partial cell retention.

In one embodiment the cultivating is a high cell density cultivating. The term "high cell density cultivating" denotes a cultivating method wherein the dry cell weight of the cultivated prokaryotic cell is at one point in the cultivating at least 10 g/L. In one embodiment the dry cell weight is at one point in the cultivating at least 20 g/L, or at least 50 g/L, or at least 100 g/L, or more than 100 g/L. In order to reach such a high cell density state the volume of feed and/or adjustment solutions added during the cultivating has to be as small as possible. Methods for the determination of dry cell weight are reported e.g. in Riesenberg, D., et al., Appl. Microbiol. Biotechnol. 34 (1990) 77-82.

The nutrients in the provided medium will be metabolized during the cultivation and have to be replenished in order to avoid a limitation.

experiment no.	**prokaryotic strain / expression plasmid**	**chemically defined minimal medium +**	**product titer**	**see example**
1	CSPZ-2/5816	Leu, Pro	16.1 g/L	3
2	CSPZ-6/5816	Leu, Pro	19.2 g/L	3
5	CSPZ-6/5816	-	20.8 g/L	5
6	MG1655 (ΔpyrF)/5816	-	10.9 g/L	5

From the comparison of experiment no. 1 with experiment no. 2 it can be seen that with the auxotrophy cured strain CSPZ-6 a higher titer can be achieved than with its parent strain CSPZ-2 under the same cultivation conditions, i.e. the auxotrophy cured strain has an increased productivity (volumetric productivity, time-space-yield).

From the comparison of experiment no. 2 with experiment no. 5 it can be seen that the auxotrophy cured strain CSPZ-6 does not require the addition of the amino acids proline and leucine for recombinant production. A comparable or even slightly increased product titer can be achieved in the absence of proline and leucine.

From the comparison of experiment no. 5 with experiment no. 6 it can be seen that with the auxotrophy cured strain CSPZ-6 a higher titer can be achieved than with a corresponding wild-type strain (MG1655 (ΔpyrF)) under the same cultivation conditions.

experiment no.	**prokaryotic strain / expression plasmid**	**chemically defined minimal medium +**	**product titer**	**see example**
7	CSPZ-6/5830	-	22.6 g/L	6
8	66C5/5830	-	2.3 g/L	6
9	66C5/5830	-	6.9 g/L	6 (elevated temperature)

The product titer obtainable with the auxotrophy cured strain CSPZ-6 has also been compared with the product titer obtainable with another corresponding wild-type strain W3110 (66C5 = W3110 ΔpyrF).

From the comparison of experiment no. 7 with experiment no. 8 it can be seen that with the auxotrophy cured strain CSPZ-6 a higher titer can be achieved than with a corresponding wild-type strain (W3110) under the same cultivation conditions.

From the comparison of experiment no. 7 with experiment no. 9 it can be seen that with the auxotrophy cured strain CSPZ-6 a higher titer can be achieved than with a corresponding wild-type strain (W3110) even if improved cultivation conditions (elevated temperature) are used for the wild-type strain but not for the auxotrophy cured strain.

experiment no.	prokaryotic strain / expression plasmid	chemically defined minimal medium +	product titer	see example
10	CSPZ-6/5836	-	33.8 g/L	7
11	CSPZ-14/5836	-	9.6 g/L	7

The product titer obtainable with the auxotrophy cured strain CSPZ-6 has also been compared with the product titer obtainable with another corresponding wild-type strain BL-21 (CSPZ-14 = BL21 ΔpyrF).

From the comparison of experiment no. 10 with experiment no. 11 it can be seen that with the auxotrophy cured strain CSPZ-6 a higher titer can be achieved than with a corresponding wild-type strain (BL-21) under the same cultivation conditions.

One aspect as reported herein is an amino acid auxotrophy cured prokaryotic strain, characterized in that

- at least one deficiency in an essential amino acid metabolic pathway has been cured,
- the strain can grow in chemically defined minimal growth medium that is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic strain, and
- the productivity of the auxotrophy cured strain is improved compared to the productivity of the parental non-auxotrophy cured strain as well as compared to corresponding wild-type strains (corresponding genotype).

In one embodiment the auxotrophy cured prokaryotic strain is an auxotrophy cured E.coli strain.

One aspect as reported herein is an amino acid auxotrophy cured prokaryotic cell, characterized in that

- at least one deficiency in an essential amino acid metabolic pathway has been cured, and

- the cell can grow in chemically defined minimal growth medium that is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell.

In one embodiment the auxotrophy cured prokaryotic cell is an auxotrophy cured E.coli cell.

The auxotrophy cured prokaryotic strain/cell as reported herein can be used in a method for the recombinant production of a therapeutic polypeptide wherein compared to the non-auxotrophy cured strain/cell the supplementation of at least one amino acid residue is not required.

One aspect as reported herein is a method for producing a polypeptide in a (recombinant) prokaryotic cell, comprising the following step:

- cultivating a (recombinant) prokaryotic cell comprising one or more nucleic acids encoding the polypeptide in a chemically defined minimal growth medium and recovering the polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium,

wherein the (recombinant) prokaryotic cell is an amino acid auxotrophy cured prokaryotic cell.

In one embodiment the growth of the amino acid auxotrophy cured prokaryotic cell compared to the non-cured prokaryotic cell (parent cell) under the same cultivation conditions and in the same growth medium requires the supplementation of fewer amino acids to the growth medium.

In one embodiment the chemically defined minimal growth medium is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell.

In one embodiment the cultivation of the auxotrophy cured prokaryotic cell requires supplementation of one or two or three or four amino acids less to the growth medium than required for the cultivation of the non-auxotrophy cured prokaryotic cell (parent cell).

In one embodiment the auxotrophy cured prokaryotic cell has at least one further (non-cured) amino acid auxotrophy.

In one embodiment the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell is not added during the cultivation.

In one embodiment the cultivation is in the absence of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell.

In one embodiment the prokaryotic cell is an E.coli cell.

In one embodiment the polypeptide is a full length antibody chain, a single chain antibody, a single domain antibody, a scFv, a scFab, or a conjugate of one of the before with a non-antibody polypeptide.

In one embodiment polypeptide is a conjugate of a scFv or scFab with a cytotoxic agent.

In one embodiment the viable cell density at 30 hours after inoculation of the cultivation is higher in the cultivation of the auxotrophy cured strain than in the cultivation of the non-cured prokaryotic cell.

One aspect as reported herein is the use of an amino acid auxotrophy cured prokaryotic strain for the (recombinant) production of a polypeptide.

In one embodiment

- in the amino acid auxotrophy cured prokaryotic strain at least one deficiency in an essential amino acid metabolic pathway has been cured, and
- the amino acid auxotrophy cured strain can grow in chemically defined minimal growth medium that is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic strain.

In one embodiment the auxotrophy cured prokaryotic strain is an auxotrophy cured E.coli strain.

In one embodiment the polypeptide is a full length antibody chain, a single chain antibody, a single domain antibody, a scFv, a scFab, or a conjugate of one of the before with a non-antibody polypeptide.

In one embodiment the polypeptide is a conjugate of a scFv or scFab with a cytotoxic agent.

In one embodiment the method for producing a polypeptide in a prokaryotic cell, comprises the following step:

- cultivating a prokaryotic cell comprising one or more nucleic acids encoding the polypeptide in a chemically defined minimal growth medium and recovering the polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium,

wherein the prokaryotic cell is an amino acid auxotrophy cured prokaryotic cell,

wherein growth of the amino acid auxotrophy cured prokaryotic cell compared to the non-cured prokaryotic cell under the same cultivation conditions and in the same growth medium requires the supplementation of fewer amino acids to the growth medium, and

wherein the chemically defined minimal growth medium is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell.

Specific embodiments

1. An amino acid auxotrophy cured prokaryotic strain, characterized in that

   - at least one deficiency in an essential amino acid metabolic pathway has been cured, and

   - the strain can grow in chemically defined minimal growth medium that is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic strain.

2. The strain according to item 1, characterized in that the auxotrophy cured prokaryotic strain is an auxotrophy cured E.coli strain.

3. An amino acid auxotrophy cured prokaryotic cell, characterized in that

   - at least one deficiency in an essential amino acid metabolic pathway has been cured, and

- the cell can grow in chemically defined minimal growth medium that is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell.

4. The strain according to item 3, characterized in that the auxotrophy cured prokaryotic cell is an auxotrophy cured E.coli cell.

5. A method for producing a polypeptide in a (recombinant) prokaryotic cell, comprising the following step:

- cultivating a (recombinant) prokaryotic cell, which has been obtained by introducing into the genome of a parent prokaryotic cell a nucleic acid curing an amino acid auxotrophy of the parent prokaryotic cell, comprising one or more nucleic acids encoding the polypeptide in a chemically defined minimal growth medium and recovering the polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium.

6. A method for producing a polypeptide in a (recombinant) prokaryotic cell, comprising the following steps:

- curing an prokaryotic cell that is auxotrophic for at least one amino acid from at least one amino acid auxotrophy,

- cultivating the amino acid auxotrophy cured (recombinant) prokaryotic cell comprising one or more nucleic acids encoding the polypeptide in a chemically defined minimal growth medium and recovering the polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium.

7. A method for producing a polypeptide in a (recombinant) prokaryotic cell, comprising the following step:

- cultivating a (recombinant) prokaryotic cell comprising one or more nucleic acids encoding the polypeptide in a chemically defined minimal growth medium and recovering the polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium,

wherein the (recombinant) prokaryotic cell is an amino acid auxotrophy cured prokaryotic cell.

8. The method according to any one of items 5 to 7, characterized in that the auxotrophy cured prokaryotic cell has at least one further (non-cured) amino acid auxotrophy.

9. The method according to any one of items 5 to 8, characterized in that the growth of the amino acid auxotrophy cured prokaryotic cell compared to the non-cured prokaryotic cell (parent cell) under the same cultivation conditions and in the same growth medium requires the supplementation of fewer amino acids to the growth medium.

10. The method according to any one of items 5 to 9, characterized in that the chemically defined minimal growth medium is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell.

11. The method according to any one of items 5 to 10, characterized in that the cultivation of the auxotrophy cured prokaryotic cell requires supplementation of one or two or three or four amino acids less to the growth medium than required for the cultivation of the non-auxotrophy cured prokaryotic cell (parent/parent cell).

12. The method according to any one of items 5 to 11, characterized in that the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell is not added during the cultivation.

13. The method according to any one of items 5 to 12, characterized in that the cultivation is in the absence of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell.

14. The method according to any one of items 5 to 13, characterized in that the prokaryotic cell is an E.coli cell.

15. The method according to any one of items 5 to 14, characterized in that the polypeptide is a full length antibody chain, a single chain antibody, a single domain antibody, a scFv, a scFab, or a conjugate of one of the before with a non-antibody polypeptide.

