(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 June 2021 (24.06.2021)

WIPO | PCT

(10) International Publication Number
WO 2021/123402 A1

(51) International Patent Classification:
C12N 15/63 (2006.01) *C12P 1/02* (2006.01)

(21) International Application Number:
PCT/EP2020/087354

(22) International Filing Date:
20 December 2020 (20.12.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
LU101582 20 December 2019 (20.12.2019) LU

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2021/123402 A1

(54) **Title:** CONTINUOUS BIOPRODUCTION BY DECOUPLING GROWTH AND PRODUCTION

(57) **Abstract:** The present invention is in the field of recombinant biotechnology, in particular in the field of protein expression and nucleotide production. The invention generally relates to systems and processes that are suitable or comprise a two stage production process, in which the growth of the bacterial host cell is spatiotemporally separated from the production of the protein or nucleic acid of interest. Accordingly, the present invention relates to a system and a process for use in continuous production of a protein of interest or a nucleotide of interest by a bacterial host cell.

CONTINUOUS BIOPRODUCTION BY DECOUPLING GROWTH AND PRODUCTION

Technical field of the Invention

[0001] The present invention is in the field of recombinant biotechnology, in particular in the field of nucleotide and protein expression. The invention generally relates to systems and processes that are suitable for or comprise a two stage production process, in which the growth of the bacterial host cell is spatially separated from the production of the protein or nucleic acid or of interest to allow a continuous production.

Background

[0002] Production of proteins of interest (POI) or nucleotides of interests has been accomplished with many prokaryotic hosts. The most prominent examples are bacteria like *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Streptomyces griseus*, or *Corynebacterium glutamicum*. A great number of biological pharmaceuticals (e.g. antibodies or functional fragments thereof) have been produced in the last decade and an increasing number is nearing approval for use in humans but their efficient production remains a challenging task. Therapeutically active doses are often in the order of milligram (mg) per administration. Thus, considerable amounts of protein are needed as active ingredients, making an efficient and cost-effective production worthwhile.

[0003] Bacterial cell expression systems have long been, and still are, one of the major tools for production of these types of molecules. The key objective of process optimization is to achieve a high yield of product having the required quality at the lowest possible cost, which is often determined by the properties of a specific expression construct or system. Very often, a batch process is used for protein expression. A batch process however is labour-intensive because the bioreactors require constant surveillance and must be cleaned thoroughly after each production. While the bioreactors are cleaned, production cannot take place making batch processes inefficient.

[0004] By applying a continuous expression system instead of a batch process, these various problems associated with a batch process can be overcome. However, also in a continuous process, high-level recombinant protein expression may overwhelm the metabolic capacity of a host cell and consequently leads to plasmid loss, reduced oxygen transfer, generation of toxic by-products, formation of inclusion bodies, and/or triggering of a stress response which often

impairs efficient protein production. It is also known that sometimes high expression of an mRNA encoding a protein of interest does not necessarily lead to high amounts of the protein.

[0005] Different approaches have been taken by scientists to deal with these problems, also for continuous expression systems. However, continuous expression systems that ensure the genetic stability of the host cells for a longer period of time to express proteins or nucleic acids of interest have not been described yet. Since a continuous expression system has many advantages including higher yield at lower costs, e.g. an improved space-time-yield (S-T-Y), there still is a need for a continuous production of proteins or nucleotides of interests in microbial host cells. Accordingly, the technical problem underlying the present invention is to comply with this need, e.g. by developing continuous manufacturing processes with an improved time-span of significant expression levels.

Summary of the Invention

[0006] The present invention provides as a solution to the technical problem new means and methods to increase the yield and/or productivity defined as yield/time but also as productivity per volume of the culture of recombinant protein or nucleotide production by applying a continuous expression system and process, which are simple and efficient and suitable for use in industrial methods. In this continuous expression, the growth of the microbial or bacterial host cells is spatially separated from the production of the protein or nucleotide of interest ("growth decoupling"). These means and methods are described herein, illustrated in the Examples, and reflected in the claims.

[0007] Accordingly, the present invention relates to a system for use in continuous production of a protein of interest or a nucleotide of interest by a bacterial host cell, wherein the bacterial host cell comprises under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell, comprising

(a) a seed reactor comprising said bacterial host cell in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, the seed reactor having at least one inlet and at least one outlet, and

(b) at least one production reactor comprising said bacterial host cells in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, each production reactor having at least one inlet and at least one outlet,

wherein an inlet of the production reactor is connected to an outlet of the seed reactor.

[0008] In one embodiment of the system of the invention, the bacterial host cell comprises a nucleotide encoding the protein of interest or the nucleotide of interest.