16. The method according to any one of items 5 to 15, characterized in that the polypeptide is a conjugate of a scFv or scFab with a cytotoxic agent.

17. Use of an amino acid auxotrophy cured prokaryotic strain for the (recombinant) production of a polypeptide.

18. The use according to item 17, characterized in that

- in the amino acid auxotrophy cured prokaryotic strain at least one deficiency in an essential amino acid metabolic pathway has been cured, and

- the amino acid auxotrophy cured strain can grow in chemically defined minimal growth medium that is free of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic strain.

19. The strain or method or use according to any one of items 1 to 18, characterized in that the auxotrophy cured prokaryotic strain is an auxotrophy cured E.coli strain.

20. The strain or method or use according to any one of items 1 to 19, characterized in that the polypeptide is a full length antibody chain, a single chain antibody, a single domain antibody, a scFv, a scFab, or a conjugate of one of the before with a non-antibody polypeptide.

21. The strain or method or use according to any one of items 1 to 20, characterized in that the polypeptide is a conjugate of a scFv or scFab with a cytotoxic agent.

22. The strain or method or use according to any one of items 1 to 21, characterized in that the auxotrophy cured prokaryotic strain is a non-lysogenic strain.

23. The strain or method or use according to any one of items 1 to 22, characterized in that the cure is a directed cure targeting only the amino acid auxotrophy.

24. The strain or method or use according to any one of items 1 to 23, characterized in that the cure is the introduction of one or more enzymes required for the synthesis of the auxotrophic amino acid.

25. The strain or method or use according to any one of items 1 to 24, characterized in that the prokaryotic strain has the genotype thi-1, ΔompT, ΔpyrF.

The following examples, sequences and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

List of Sequences

SEQ ID NO: 01	Human interferon derived sequence.
SEQ ID NO: 02	Hexa-histidine affinity tag.
SEQ ID NO: 03	IgA protease cleavage site.
SEQ ID NO: 04	Tetranectin apolipoprotein A-I fusion polypeptide amino acid sequence.
SEQ ID NO: 05	N-terminal extended Tetranectin apolipoprotein A-I fusion polypeptide amino acid sequence obtained after IgA protease cleavage.
SEQ ID NO: 06	Tetranectin apolipoprotein A-I fusion polypeptide amino acid sequence without affinity tag and IgA protease cleavage (N-terminal shortened fusion polypeptide).

Description of the Figures

Figure 1	Growth comparison of parental strain CSPZ-2 (diamonds) and auxotrophy cured strain CSPZ-6 (triangles).
Figure 2	Product yield comparison of parental strain CSPZ-2 (diamonds) and auxotrophy cured strain CSPZ-6 (triangles).
Figure 3	A: Trend plot of the L-leucine concentration (CSPZ-2: diamonds; CSPZ-6: triangles). B: Trend plot of the L-proline concentration (CSPZ-2: diamonds; CSPZ-6: triangles).
Figure 4	Growth comparison of auxotrophy cured and wild-type strain on medium with and without amino acid supplementation (CSPZ-6

on CDM supplemented with L-leucine and L-proline: triangle; CSPZ-6 on CDM: squares; MG1655 derivative strain on CDM: "X").

Figure 5 Product yield comparison of auxotrophy cured and wild-type strain on medium with and without amino acid supplementation (CSPZ-6 on CDM supplemented with L-leucine and L-proline: triangle; CSPZ-6 on CDM: squares; MG1655 derivative strain on CDM: "X").

Figure 6 Growth comparison of auxotrophy cured strain CSPZ-6 (diamonds) and wild-type strain 66C5 (squares) on medium without amino acid supplementation.

Figure 7 Growth comparison of auxotrophy cured strain CSPZ-6 (diamonds, normal temperature), wild-type strain 66C5 (squares, normal temperature), and wild-type strain 66C5 at elevated temperature (triangles) on medium without amino acid supplementation.

Figure 8 Product yield comparison of auxotrophy cured strain CSPZ-6 (diamonds) and wild-type strain 66C5 (squares) on medium without amino acid supplementation.

Figure 9 Product yield comparison of auxotrophy cured strain CSPZ-6 (diamonds, normal temperature), wild-type strain 66C5 (squares, normal temperature), and wild-type strain 66C5 at elevated temperature (triangles) on medium without amino acid supplementation.

Figure 10 Growth comparison of auxotrophy cured strain CSPZ-6 (diamonds) and wild-type strain BL21 on medium without amino acid supplementation.

Figure 11 Product yield comparison of auxotrophy cured strain CSPZ-6 (diamonds) and wild-type strain BL21 (squares) on medium without amino acid supplementation.

Figure 12 Growth comparison of auxotrophy cured strain CSPZ-6 (diamonds) and wild-type strain MG1655 derivate (triangles).

Figure 13 Product yield comparison of auxotrophy cured strain CSPZ-6 (diamonds) and wild-type strain MG1655 derivate (triangles).

Examples

Materials and Methods

Recombinant DNA technique:

Standard methods were used to manipulate DNA as described in Sambrook, J., et al., Molecular cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturer's instructions.

Example 1

Cure of auxotrophy

The E.coli K12 strain CSPZ-2 (leuB, proC, trpE, thi-1, ΔpyrF) was cured from its genetic defects in the genes leuB, proC, proBA and trpE. This was done by using a method for directed genetic engineering of E.coli chromosomal genes (Gene Bridges, Heidelberg, Germany; http://www.genebridges.com; see e.g. WO 99/29837, WO 01/04288, US 6,355,412). In detail the following stepwise changes were made:

Repair of leuB locus:

The E.coli CSPZ-2 has a TCG to TTG point mutation in the locus leuB which results in a Serine to Leucine amino acid exchange at Position 286 in the corresponding 3-isopropylmalate dehydrogenase making this enzyme defective. The strain is therefore not able to grow on cultivation medium not supplemented with L-Leucine. With an oligonucleotide encoding the wild-type Ser286 with flanking sequences the genomic mutation in strain CSPZ-2 was exchanged to generate the cured strain CSPZ-3 (CSPZ-2 $leuB^{+}$) which can grow on M9 medium plates supplemented with L-Proline but does not require L-Leucine.

Repair of proC (b0386) and proBA (b0242 and b0243) loci:

The strain CSPZ-3 still requires the supplementation of Proline to grow on M9 minimal medium. A PCR-amplification of the proC locus resulted in an approx. 2.4 kb fragment. For E.coli K12 (wild-type strain) a 1,024 bp fragment was expected (Blattner, F.R., et al., Science 277 (1997) 1453-1474). Sequencing of the PCR product revealed the disruption of the proC locus by IS186 (1,335 bp) transposition

into the genome. The proBA locus was characterized by PCR, but the amplification failed indicating the absence of the proBA operon.

A PCR product amplified from E.coli strain MG1655 genomic DNA was used for Red/ET recombination to cure the deficiency in the proC locus.

In a consecutive step the proBA operon was inserted via Red/ET recombination into the infA-serW intergenic region of the genome to restore prototrophic phenotype in CSPZ-4. The integration was verified by PCR amplifying and sequencing of the integration site and by plating on M9 minimal medium agar plates. The Proline prototrophic, auxotrophy cured strain was named CSPZ-5.

Repair of the trpE (b1264) locus:

DNA sequencing of the trpE locus of the parental strain CSPZ-2 revealed the deletion of 9 nucleotides resulting in the loss of Glu139, Glu 140 and Arg141. This mutation does not lead to a Tryptophan auxotrophic phenotype but may affect enzyme activity and lead to reduced growth. Therefor the mutated trpE locus was exchanged by the wild-type locus via Red/ET recombination. Successful restoration of the trpE gene was confirmed by sequencing and the resulting strain was named CSPZ-6.

Example 2

Making of the expression plasmids

Tetranectin-apolipoprotein A-I fusion polypeptide

The tetranectin-apolipoprotein A-I fusion polypeptide was prepared by recombinant means. The amino acid sequence of the expressed fusion polypeptide in N- to C-terminal direction is as follows:

- the amino acid methionine (M),
- a fragment of an interferon sequence that has the amino acid sequence of CDLPQTHSL (SEQ ID NO: 01),
- a GS linker,
- a hexa-histidine tag that has the amino acid sequence of HHHHHH (SEQ ID NO: 02),
- a GS linker,
- an IgA protease cleavage site that has the amino acid sequence of VVAPPAP (SEQ ID NO: 03), and

- a tetranectin-apolipoprotein A-I that has the amino acid sequence of SEQ ID NO: 04.

The tetranectin-apolipoprotein A-I fusion polypeptides as described above are precursor polypeptides from which the tetranectin-apolipoprotein A-I fusion polypeptides was released by enzymatic cleavage in vitro using IgA protease.

The precursor polypeptide encoding fusion gene was assembled with known recombinant methods and techniques by connection of appropriate nucleic acid segments. Nucleic acid sequences made by chemical synthesis were verified by DNA sequencing. The expression plasmid for the production of tetranectin-apolipoprotein A-I was prepared as follows.

Making of the E.coli expression plasmids

a) Plasmid 5816

Plasmid 4980 (4980-pBRori-URA3-LACI-SAC) is an expression plasmid for the expression of core-streptavidin in E.coli. It was generated by ligation of the 3142 bp long EcoRI/CelII-vector fragment derived from plasmid 1966 (1966-pBRori-URA3-LACI-T-repeat; reported in EP-B 1 422 237) with a 435 bp long core-streptavidin encoding EcoRI/CelII-fragment.

The core-streptavidin E.coli expression plasmid comprises the following elements:

- the origin of replication from vector pBR322 for replication in E.coli (corresponding to bp position 2517-3160 according to Sutcliffe, G., et al., Quant. Biol. 43 (1979) 77-90),
- the URA3 gene of Saccharomyces cerevisiae coding for orotidine 5'-phosphate decarboxylase (Rose, M. et al. Gene 29 (1984) 113-124) which allows plasmid selection by complementation of E.coli pyrF mutant strains (uracil auxotrophy),
- the core-streptavidin expression cassette comprising
  - the T5 hybrid promoter (T5-PN25/03/04 hybrid promoter according to Bujard, H., et al., Methods Enzymol. 155 (1987) 416-433 and Stueber, D., et al., Immunol. Methods IV (1990) 121-152) including a synthetic ribosomal binding site according to Stueber, D., et al. (see before),
  - the core-streptavidin gene,

- two bacteriophage-derived transcription terminators, the λ-T0 terminator (Schwarz, E., et al., Nature 272 (1978) 410-414) and the fd-terminator (Beck E. and Zink, B. Gene 1-3 (1981) 35-58),
- the lacI repressor gene from E.coli (Farabaugh P.J. Nature 274 (1978) 765-769).

The final expression plasmid 5816 for the expression of the tetranectin-apolipoprotein A-I precursor polypeptide was prepared by excising the core-streptavidin structural gene from plasmid 4980 using the singular flanking EcoRI and CelII restriction endonuclease cleavage site and inserting the EcoRI/CelII restriction site flanked nucleic acid encoding the precursor polypeptide into the 3142 bp long EcoRI/CelII-4980 vector fragment.

b) Plasmid 5830

Plasmid 5830 is identical to plasmid 5816 except that the codons encoding the tripeptide QKK are changed from caa aaa aag (plasmid 5816) to cag aag aag (plasmid 5830).

c) Plasmid 5836

The encoding fusion gene for expression of a shortened tetranectin-apolipoprotein A-I fusion protein is assembled with known recombinant methods and techniques by connection of appropriate nucleic acid segments. Nucleic acid sequences made by chemical synthesis are verified by DNA sequencing. The expression plasmid for the production of the fusion protein of SEQ ID NO: 06 can be prepared as follows:

Plasmid 4980 (4980-pBRori-URA3-LACI-SAC) is an expression plasmid for the expression of core-streptavidin in E.coli. It was generated by ligation of the 3142 bp long EcoRI/CelII-vector fragment derived from plasmid 1966 (1966-pBRori-URA3-LACI-T-repeat; reported in EP-B 1 422 237) with a 435 bp long core-streptavidin encoding EcoRI/CelII-fragment.

The core-streptavidin E.coli expression plasmid comprises the following elements:

- the origin of replication from vector pBR322 for replication in E.coli (corresponding to bp position 2517-3160 according to Sutcliffe, G., et al., Quant. Biol. 43 (1979) 77-90),
- the URA3 gene of Saccharomyces cerevisiae coding for orotidine 5'-phosphate decarboxylase (Rose, M., et al., Gene 29 (1984) 113-124) which

allows plasmid selection by complementation of E.coli ΔpyrF mutant strains (uracil auxotrophy),

- the core-streptavidin expression cassette comprising
  - the T5 hybrid promoter (T5-PN25/03/04 hybrid promoter according to Bujard, H., et al., Methods. Enzymol. 155 (1987) 416-433 and Stueber, D., et al., Immunol. Methods IV (1990) 121-152) including a synthetic ribosomal binding site according to Stueber, D., et al. (see before),
  - the core-streptavidin gene,
  - two bacteriophage-derived transcription terminators, the λ-T0 terminator (Schwarz, E., et al., Nature 272 (1978) 410-414) and the fd-terminator (Beck, E. and Zink, B., Gene 1-3 (1981) 35-58),
- the lacI repressor gene from E.coli (Farabaugh, P.J., Nature 274 (1978) 765-769).

The final expression plasmid 5836 for the expression of the shortened tetranectin-apolipoprotein A-I fusion protein can be prepared by excising the core-streptavidin structural gene from plasmid 4980 using the singular flanking EcoRI and CelII restriction endonuclease cleavage site and inserting the EcoRI/CelII restriction site flanked nucleic acid encoding the fusion protein into the 3142 bp long EcoRI/CelII-1 plasmid fragment.

d) Plasmid 3036

The expression plasmid 3036 for recombinant production of IgA-Protease is based on the vector OripBR-URA3-EK-IFN (see e.g. US 6,291,245). Plasmid 3036 differs from OripBR-URA3-EK-IFN by the presence of a lacI repressor gene and the target gene encoding the IgA protease protein. The lacI repressor gene was derived from plasmid pUHA1 (Stueber, D., et al., In: Immunological Methods IV (1990) 121-152). It was amplified by polymerase chain reaction (PCR) according to the method described by Mullis, K.B. and Faloona, F.A. (Methods Enzymology 155 (1987) 335-350), and cloned into plasmid 3036 precursor. The target gene encoding the IgA-protease protein from Neisseria gonorrhoeae was generated by PCR and cloned into the expression vector.

The IgA-Protease expression plasmid 3036 comprises the following elements:

- the origin of replication from vector pBR322 for replication in E.coli (corresponding to bp position 2517-3160 according to Sutcliffe, G., et al., Quant. Biol. 43 (1979) 77-90),

- the URA3 gene of Saccharomyces cerevisiae coding for orotidine 5'-phosphate decarboxylase (Rose, M., et al., Gene 29 (1984) 113-124) which allows plasmid selection by complementation of E.coli ΔpyrF mutant strains (uracil auxotrophy),
- the IgA-Protease expression cassette comprising
  - the T5 hybrid promoter (T5-PN25/03/04 hybrid promoter according to Bujard, H., et al., Methods. Enzymol. 155 (1987) 416-433 and Stueber, D., et al., Immunol. Methods IV (1990) 121-152) including a synthetic ribosomal binding site according to Stueber, D., et al. (see before),
  - Open-reading-frame of IgA Protease (Neisseria gonorrhoeae, GenBank Accession No. P09790),
  - two bacteriophage-derived transcription terminators, the λ-T0 terminator (Schwarz, E., et al., Nature 272 (1978) 410-414) and the fd-terminator (Beck, E. and Zink, B., Gene 1-3 (1981) 35-58),
- the lacI repressor gene from E.coli (Farabaugh, P.J., Nature 274 (1978) 765-769).

Example 3

Expression of a fusion polypeptide by cultivation on a chemically defined minimal growth medium that has been supplemented with the auxotrophy complementing amino acids leucine and proline

For the expression of the tetranectin-apolipoprotein A-I fusion protein as reported in WO 2012/028526 an E.coli host/vector system which enables an antibiotic-free plasmid selection by complementation of an E.coli auxotrophy (pyrF) was employed (see EP 0 972 838 and US 6,291,245).

The E.coli K12 strains CSPZ-2 (leuB, proC, trpE, thi-1, ΔpyrF) and CSPZ-6 (thi-1, ΔpyrF) were transformed by electroporation with the expression plasmid 5816 (see Example 2). The transformed E.coli cells were first grown at 37 °C on agar plates. A colony picked from this plate was transferred to a 3 mL roller culture and grown at 37 °C to an optical density of 1-2 (measured at 578 nm). Then 1000 µL culture were mixed with 1000 µL sterile 86%-glycerol and immediately frozen at -80 °C for long time storage.

The correct product expression of this clone was first verified in small scale shake flask experiments and analyzed with SDS-Page prior to the transfer to the 10 L fermenter.

Pre-cultivation:

For pre-cultivation a chemical defined medium (CDM) has been used:

NH4Cl 1.0 g/L, K2HPO4*3H2O 18.3 g/L, citrate 1.6 g/L, glycine 0.78 g/L, L-alanine 0.29 g/L, L-arginine 0.41 g/L, L-asparagine*H2O 0.37 g/L, L-aspartate 0.05 g/L, L-cysteine*HCl*H2O 0.05 g/L, L-histidine 0.05 g/L, L-isoleucine 0.31 g/L, L-leucine 0.38 g/L, L-lysine*HCl 0.40 g/L, L-methionine 0.27 g/L, L-phenylalanine 0.43 g/L, L-proline 0.36 g/L, L-serine 0.15 g/L, L-threonine 0.40 g/L, L-tryptophan 0.07 g/L, L-valine 0.33 g/L, L-tyrosine 0.51 g/L, L-glutamine 0.12 g/L, Na-L-glutamate*H2O 0.82 g/L, glucose*H2O 5.0 g/L, trace elements solution 5 mL/L, MgSO4*7H2O 0.86 g/L, thiamin*HCL 17.5 mg/L.

The trace elements solution contains MnSO4*H2O 1.28 g/1, ZnSO4*7H2O 1.70 g/L, H3BO3 0.30 g/L, (NH4)6Mo7O24*4H2O 0.18 g/L, CoC12*6H2O 0.25 g/L, CuSO4*5H2O 0.22 g/L, EDTA 0.75 g/L.

For pre-cultivation 300 mL of the CDM in a 1000 mL Erlenmeyer-flask with four baffles was inoculated with 0.9 mL out of a primary seed bank ampoule. The cultivation was performed on a rotary shaker for 9 hours at 37 °C and 170 rpm until an optical density (578 nm) of 7-13 was obtained. 100 g of this pre-cultivation was then transferred to the 10 L bioreactor to inoculate the batch medium.

Fermentation with amino acids L-Leucine and L-Proline (experiment no. 1 and experiment no. 2):

For fermentation in a 10 L Biostat C, DCU3 fermenter (Sartorius, Melsungen, Germany) the following batch medium was used: KH2PO4 4.73 g/L, (NH4)2HPO4 7.47 g/L, K2HPO4*3H2O 14.94 g/L, citrate 2.07 g/1, L-methionine 1.22 g/L, L-proline 4.08 g/L, L-leucine 2.57 g/L, ammonium ferric citrate 0.08 g/L, NaHCO3 0.82 g/L, trace elements solution 7.3 mL/L, MgSO4*7H2O 1.42 g/L, thiamine*HC1 20.9 mg/L, glucose*H2O 29.3 g/L, biotin 0.2 mg/L, 1.2 mL/L Synperonic 10% anti foam agent.

The trace elements solution contains FeSO4*7H2O 10 g/L, ZnSO4*7H2O 2.25 g/L, MnSO4*H2O 2.13 g/L, CuSO4*5H2O 1.0 g/L, CoC12*6H2O 0.42 g/L, (NH4)6Mo7O24*4H2O 0.3 g/L, H3BO3 0.50 g/L, thiamine-HCL 2.0 g/L solubilized in 1N HCl solution.

The feed 1 solution contained 700 g/L glucose*H2O, KH2PO4 8.43 g/L and K2HPO4*3H2O 22.38 g/L.

Feed 2 comprises 585 g/L L-proline and 150.3 g/L MgSO4*7H2O.

All components were dissolved in deionized water.

The alkaline solution for pH regulation was an aqueous 12.5 % (w/v) NH3 solution supplemented with 11.25 g/L L-methionine, 51.5 g/L L-proline and 56.25 g/L L-leucine (see WO 2012/028522).

Starting with 4.2 L sterile batch medium plus 100 mL inoculum from the pre-cultivation the batch fermentation was performed at 37 °C, pH 6.9 ± 0.2, 800 mbar back pressure and an initial aeration rate of 10 L/min. The relative value of dissolved oxygen (pO2) was kept at 50 % throughout the fermentation by increasing the stirrer speed up to 1500 rpm. After the initially supplemented glucose was depleted, indicated by a steep increase in dissolved oxygen values, the temperature was shifted to 25 °C and 15 minutes later the fermentation entered the fed-batch mode with the start of both feeds (50 and 13 g/h respectively). The rate of feed 2 is kept constant, while the rate of feed 1 is increased stepwise with a predefined feeding profile from 50 to finally 160 g/h within 8.5 hours. When carbon dioxide off gas concentration leveled above 2% the aeration rate was constantly increased from 10 to 20 L/min within 5 hours. The expression of recombinant tetranectin-apolipoprotein A-1 fusion protein was induced by the addition of 2.4 g IPTG at an optical density of 150.

At the end of fermentation the within the cytoplasm soluble expressed tetranectin-apolipoprotein A-1 fusion protein was transferred to insoluble protein aggregates, the so called inclusion bodies, with a heat step where the whole culture broth in the fermenter is heated to 50 °C for 1 hour before harvest (see e.g. EP-B 1 486 571). Thereafter, the content of the fermenter was centrifuged with a flow-through centrifuge (13,000 rpm, 13 L/h) and the harvested biomass was stored at -20 °C until further processing. The synthesized tetranectin-apolipoprotein A-1 fusion proteins were found exclusively in the insoluble cell debris fraction in the form of insoluble protein aggregates, so-called inclusion bodies (IBs).

Analysis of product formation:

Samples drawn from the fermenter, one prior to induction and the others at dedicated time points after induction of protein expression are analyzed with SDS-Polyacrylamide gel electrophoresis. From every sample the same amount of cells (OD_{target} = 5) are suspended in 5 mL PBS buffer and disrupted via sonication on ice. Then 100 µL of each suspension are centrifuged (15,000 rpm, 5 minutes) and each supernatant is withdrawn and transferred to a separate vial. This is to discriminate between soluble and insoluble expressed target protein. To each supernatant (=soluble) fraction 300 µL and to each pellet (=insoluble) fraction 400 µL of SDS sample buffer (Laemmli, U.K., Nature 227 (1970) 680-685) are added. Samples are heated for 15 minutes at 95 °C under intense mixing to solubilize and reduce all proteins in the samples. After cooling to room temperature 5 µL of each sample are transferred to a 4-20% TGX Criterion Stain Free polyacrylamide gel (Bio-Rad). Additionally 5 µL molecular weight standard (Precision Plus Protein Standard, Bio-Rad) and 3 amounts (0.3 µL, 0.6 µL and 0.9 µL) quantification standard with known product protein concentration (0.1 µg/µL) are positioned on the gel.

The electrophoresis was run for 60 min. at 200 V and thereafter the gel was transferred to a GelDOC EZ Imager (Bio-Rad) and processed for 5 min. with UV radiation. Gel images were analyzed using Image Lab analysis software (Bio-Rad). With the three standards a linear regression curve was calculated with a coefficient of >0.99 and thereof the concentrations of target protein in the original sample was calculated.

The activity of the purified and lipidated TN-ApoA1 was measured in an eight day radioactive efflux assay. Cells (THP-1) were differentiated by PMA (phorbol myristate acetate) to macrophages. These cells were loaded with acetylated LDL and ^{3}H-labeled cholesterol. The supernatant was discarded and cells were incubated for 5 hours with equilibration medium to remove non-specifically bound cholesterol. The lipidated TN-ApoA1 was added which enabled the export of the ^{3}H-labeled cholesterol out of the cells during the following 18 hours. Radioactivity was measured in the supernatant and in the cell lysate.

Results:

The above mentioned fermentation process was used to express tetranectin-apolipoprotein A-1 in the parental strain CSPZ-2 which is auxotrophic against

L-leucine and L-proline and in the auxotrophy cured strain CSPZ-6. Despite the latter strain does not need the feeding of the amino acids L-leucine and L-proline any more, the performance of this auxotrophy cured strain was tested with the supplemented fermentation medium for direct performance comparison to strain CSPZ-2.

The auxotrophy cured strain is growing faster than the parental strain despite the same medium and fermentation process was used (see Figure 1).

The auxotrophy cured strain is faster expressing more product than the parental strain despite the same medium and fermentation process was used (see Figure 2).

As can be seen from the concentration trends of the amino acids leucine and proline (see Figure 3 A and B) the auxotrophy cured strain can now metabolize L-leucine and L-proline due to intact metabolic pathways. Therefore the growth of the auxotrophy cured strain is faster under glucose limited cultivation conditions of the applied fed-batch process.

Surprisingly the auxotrophy cured strain had a significant better performance than the parental strain on the same chemical defined fermentation medium supplemented with the amino acids L-leucine and L-proline. The growth is accelerated due to the possibility to metabolize the contained amino acids and this may also improve product formation. The conclusion from this is to use a prototroph E.coli production strain.

Example 4

Expression of IgA protease by cultivation on a chemically defined minimal growth medium that has been supplemented with the auxotrophy complementing amino acids leucine and proline

For the expression of IgA-Protease (106 kDa) the E.coli host/vector system which enables an antibiotic-free plasmid selection by complementation of an E.coli auxotrophy (PyrF) was employed (see EP 0 972 838 and US 6,291,245).

The E.coli K12 strains CSPZ-2 (leuB, proC, trpE, thi-1, ΔpyrF) and CSPZ-6 (thi-1, ΔpyrF) were transformed by electroporation with the expression plasmid 3036. The transformed E.coli cells were first grown at 37 °C on agar plates. A colony picked from this plate was transferred to a 3 mL roller culture and grown at 37 °C to an optical density of 1-2 (measured at 578 nm). Then 1000 µL culture were mixed

with 1000 µL sterile 86%-glycerol and immediately frozen at -80 °C for long time storage. The correct product expression of this clone was first verified in small scale shake flask experiments and analyzed with SDS-Page prior to the transfer to the 10 L fermenter.

Pre-cultivation:

For pre-cultivation a chemical defined medium (CDM) has been used:

NH4Cl 1.0 g/L, K2HPO4*3H2O 18.3 g/L, citrate 1.6 g/L, glycine 0.78 g/L, L-alanine 0.29 g/L, L-arginine 0.41 g/L, L-asparagine*H2O 0.37 g/L, L-aspartate 0.05 g/L, L-cysteine*HCl*H2O 0.05 g/L, L-histidine 0.05 g/L, L-isoleucine 0.31 g/L, L-leucine 0.38 g/L, L-lysine*HCl 0.40 g/L, L-methionine 0.27 g/L, L-phenylalanine 0.43 g/L, L-proline 0.36 g/L, L-serine 0.15 g/L, L-threonine 0.40 g/L, L-tryptophan 0.07 g/L, L-valine 0.33 g/L, L-tyrosine 0.51 g/L, L-glutamine 0.12 g/L, Na-L-glutamate*H2O 0.82 g/L, glucose*H2O 6.0 g/L, trace elements solution 0.5 mL/L, MgSO4*7H2O 0.86 g/L, thiamin*HCl 17.5 mg/L.

The trace elements solution contains FeSO4*7H2O 10.0 g/L, ZnSO4 * 7H2O 2.25 g/L, MnSO4 * H2O 2.13 g/L, H3BO3 0.50 g/L, (NH4)6Mo7O24 * 4H2O 0.3 g/L, CoCl2*6H2O 0.42 g/L, CuSO4 * 5H2O 1.0 g/L dissolved in 0.5M HCl.

For pre-cultivation 220 ml of CDM in a 1000 mL Erlenmeyer-flask with four baffles was inoculated with 1.0 mL out of a primary seed bank ampoule. The cultivation was performed on a rotary shaker for 9 hours at 37 °C and 170 rpm until an optical density (578 nm) of 11-13 was obtained. The inoculum volume was calculated with $V_{inoc.}$ = 100 mL * 5 / OD_{PC} and is dependent on the optical density of the pre-cultivation to inoculate the batch medium of the 10 L bioreactor with equal amount of cells.

Fermentation with amino acids L-Leucine and L-Proline (experiment no. 3 and experiment no. 4):

For fermentation in a 10 L Biostat C, DCU3 fermenter (Sartorius, Melsungen, Germany) the same process as described in Example 3 was performed except that no heat step was conducted at the end of the fermentations.

Analysis of product formation:

Samples drawn from the fermenter, one prior to induction and the others at dedicated time points after induction of protein expression are analyzed with SDS-Polyacrylamide gel electrophoresis. From every sample the same amount of cells (OD_{target} = 10) are suspended in 5 mL PBS buffer and disrupted via sonication on ice. Then 100 µL of each suspension are centrifuged (15,000 rpm, 5 minutes) and each supernatant is withdrawn and transferred to a separate vial. This is to discriminate between soluble and insoluble expressed target protein. To each supernatant (= soluble) fraction 100 µL and to each pellet (= insoluble) fraction 200 µL of SDS sample buffer (Laemmli, U.K., Nature 227 (1970) 680-685) are added. Samples are heated for 15 min. at 95°C under intense mixing to solubilize and reduce all proteins in the samples. After cooling to room temperature 5 µL of each sample are transferred to a 4-20 % TGX Criterion Stain Free polyacrylamide gel (Bio-Rad). Additionally 5 µL molecular weight standard (Precision Plus Protein Standard, Bio-Rad) and 3 amounts (0.3 µL, 0.6 µL and 0.9 µL) quantification standard with known product protein concentration (0.1 µg/µL) are positioned on the gel.

The electrophoresis was run for 60 min. at 200 V and thereafter the gel was transferred the GelDOC EZ Imager (Bio-Rad) and processed for 5 min. with UV radiation. Gel images were analyzed using Image Lab analysis software (Bio-Rad). With the three standards a linear regression curve was calculated with a coefficient of >0.99 and thereof the concentrations of target protein in the original sample was calculated.

Results:

The above mentioned fermentation process was used to express IgA protease in the parental strain CSPZ-2 which is auxotrophic against L-Leucine and L-Proline and in the auxotrophy cured strain CSPZ-6. Despite the latter strain does not need the feeding of the amino acids L-Leucine and L-Proline any more, the performance of this auxotrophy cured strain on the supplemented fermentation medium for direct performance comparison to parental strain CSPZ-2 has been tested.

The growth of the auxotrophy cured strain on amino acid supplemented batch medium was significant faster compared to the parental strain.

Example 5

Cultivation of an auxotrophy cured E.coli strain and the wild-type strain MG1655 on a chemically defined minimal growth medium

One clear benefit of an auxotrophy cured strain is that no more expensive amino acids are needed to be fed during cultivation on chemical defined medium due to the prototrophic phenotype. This would dramatically reduce costs of goods for the process. To test an identical fermentation with the auxotrophy cured strain CSPZ-6 was conducted where simply the amino acids L-leucine and L-proline were excluded from the medium and feeds. To demonstrate that the auxotrophy cured strain has significant advantages over a wild-type E.coli strain which is also prototrophic growth and product formation of the K12 strain MG1655 (with deleted pyrF gene to fit to the antibiotic free selection system) within the same fermentation process was explored.

The E.coli K12 strains CSPZ-6 (thi-1, ΔpyrF) and MG1655 derivative (ΔpyrF, ΔompT) were transformed by electroporation with the expression plasmid 5816 (see Example 2). The transformed E.coli cells were first grown at 37 °C on agar plates. A colony picked from this plate was transferred to a 3 mL roller culture and grown at 37 °C to an optical density of 1-2 (measured at 578nm). Then 1000 µL culture were mixed with 1000 µL sterile 86%-glycerol and immediately frozen at -80 °C for long time storage.

The correct product expression of this clone was first verified in small scale shake flask experiments and analyzed with SDS-Page prior to the transfer to the 10 L fermenter.

Pre-cultivation:

For pre-cultivation a chemical defined medium (CDM) has been used:

NH4Cl 1.0 g/L, K2HPO4*3H2O 18.3 g/L, citrate 1.6 g/L, glycine 0.78 g/L, L-alanine 0.29 g/L, L-arginine 0.41 g/L, L-asparagine*H2O 0.37 g/L, L-aspartate 0.05 g/L, L-cysteine*HCl*H2O 0.05 g/L, L-histidine 0.05 g/L, L-isoleucine 0.31 g/L, L-leucine 0.38 g/L, L-lysine*HCl 0.40 g/L, L-methionine 0.27 g/L, L-phenylalanine 0.43 g/L, L-proline 0.36 g/L, L-serine 0.15 g/L, L-threonine 0.40 g/L, L-tryptophan 0.07 g/L, L-valine 0.33 g/L, L-tyrosine 0.51 g/L, L-glutamine 0.12 g/L, Na-L-glutamate*H2O 0.82 g/L, glucose*H2O 5.0 g/L, trace elements solution 5 mL/L, MgSO4*7H2O 0.86 g/L, thiamin*HCl 17.5 mg/L.

The trace elements solution contains MnSO4*H2O 1.28 g/L, ZnSO4*7H2O 1.70 g/L, H3BO3 0.30 g/L, (NH4)6Mo7O24*4H2O 0.18 g/L, CoC12*6H2O 0.25 g/L, CuSO4*5H2O 0.22 g/L, EDTA 0.75 g/L.

For pre-cultivation 300 mL of CDM medium in a 1000 mL Erlenmeyer-flask with four baffles was inoculated with 0.9 mL out of a primary seed bank ampoule. The cultivation was performed on a rotary shaker for 9 hours at 37 °C and 170 rpm until an optical density (578 nm) of 6-13 was obtained. 100 g of this pre-cultivation was then transferred to the 10 L bioreactor to inoculate the batch medium.

Fermentation without amino acids L-Leucine and L-Proline (experiment no. 5 and experiment no. 6):

For fermentation in a 10 L Biostat C, DCU3 fermenter (Sartorius, Melsungen, Germany) the following batch medium was used: KH2PO4 4.73 g/L, (NH4)2HPO4 7.47 g/L, K2HPO4*3H2O 14.94 g/L, citrate 2.07 g/L, L-methionine 1.22 g/L, ammonium ferric citrate 0.08 g/L, NaHCO3 0.82 g/L, trace elements solution 7.3 mL/L, MgSO4*7H2O 1.42 g/L, thiamine*HCl 20.9 mg/L, glucose*H2O 29.3 g/L, biotin 0.2 mg/L, 1.2 mL/L Synperonic 10% anti foam agent.

The trace elements solution contains FeSO4*7H2O 10 g/L, ZnSO4*7H2O 2.25 g/L, MnSO4*H2O 2.13 g/L, CuSO4*5H2O 1.0 g/L, CoC12*6H2O 0.42 g/L, (NH4)6Mo7O24*4H2O 0.3 g/L, H3BO3 0.50 g/L, thiamine-HCl 2.0 g/L solubilized in 1N HCl solution.

The feed 1 solution contained 700 g/L glucose*H2O, KH2PO4 8.43 g/L and K2HPO4*3H2O 22.38 g/L.

Feed 2 comprises only 150.3 g/L MgSO4*7H2O.

All components were dissolved in deionized water.

The alkaline solution for pH regulation was an aqueous 12.5 % (w/v) NH3 solution supplemented with 11.25 g/L L-methionine.

Starting with 4.2 L sterile batch medium plus 100 mL inoculum from the pre-cultivation the batch fermentation was performed at 37 °C, pH 6.9 ± 0.2, 800 mbar back pressure and an initial aeration rate of 10 L/min. The relative value of dissolved oxygen (pO2) was kept at 50 % throughout the fermentation by increasing the stirrer speed up to 1500 rpm. After the initially supplemented

glucose was depleted, indicated by a steep increase in dissolved oxygen values, the temperature was shifted to 25 °C and 15 minutes later the fermentation entered the fed-batch mode with the start of both feeds (50 and 13 g/h respectively). The rate of feed 2 is kept constant, while the rate of feed 1 is increased stepwise with a predefined feeding profile from 50 to finally 160 g/h within 8.5 hours. When carbon dioxide off gas concentration leveled above 2% the aeration rate was constantly increased from 10 to 20 L/min within 5 hours. The expression of recombinant tetranectin-apolipoprotein A-1 fusion protein was induced by the addition of 2.4 g IPTG at an optical density of 150. At the end of fermentation the within the cytoplasm soluble expressed tetranectin-apolipoprotein A-I is transferred to insoluble protein aggregates, the so called inclusion bodies, with a heat step where the whole culture broth in the fermenter is heated to 50 °C for one hour before harvest (see e.g. EP-B 1 486 571). Thereafter, the content of the fermenter was centrifuged with a flow-through centrifuge (13,000 rpm, 13 l/h) and the harvested biomass was stored at -20 °C until further processing. The synthesized tetranectin-apolipoprotein A-I fusion proteins were found exclusively in the insoluble cell debris fraction in the form of insoluble protein aggregates, so-called inclusion bodies (IBs).

Analysis of product formation:

Samples drawn from the fermenter, one prior to induction and the others at dedicated time points after induction of protein expression are analyzed with SDS-Polyacrylamide gel electrophoresis. From every sample the same amount of cells (OD_{target} = 5) are suspended in 5 mL PBS buffer and disrupted via sonication on ice. Then 100 µL of each suspension are centrifuged (15,000 rpm, 5 minutes) and each supernatant is withdrawn and transferred to a separate vial. This is to discriminate between soluble and insoluble expressed target protein. To each supernatant (=soluble) fraction 300 µL and to each pellet (= insoluble) fraction 400 µL of SDS sample buffer (Laemmli, U.K., Nature 227 (1970) 680-685) are added. Samples are heated for 15 minutes at 95°C under intense mixing to solubilize and reduce all proteins in the samples. After cooling to room temperature 5 µL of each sample are transferred to a 4-20% TGX Criterion Stain Free polyacrylamide gel (Bio-Rad). Additionally 5 µL molecular weight standard (Precision Plus Protein Standard, Bio-Rad) and 3 amounts (0.3 µL, 0.6 µL and 0.9 µL) quantification standard with known product protein concentration (0.1 µg/µL) are positioned on the gel.

The electrophoresis was run for 60 min. at 200 V and thereafter the gel was transferred the GelDOC EZ Imager (Bio-Rad) and processed for 5 min. with UV radiation. Gel images were analyzed using Image Lab analysis software (Bio-Rad). With the three standards a linear regression curve was calculated with a coefficient of >0.99 and thereof the concentrations of target protein in the original sample was calculated.

The activity of the purified and lipidated TN-ApoA1 was measured in an eight day radioactive efflux assay. Cells (THP-1) were differentiated by PMA (phorbol myristate acetate) to macrophages. These cells were loaded with acetylated LDL and ^{3}H-labeled cholesterol. The supernatant was discarded and cells were incubated for 5 hours with equilibration medium to remove non-specifically bound cholesterol. The lipidated TN-ApoA1 was added which enabled the export of the ^{3}H-labeled cholesterol out of the cells during the following 18 hours. Radioactivity was measured in the supernatant and in the cell lysate.

Results:

The above mentioned fermentation process was used to express tetranectin-apolipoprotein A-I in the auxotrophy cured strain CSPZ-6 and the prototrophic MG1655 derivative strain representing the K12 wild-type strain MG1655. Both strains do not need the feeding of the amino acids L-leucine and L-proline which where consequently excluded from the medium and feeds. This distinctly reduces cost of goods for the process.

Both, the auxotrophy cured and the wild-type strain are growing faster on medium without the amino acids L-leucine and L-proline due to the lack of growth inhibitory effects of higher concentrations of L-leucine (see Example 3). Final optical density of the auxotrophy cured strain is well improved in this medium lacking the amino acids, while the growth of the wild-type strain is significant reduced after induction of product formation and in the late phase of fermentation (see Figure 4).

Despite the product formation of the auxotrophy cured strain in the first few hours after inductor addition is faster in the fermentation medium supplemented with amino acids the final yield is nearly the same compared to the fermentation without amino acids addition (see Figure 5). Therefore the leave of the amino acids from the medium saves significant costs but does not reduce product yields. The

prototroph wild-type E.coli MG1655 derivative strain has significant deficiencies in yields of growth and product.

Summary:

The auxotrophy cured strain showed a better performance than a prototroph wild-type E.coli strain on the same chemical defined fermentation medium even if the amino acids L-leucine and L-proline are not supplemented. Therefore it is advantageous to cure high producing E.coli strains from their amino acid auxotrophies in order to allow a cultivation on chemically defined minimal growth medium and profit from the advantages of using such a chemically defined minimal growth medium instead of using a prototrophic wild-type E.coli strain like MG1655 or the closely related W3110 (Vijayendran, C., et al., J. Biotechnol. 128 (2007) 747-761) as can further be seen in Example 6.

Example 6

Cultivation of an auxotrophy cured E.coli strain and the wild-type strain W3110 on a chemically defined minimal growth medium

To demonstrate that the auxotrophy cured strain CSPZ-6 has significant advantages over the prototrophic wild-type E.coli strain W3110 (with deleted pyrF gene to fit to the antibiotic free selection system) growth and product formation within the same fermentation process was explored.

The E.coli K12 strains CSPZ-6 (thi-1, ΔpyrF) and 66C5 (= W3110 ΔpyrF derivate) were transformed by electroporation with the expression plasmid 5830 (see Example 2). The transformed E.coli cells were first grown at 37 °C on agar plates. A colony picked from this plate was transferred to a 3 mL roller culture and grown at 37 °C to an optical density of 1-2 (measured at 578nm). Then 1000 µL culture were mixed with 1000 mL sterile 86%-glycerol and immediately frozen at -80 °C for long time storage.

The correct product expression of this clone was first verified in small scale shake flask experiments and analyzed with SDS-Page prior to the transfer to the 10 L fermenter.

Pre-cultivation:

For pre-cultivation a chemical defined medium (CDM) has been used:

NH4Cl 1.0 g/L, K2HPO4*3H2O 18.3 g/L, citrate 1.6 g/L, glycine 0.78 g/L, L-alanine 0.29 g/L, L-arginine 0.41 g/L, L-asparagine*H2O 0.37 g/L, L-aspartate 0.05 g/L, L-cysteine*HCl*H2O 0.05 g/L, L-histidine 0.05 g/L, L-isoleucine 0.31 g/L, L-leucine 0.38 g/L, L-lysine*HCl 0.40 g/L, L-methionine 0.27 g/L, L-phenylalanine 0.43 g/L, L-proline 0.36 g/L, L-serine 0.15 g/L, L-threonine 0.40 g/L, L-tryptophan 0.07 g/L, L-valine 0.33 g/L, L-tyrosine 0.51 g/L, L-glutamine 0.12 g/L, Na-L-glutamate*H2O 0.82 g/L, glucose*H2O 5.0 g/L, trace elements solution 5 mL/L, MgSO4*7H2O 0.86 g/L, thiamin*HCl 17.5 mg/L.

The trace elements solution contains FeSO4*7H2O 10.0 g/L, ZnSO4*7H2O 2.25 g/L, MnSO4*H2O 2.13 g/L, H3BO3 0.50 g/L, (NH4)6Mo7O24*4H2O 0.3 g/L, CoC12*6H2O 0.42 g/L, CuSO4*5H2O 1.0 g/L dissolved in 0.5 M HCl.

For pre-fermentation 300 ml of CDM-medium in a 1000 ml Erlenmeyer-flask with four baffles was inoculated with 0.9 ml out of a primary seed bank ampoule. The cultivation was performed on a rotary shaker for 8 hours at 32°C and 170 rpm until an optical density (578 nm) of 4-9 was obtained. The inoculum volume was calculated with $V_{inoc.}$ = 100 ml * 5 / ODPC and is dependent on the optical density of the pre cultivation to inoculate the batch medium of the 10L bioreactor with equal amount of cells.

Fermentation without amino acids L-Leucine and L-Proline (experiment no. 7 and experiment no. 8):

For fermentation in a 10 L Biostat C, DCU3 fermenter (Sartorius, Melsungen, Germany) the following batch medium was used: KH2PO4 1.58 g/L, (NH4)2HPO4 7.47 g/L, K2HPO4*3H2O 13.32 g/L, citrate 2.07 g/L, L-methionine 1.22 g/L, NaHCO3 0.82 g/L, trace elements solution 7.3 mL/L, MgSO4*7H2O 0.99 g/L, thiamine*HCl 20.9 mg/L, glucose*H2O 29.3 g/L, biotin 0.2 mg/L, 1.2 mL/L Synperonic 10 % anti foam agent.

The trace elements solution contains FeSO4*7H2O 10 g/L, ZnSO4*7H2O 2.25 g/L, MnSO4*H2O 2.13 g/L, CuSO4*5H2O 1.0 g/L, CoCl2*6H2O 0.42 g/L, (NH4)6Mo7O24 * 4H2O 0.3 g/L, H3BO3 0.50 g/L solubilized in 0.5M HCl solution.

The feed 1 solution contained 700 g/L glucose*H2O, 12.3 g/L MgSO4*7H2O and 0.1 g/L FeSO4*7H2O.

Feed 2 comprisesKH2PO4 52.7 g/L, K2HPO4*3H2O 139.9 g/L and (NH4)2HPO4 132.1 g/L.

All components were dissolved in deionized water.

The alkaline solution for pH regulation was an aqueous 12.5 % (w/v) NH3 solution supplemented with 11.25 g/L L-methionine.

Starting with 4.2 L sterile batch medium plus 100 mL inoculum from the pre cultivation the batch fermentation was performed at 31 °C, pH 6.9 ± 0.2, 800 mbar back pressure and an initial aeration rate of 10 L/min. The relative value of dissolved oxygen (pO2) was kept at 50 % throughout the fermentation by increasing the stirrer speed up to 1500 rpm. After the initially supplemented glucose was depleted, indicated by a steep increase in dissolved oxygen values, the temperature was shifted to 25 °C and 15 minutes later the fermentation entered the fed-batch mode with the start of both feeds (60 and 14 g/h respectively). The rate of feed 2 is kept constant, while the rate of feed 1 is increased stepwise with a predefined feeding profile from 60 to finally 160 g/h within 7 hours. When carbon dioxide off gas concentration leveled above 2 % the aeration rate was constantly increased from 10 to 20 L/min within 5 hours. The expression of recombinant tetranectin-apolipoprotein A-I fusion protein was induced by the addition of 2.4 g IPTG at an optical density of 120. At the end of fermentation the within the cytoplasm soluble expressed tetranectin-apolipoprotein A-I is transferred to insoluble protein aggregates, the so called inclusion bodies, with a heat step where the whole culture broth in the fermenter is heated to 50 °C for 1 hour before harvest (see e.g. EP-B 1 486 571). Thereafter, the content of the fermenter was centrifuged with a flow-through centrifuge (13,000 rpm, 13 L/h) and the harvested biomass was stored at -20 °C until further processing. The synthesized tetranectin-apolipoprotein A-I fusion proteins were found exclusively in the insoluble cell debris fraction in the form of insoluble protein aggregates, so-called inclusion bodies (IBs).

Analysis of product formation:

Samples drawn from the fermenter, one prior to induction and the others at dedicated time points after induction of protein expression are analyzed with SDS-

Polyacrylamide gel electrophoresis. From every sample the same amount of cells (OD_{target} = 5) are suspended in 5 mL PBS buffer and disrupted via sonication on ice. Then 100 µL of each suspension are centrifuged (15,000 rpm, 5 minutes) and each supernatant is withdrawn and transferred to a separate vial. This is to discriminate between soluble and insoluble expressed target protein. To each supernatant (= soluble) fraction 300 µL and to each pellet (= insoluble) fraction 400 µL of SDS sample buffer (Laemmli, U.K., Nature 227 (1970) 680-685) are added. Samples are heated for 15 minutes at 95 °C under intense mixing to solubilize and reduce all proteins in the samples. After cooling to room temperature 5 µL of each sample are transferred to a 4-20 % TGX Criterion Stain Free polyacrylamide gel (Bio-Rad). Additionally 5 µL molecular weight standard (Precision Plus Protein Standard, Bio-Rad) and 3 amounts (0.3 µL, 0.6 µL and 0.9 µL) quantification standard with known product protein concentration (0.1 µg/µL) are positioned on the gel.

The electrophoresis was run for 60 min. at 200 V and thereafter the gel was transferred the GelDOC EZ Imager (Bio-Rad) and processed for 5 min. with UV radiation. Gel images were analyzed using Image Lab analysis software (Bio-Rad). With the three standards a linear regression curve was calculated with a coefficient of >0.99 and thereof the concentrations of target protein in the original sample was calculated.

The activity of the purified and lipidated TN-ApoA1 was measured in an eight day radioactive efflux assay. Cells (THP-1) were differentiated by PMA (phorbol myristate acetate) to macrophages. These cells were loaded with acetylated LDL and ^{3}H-labeled cholesterol. The supernatant was discarded and cells were incubated for 5 hours with equilibration medium to remove non-specifically bound cholesterol. The lipidated TN-ApoA1 was added which enabled the export of the ^{3}H-labeled cholesterol out of the cells during the following 18 hours. Radioactivity was measured in the supernatant and in the cell lysate.

Results:

The above mentioned fermentation process was used to express tetranectin-apolipoprotein A-I in the auxotrophy cured strain CSPZ-6 and the prototrophic strain 66C5 representing the K12 wild-type strain W3110. Both strains do not need the feeding of the amino acids L-leucine and L-proline which where consequently excluded from the medium and feeds. This reduces cost of goods for the process.

Surprisingly the wild-type strain 66C5 despite being prototrophic and inoculated with the equal amount of cells grew extremely slow from the start when compared to the auxotrophy cured strain CSPZ-6 on the same chemically defined medium and under the same cultivation conditions (see Figure 6). As a consequence the glucose feed rates had to be adapted to avoid overfeeding and glucose accumulation in the medium. Also the final optical densities differ greatly between both strains. While the auxotrophy cured strain reached more than 300 the wild-type strain only obtained 143 and fermentation was terminated due to growth stagnation. The fermentation with the strain 66C5 was repeated with elevated temperatures (experiment no. 9) for batch phase (37 °C) and fed-batch phase (30°C), but growth yield could not be improved very much (see Figure 7).

Because the growth of the wild-type strain 66C5 was way behind the growth of the auxotrophy cured strain CSPZ-6 when cultivated on the same chemically defined medium and under the same conditions, also product formation was much lower (see Figure 8). While the auxotrophy cured strain finally obtained 22.6 g/L after 31 hours of expression the fermentation of wild-type strain 66C5 was terminated after 38 hours of cultivation due to stagnation of growth and consequently product yield was quantified at only 2.3 g/L. Also the specific productivity was lower (21.9 mg/OD vs. 71.1 mg/OD). The fermentation with the wild-type strain 66C5 was repeated with elevated temperatures for batch phase (37°C) and fed-batch phase (30°C), but product yield could only be improved to 6.9 g/L (see Figure 9). The prototroph wild-type E.coli strain has significant deficiencies in direct comparison with the auxotrophy cured strain.

Summary:

The auxotrophy cured strain CSPZ-6 had a much better performance than a prototroph wild-type E.coli strain W3110 derivate on the same chemical defined fermentation medium without the supplementation of other amino acids than methionine. Therefore it is advantageous to cure E.coli strains from their amino acids auxotrophies in order to use a chemically defined minimal medium for cultivation and to profit from the advantages of using such a medium instead of using a prototrophic wild-type E.coli strain, such as for example MG1655 or W3110.

Example 7

Cultivation of auxotrophy cured strain CSPZ-6 and the prototrophic wild-type strain BL21 ΔpyrF derivate on a chemically defined medium

To demonstrate that the auxotrophy cured strain CSPZ-6 has significant advantages over the prototrophic wild-type E.coli B strain BL21 (with deleted pyrF gene to fit to the antibiotic free selection system, named CSPZ-14) the growth and product formation within the same fermentation process has been explored.

The E.coli K12 strain CSPZ-6 (thi-1, ΔpyrF) and the E.coli B strain CSPZ-14 (= BL21 ΔpyrF derivate) were transformed by electroporation with the plasmid no. 5836 (see Example 2) to express a shortened tetranectin-apolipoprotein A-I fusion protein derivate. The transformed E.coli cells were first grown at 37 °C on agar plates. A colony picked from this plate was transferred to a 3 mL roller culture and grown at 37 °C to an optical density of 1-2 (measured at 578 nm). Then 1000 µL culture were mixed with 1000 µL sterile 86%-glycerol and immediately frozen at -80 °C for long time storage. The correct product expression of this clone was first verified in small scale shake flask experiments and analyzed with SDS-Page prior to the transfer to the 10 L fermenter.

Pre-cultivation:

For pre-cultivation a chemical defined medium (CDM) has been used:

NH4Cl 1.0 g/L, K2HPO4*3H2O 18.3 g/L, citrate 1.6 g/L, glycine 0.78 g/L, L-alanine 0.29 g/L, L-arginine 0.41 g/L, L-asparagine*H2O 0.37 g/L, L-aspartate 0.05 g/L, L-cysteine*HCl*H2O 0.05 g/L, L-histidine 0.05 g/L, L-isoleucine 0.31 g/L, L-leucine 0.38 g/L, L-lysine*HCl 0.40 g/L, L-methionine 0.27 g/L, L-phenylalanine 0.43 g/L, L-proline 0.36 g/L, L-serine 0.15 g/L, L-threonine 0.40 g/L, L-tryptophan 0.07 g/L, L-valine 0.33 g/L, L-tyrosine 0.51 g/L, L-glutamine 0.12 g/L, Na-L-glutamate*H2O 0.82 g/L, glucose*H2O 6.0 g/L, trace elements solution 0.5 mL/L, MgSO4*7H2O 0.86 g/L, thiamin*HCl 17.5 mg/L.

The trace elements solution contains FeSO4*7H2O 10.0 g/L, ZnSO4 * 7H2O 2.25 g/L, MnSO4 * H2O 2.13 g/L, H3BO3 0.50 g/L, (NH4)6Mo7O24 * 4H2O 0.3 g/L, CoCl2*6H2O 0.42 g/L, CuSO4 * 5H2O 1.0 g/L dissolved in 0.5M HCl.

For pre-cultivation 300 ml of CDM in a 1000 mL Erlenmeyer-flask with four baffles was inoculated with 0.9 mL out of a primary seed bank ampoule. The

cultivation was performed on a rotary shaker for 8 hours at 32 °C and 170 rpm until an optical density (578 nm) of 4-9 was obtained. 100 g of this pre-cultivation was then transferred to the 10 L bioreactor to inoculate the batch medium.

Fermentation without amino acids L-leucine and L-proline (experiment no. 10 and experiment no. 11):

For fermentation in a 10 L Biostat C, DCU3 fermenter (Sartorius, Melsungen, Germany) the following batch medium was used:

KH_2PO_4 1.58 g/L, $(NH_4)_2HPO_4$ 7.47 g/L, K2HPO4*3H2O 13.32 g/L, citrate 2.07 g/L, L-methionine 1.22 g/L, $NaHCO_3$ 0.82 g/L, trace elements solution 7.3 mL/L, $MgSO_4*7H_2O$ 0.99 g/L, thiamine*HCl 20.9 mg/L, glucose*H_2O 29.3 g/L, biotin 0.2 mg/L, 1.2 mL/L Synperonic 10 % anti foam agent.

The trace elements solution contains FeSO4*7H2O 10 g/L, ZnSO4*7H2O 2.25 g/L, MnSO4*H2O 2.13 g/L, CuSO4*5H2O 1.0 g/L, CoCl2*6H2O 0.42 g/L, (NH4)6Mo7O24*4H2O 0.3 g/L, H3BO3 0.50 g/L solubilized in 0.5M HCl solution.

The feed 1 solution contained 700 g/L glucose*H2O, 7.4 g/L $MgSO_4*7H_2O$ and 0.1 g/L FeSO4*7H2O.

Feed 2 comprises KH_2PO_4 52.7 g/L, K2HPO4*3H2O 139.9 g/L and (NH4)2HPO4 66.0 g/L.

All components were dissolved in deionized water.

The alkaline solution for pH regulation was an aqueous 12.5 % (w/v) NH_3 solution supplemented with 11.25 g/L L-methionine.

Starting with 4.2 L sterile batch medium plus 100 mL inoculum from the pre cultivation the batch fermentation was performed at 31 °C, pH 6.9 ± 0.2, 800 mbar back pressure and an initial aeration rate of 10 L/min. The relative value of dissolved oxygen (pO2) was kept at 50 % throughout the fermentation by increasing the stirrer speed up to 1500 rpm. After the initially supplemented glucose was depleted, indicated by a steep increase in dissolved oxygen values, the temperature was shifted to 25 °C and 15 minutes later the fermentation entered the fed-batch mode with the start of both feeds (60 and 14 g/h respectively). The rate of feed 2 is kept constant, while the rate of feed 1 is increased stepwise with a

predefined feeding profile from 60 to finally 160 g/h within 7 hours. When carbon dioxide off gas concentration leveled above 2 % the aeration rate was constantly increased from 10 to 20 L/min within 5 hours. The expression of recombinant tetranectin-apolipoprotein A-I fusion protein was induced by the addition of 2.4 g IPTG at an optical density of approx. 150. At the end of fermentation the within the cytoplasm soluble expressed tetranectin-apolipoprotein A-I is transferred to insoluble protein aggregates, the so called inclusion bodies, with a heat step where the whole culture broth in the fermenter is heated to 50 °C for 1 hour before harvest (see e.g. EP-B 1 486 571). Thereafter, the content of the fermenter was centrifuged with a flow-through centrifuge (13,000 rpm, 13 L/h) and the harvested biomass was stored at -20 °C until further processing. The synthesized tetranectin-apolipoprotein A-I fusion proteins were found exclusively in the insoluble cell debris fraction in the form of insoluble protein aggregates, so-called inclusion bodies (IBs).

Analysis of product formation:

Samples drawn from the fermenter, one prior to induction and the others at dedicated time points after induction of protein expression are analyzed with SDS-Polyacrylamide gel electrophoresis. From every sample the same amount of cells (OD_{target} = 5) are suspended in 5 mL PBS buffer and disrupted via sonication on ice. Then 100 µL of each suspension are centrifuged (15,000 rpm, 5 minutes) and each supernatant is withdrawn and transferred to a separate vial. This is to discriminate between soluble and insoluble expressed target protein. To each supernatant (= soluble) fraction 300 µL and to each pellet (= insoluble) fraction 400 µL of SDS sample buffer (Laemmli, U.K., Nature 227 (1970) 680-685) are added. Samples are heated for 15 minutes at 95 °C under intense mixing to solubilize and reduce all proteins in the samples. After cooling to room temperature 5 µL of each sample are transferred to a 4-20 % TGX Criterion Stain Free polyacrylamide gel (Bio-Rad). Additionally 5 µL molecular weight standard (Precision Plus Protein Standard, Bio-Rad) and 3 amounts (0.3 µL, 0.6 µL and 0.9 µL) quantification standard with known product protein concentration (0.1 µg/µL) are positioned on the gel.

The electrophoresis was run for 60 min. at 200 V and thereafter the gel was transferred the GelDOC EZ Imager (Bio-Rad) and processed for 5 min. with UV radiation. Gel images were analyzed using Image Lab analysis software (Bio-Rad). With the three standards a linear regression curve was calculated with a coefficient

of >0.99 and thereof the concentrations of target protein in the original sample was calculated.

The activity of the purified and lipidated shortened TN-ApoA1 was measured in an eight day radioactive efflux assay. Cells (THP-1) were differentiated by PMA (phorbol myristate acetate) to macrophages. These cells were loaded with acetylated LDL and ^{3}H-labeled cholesterol. The supernatant was discarded and cells were incubated for 5 hours with equilibration medium to remove non-specifically bound cholesterol. The lipidated shortened TN-ApoA1 was added which enabled the export of the ^{3}H-labeled cholesterol out of the cells during the following 18 hours. Radioactivity was measured in the supernatant and in the cell lysate.

Results:

The above mentioned fermentation process was used to express a shortened tetranectin-apolipoprotein A-I fusion protein in the auxotrophy cured strain CSPZ-6 and in the prototrophic strain CSPZ-14 representing the E.coli B wild-type strain BL21. Both strains do not need the feeding of the amino acids L-leucine and L-proline which where consequently excluded from the medium and feeds. This reduces cost of goods for the process.

Surprisingly the wild-type strain and BL21 derivate CSPZ-14, despite being prototrophic and the fermentation was inoculated with an equal amount of cells, grew significantly slower from the start when compared to the auxotrophy cured strain CSPZ-6 on the same chemically defined medium and under the same cultivation conditions (see Figure 10). As a consequence the glucose feeding started 1.2 hours later, but the increase of the feed rate followed the same profile as in the comparative fermentation. The final optical densities differ greatly between both strains. While the auxotrophy cured strain reached an optical density of 266 before the final heat step the optical density of the wild-type strain was decreasing form a maximum at hour 33 to only 143 at the end of the fermentation (hour 47).

Product formation was induced by the addition of 2.4 g IPTG at an optical density of approx. 150 in both cultivations. Due to the reduced growth of the strain CSPZ-14 this OD was reached at hour 22.2 in comparison to hour 19.5 with strain CSPZ-6 when cultivated on the same chemically defined medium and under the same conditions. Despite that product formation rate of CSPZ-14 was significant lower and therefore the final yield reached only 9.6 g/L after 23 hours of expression (see

Figure 11). In comparison to that the auxotrophy cured strain yielded in 33.8 g/L fusion protein after 24 hours of induced expression. Also the specific productivity was lower (67.1 mg/OD vs. 135.7 mg/OD). The prototroph wild-type E.coli strain has significant deficits in direct comparison with the auxotrophy cured strain.

Summary:

The auxotrophy cured strain CSPZ-6 had a much better performance than a prototroph wild-type E.coli strain CSPZ-14 (BL21 ΔpyrF derivate) on the same chemical defined fermentation medium without the supplementation of other amino acids than methionine. Therefore it is advantageous to cure E.coli strains from their amino acids auxotrophies in order to culture them on chemically defined minimal medium and profit from the advantages of using such a medium instead of using a prototrophic wild-type E.coli strain, such as for example MG1655, W3110 or BL21.

Example 8

Cultivation of auxotrophy cured strain CSPZ-6 and the prototrophic wild-type strain MG1655 on a chemically defined medium

To demonstrate that the auxotrophy cured strain CSPZ-6 has significant advantages over the prototrophic wild-type E.coli K12 strain MG1655 (with deleted pyrF gene to fit to the antibiotic free selection system, named CSPZ-9) growth and IgA-protease product formation within the same fermentation process between the two strains has been explored.

The E.coli K12 strains CSPZ-6 (thi-1, ΔpyrF) and CSPZ-9 (= MG1655 ΔpyrF derivate) were transformed by electroporation with the plasmid 3036 to express IgA-protease. The transformed E.coli cells were first grown at 37 °C on agar plates. A colony picked from this plate was transferred to a 3 mL roller culture and grown at 37 °C to an optical density of 1-2 (measured at 578 nm). Then 1000 µL culture were mixed with 1000 µL sterile 86%-glycerol and immediately frozen at -80 °C for long time storage. The correct product expression of this clone was first verified in small scale shake flask experiments and analyzed with SDS-Page prior to the transfer to the 10 L fermenter.

Pre-cultivation:

For pre-cultivation a chemical defined medium (CDM) has been used:

NH4Cl 1.0 g/L, K2HPO4*3H2O 18.3 g/L, citrate 1.6 g/L, glycine 0.78 g/L, L-alanine 0.29 g/L, L-arginine 0.41 g/L, L-asparagine*H2O 0.37 g/L, L-aspartate 0.05 g/L, L-cysteine*HCl*H2O 0.05 g/L, L-histidine 0.05 g/L, L-isoleucine 0.31 g/L, L-leucine 0.38 g/L, L-lysine*HCl 0.40 g/L, L-methionine 0.27 g/L, L-phenylalanine 0.43 g/L, L-proline 0.36 g/L, L-serine 0.15 g/L, L-threonine 0.40 g/L, L-tryptophan 0.07 g/L, L-valine 0.33 g/L, L-tyrosine 0.51 g/L, L-glutamine 0.12 g/L, Na-L-glutamate*H2O 0.82 g/L, glucose*H2O 6.0 g/L, trace elements solution 0.5 mL/L, MgSO4*7H2O 0.86 g/L, thiamin*HCl 17.5 mg/L.

The trace elements solution contains FeSO4*7H2O 10.0 g/L, ZnSO4 * 7H2O 2.25 g/L, MnSO4 * H2O 2.13 g/L, H3BO3 0.50 g/L, (NH4)6Mo7O24 * 4H2O 0.3 g/L, CoCl2*6H2O 0.42 g/L, CuSO4 * 5H2O 1.0 g/L dissolved in 0.5M HCl.

For pre-cultivation 220 ml of CDM in a 1000 mL Erlenmeyer-flask with four baffles was inoculated with 0.9 mL out of a primary seed bank ampoule. The cultivation was performed on a rotary shaker for 8 hours at 37 °C and 170 rpm until an optical density (578 nm) of 10-12 was obtained. To inoculate the batch medium of the 10 L bioreactor with equal amount of cells, the inoculum volume was calculated with $V_{inoc.}$ = 100 mL * 5 / OD_{PC} and is therefore dependent on the optical density of the pre cultivation.

Fermentation without amino acids L-leucine and L-proline (experiment no. 12 and experiment no. 13):

For fermentation in a 10 L Biostat C, DCU3 fermenter (Sartorius, Melsungen, Germany) the following batch medium was used:

KH_2PO_4 1.58 g/L, $(NH_4)_2HPO_4$ 7.47 g/L, K2HPO4*3H2O 13.32 g/L, citrate 2.07 g/L, L-methionine 1.22 g/L, $NaHCO_3$ 0.82 g/L, trace elements solution 7.3 mL/L, $MgSO_4*7H_2O$ 0.99 g/L, thiamine*HCl 20.9 mg/L, glucose*H_2O 29.3 g/L, biotin 0.2 mg/L, 1.2 mL/L Synperonic 10 % anti foam agent.

The trace elements solution contains FeSO4*7H2O 10 g/L, ZnSO4*7H2O 2.25 g/L, MnSO4*H2O 2.13 g/L, CuSO4*5H2O 1.0 g/L, CoCl2*6H2O 0.42 g/L, (NH4)6Mo7O24*4H2O 0.3 g/L, H3BO3 0.50 g/L solubilized in 0.5 M HCl solution.

The feed 1 solution contained 700 g/L glucose*H2O, 7.4 g/L $MgSO_4*7H_2O$ and 0.1 g/L FeSO4*7H2O.

Feed 2 comprises KH_2PO_4 52.7 g/L, K2HPO4*3H2O 139.9 g/L and (NH4)2HPO4 66.0 g/L.

All components were dissolved in deionized water.

The alkaline solution for pH regulation was an aqueous 12.5 % (w/v) NH_3 solution supplemented with 11.25 g/L L-methionine.

Starting with 4.2 L sterile batch medium plus the respective inoculum volume the batch fermentation was performed at 31 °C, pH 6.9 ± 0.2, 800 mbar back pressure and an initial aeration rate of 10 L/min. The relative value of dissolved oxygen (pO2) was kept at 50 % throughout the fermentation by increasing the stirrer speed up to 1500 rpm. After the initially supplemented glucose was depleted, indicated by a steep increase in dissolved oxygen values, the temperature was shifted to 25 °C and 15 minutes later the fermentation entered the fed-batch mode with the start of both feeds (60 and 14 g/h respectively). The rate of feed 2 is kept constant, while the rate of feed 1 is increased stepwise with a predefined feeding profile from 60 to finally 160 g/h within 7 hours. When carbon dioxide off gas concentration leveled above 2 % the aeration rate was constantly increased from 10 to 20 L/min within 5 hours. The expression of recombinant IgA-protease was induced by the addition of 2.4 g IPTG at an optical density of approx. 150. The fermentation is conducted up to 48 hours despite there is no significant decrease in optical density. In the end the culture broth is cooled to 4-8 °C and stored overnight in the fermenter vessel. The content of the fermenter was centrifuged the next day with a flow-through centrifuge (13,000 rpm, 13 L/h) and the harvested biomass was stored at -20 °C until further processing. The synthesized IgA-protease is found exclusively in the insoluble cell debris fraction in the form of insoluble protein aggregates the so-called inclusion bodies (IBs).

Analysis of product formation:

Samples drawn from the fermenter, one prior to induction and the others at dedicated time points after induction of protein expression are analyzed with SDS-Polyacrylamide gel electrophoresis. From every sample the same amount of cells (OD_{target} = 10) are suspended in 5 mL PBS buffer and disrupted via sonication on ice. Then 100 µL of each suspension are centrifuged (15,000 rpm, 5 minutes) and

each supernatant is withdrawn and transferred to a separate vial. This is to discriminate between soluble and insoluble expressed target protein. To each supernatant (= soluble) fraction 100 µL and to each pellet (= insoluble) fraction 200 µL of SDS sample buffer (Laemmli, U.K., Nature 227 (1970) 680-685) are added. Samples are heated for 15 minutes at 95 °C under intense mixing to solubilize and reduce all proteins in the samples. After cooling to room temperature 5 µL of each sample are transferred to a 4-20 % TGX Criterion Stain Free polyacrylamide gel (Bio-Rad). Additionally 5 µL molecular weight standard (Precision Plus Protein Standard, Bio-Rad) and 3 amounts (0.3 µL, 0.6 µL and 0.9 µL) quantification standard with known product protein concentration (0.15 µg/µL) are positioned on the gel.

The electrophoresis was run for 60 min. at 200 V and thereafter the gel was transferred the GelDOC EZ Imager (Bio-Rad) and processed for 5 min. with UV radiation. Gel images were analyzed using Image Lab analysis software (Bio-Rad). With the three standards a linear regression curve was calculated with a coefficient of >0.99 and thereof the concentrations of target protein in the original sample was calculated.

The activity of the refolded and purified IgA-protease was tested with the molecule tetranectin-apolipoprotein A-I fusion polypeptides as described above which is a precursor polypeptide from which the tetranectin-apolipoprotein A-I fusion polypeptides was released by enzymatic cleavage in vitro using this IgA protease. Cleavage activity was in the expected range.

Results:

The above mentioned fermentation process was used to express IgA-Protease protein in the auxotrophy cured strain CSPZ-6 and in the prototrophic strain CSPZ-9 representing the E.coli K12 wild-type strain MG1655. Both strains do not need the feeding of the amino acids L-leucine and L-proline which where consequently excluded from the medium and feeds. This dramatically reduces cost of goods for the process.

The wild-type strain and MG1655 derivate CSPZ-9 showed a faster growth in the beginning of the fermentation when compared to the auxotrophy cured strain CSPZ-6 on the same chemically defined medium and under the same cultivation conditions. 24 hours after inoculation the optical densities were almost the same. But thereafter the optical density of both strains was surprisingly different. The

growth of strain CSPZ-9 had significantly slowed down after 30 hours of cultivation and as a consequence of the continuous feeding of feed 1 the glucose concentration in the medium increased to 16 g/L. Therefore the fermentation was terminated after 37 hours of cultivation. Metabolite analysis revealed a significant increase in concentrations of ammonia, glutamate, iron, magnesium and acetate in the medium due to the significant decrease in growth rate. The final optical densities differ greatly between both strains. While the cured strain reached 362 the optical density of the wild-type strain was decreasing form a maximum at hour 24 of 256 to only 177 at the end of the fermentation (hour 37) (see Figure 12).

After the induction of protein expression with the addition of 2.4 g IPTG at an optical density of approx. 150 significantly more IgA-9rotease is produced with strain CSPZ-6 when compared to the wild-type strain CSPZ-9 (see Figure 13).

Summary:

The auxotrophy cured strain CSPZ-6 showed improved growth characteristics compared to the prototroph wild-type E.coli strain CSPZ-9 (MG1655 ΔpyrF derivate) on the same chemical defined fermentation medium without the supplementation of other amino acids. Therefore it is useful to cure highly productive E.coli strains from its amino acids auxotrophies in order to be able to culture them on chemically defined minimal medium and profit from the advantages of using such a medium and instead of using a prototrophic wild-type E.coli strain derivate from MG1655, W3110 or BL21.

Patent Claims

1. A method for producing a polypeptide in a prokaryotic cell, comprising the following step:

   - curing a prokaryotic cell that is auxotrophic for at least one amino acid from at least one amino acid auxotrophy by introducing into the genome of a parent prokaryotic cell a nucleic acid curing an amino acid auxotrophy of the parent prokaryotic cell,

   - cultivating the the amino acid auxotrophy cured prokaryotic cell comprising one or more nucleic acids encoding the polypeptide in a chemically defined minimal growth medium and recovering the polypeptide from the prokaryotic cell or the periplasm of the prokaryotic cell or from the medium,

   wherein the product titer when using the auxotrophy cured prokaryotic strain is higher than the product titer that can be obtained with a corresponding prototrophic wild-type strain.

2. The method according to claim 1, characterized in that the cultivation of the auxotrophy cured prokaryotic cell requires supplementation of one or two or three or four amino acids less to the growth medium than required for the cultivation of the parent non-auxotrophy cured prokaryotic cell.

3. The method according to any one of claims 1 to 2, characterized in that the auxotrophy cured prokaryotic cell has at least one further amino acid auxotrophy.

4. The method according to any one of claims 1 to 3, characterized in that the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell is not added during the cultivation.

5. The method according to any one of claims 1 to 3, characterized in that the cultivation is in the absence of the amino acid corresponding to the auxotrophy that has been cured in the auxotrophy cured prokaryotic cell.

6. The method according to any one of claims 1 to 5, characterized in that the prokaryotic cell is an E.coli cell.

7. The method according to any one of claims 1 to 6, characterized in that the polypeptide is a full length antibody chain, a single chain antibody, a single domain antibody, a scFv, a scFab, or a conjugate of one of the before with a non-antibody polypeptide.

8. The method according to claim 7, characterized in that the polypeptide is a conjugate of a scFv or scFab with a cytotoxic agent.