[0009] In one embodiment of the system of the invention, the protein of interest or the nucleotide of interest is produced in the at least one production reactor.

[0010] In one embodiment of the system of the invention, said nucleotide of interest encodes one or more proteins of interest.

[0011] In one embodiment of the system of the invention, said nucleotide is a nucleic acid molecule.

[0012] In one embodiment of the system of the invention, said nucleic acid molecule is a plasmid, minichromosome, or RNA.

[0013] In one embodiment of the system of the invention, said nucleotide of interest or said nucleotide encoding the protein of interest is under the control of a second inducible promoter or under the control of a constitutive promoter.

[0014] In one embodiment of the system of the invention, the promoter of said heterologous nucleotide of interest is recognized by a RNA polymerase which is heterologous for said bacterial host cell, said heterologous RNA polymerase is encoded by a nucleotide sequence comprised by said bacterial host cell.

[0015] In one embodiment of the system of the invention, said RNA polymerase is bacteriophage T3 RNA polymerase, T7 bacteriophage RNA polymerase, engineered orthogonal T7 RNA polymerase, bacteriophage SP6 RNA polymerase or bacteriophage Xp10 RNA polymerase.

[0016] In one embodiment of the system of the invention, said nucleotide sequence encoding said RNA polymerase is under the control of a third inducible promoter or under the control of a constitutive promoter.

[0017] In one embodiment of the system of the invention, said first, second or third inducible promoter is regulated by arabinose, IPTG, tryptophan, xylose, lactose, rhamnose, phosphate, propionate, benzoic acid, phage lambda cI protein or heat.

[0018] In one embodiment of the system of the invention, said first, second or third promoter are different.

[0019] In one embodiment of the system of the invention, said bacterial host cell has a non-functional arabinose operon.

[0020] In one embodiment of the system of the invention, said bacterial host cell is *E. coli*, preferably *E. coli* B-lineage.

[0021] In one embodiment of the system of the invention, growth is inhibited by inhibiting transcription, DNA-replication and/or cell division.

[0022] In one embodiment of the system of the invention, said phage protein is

(i) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 1 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 1 and which inhibits bacterial host cell RNA polymerase;

(ii) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 2 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 2 and which inhibits bacterial host cell RNA polymerase;

(iii) a protein which phosphorylates bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 3 or a fragment thereof which phosphorylates bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 3 and which phosphorylates bacterial host cell RNA polymerase;

(iv) a protein which inhibits bacterial host cell DNA replication, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 4 or a fragment thereof which inhibits bacterial host cell DNA replication; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 4 and which inhibits bacterial host cell DNA replication;

(v) a protein which inhibits bacterial host cell DNA replication, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 5 or a fragment thereof which inhibits bacterial host cell DNA replication; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 5 and which inhibits bacterial host cell DNA replication; or

(vi) a protein which inhibits bacterial host cell DNA replication, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 6 or a fragment thereof which inhibits bacterial host cell DNA replication; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 6 and which inhibits bacterial host cell DNA replication;

(vii) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 7 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 7 and which inhibits bacterial host cell RNA polymerase;

(viii) a protein which causes host transcription shut-off, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 8, 9, 10, 11, 12, 13, 14 or a fragment thereof which causes host transcription shut-off; or

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 8, 9, 10, 11, 12, 13 or 14 and which causes host transcription shut-off; or

(ix) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 17 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 17 and which inhibits bacterial host cell RNA polymerase.

[0023] In one embodiment of the system of the invention, the bacterial host cell is cultured in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in the seed reactor for biomass production.

[0024] In one embodiment of the system of the invention, the bacterial host cell is cultured in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in the production reactor for production of said nucleotide sequence of interest by said bacterial host cells.

[0025] In one embodiment of the system of the invention, the system further comprises (c) a means for operating the seed and production reactors as linked chemostats or turbidostats.

[0026] In one embodiment of the system of the invention, the seed reactor outflow serves as inflow to the production reactor.

[0027] In one embodiment of the system of the invention, the seed reactor does not comprise or does not essentially comprise an inducer for the first inducible promoter.

[0028] In one embodiment of the system of the invention, at least one of the at least one production reactor comprises an inducer for the first inducible promoter.

[0029] In one embodiment of the system of the invention, at least one of the at least one of the at least one production reactor comprises an inducer for the second inducible promoter.

[0030] In one embodiment of the system of the invention, the first inducible promoter and the second inducible promoter can be induced by the same inducer.

[0031] In one embodiment of the system of the invention, the seed reactor comprises a means for regulating pH.

[0032] In one embodiment of the system of the invention, the seed reactor comprises a means for regulating dissolved oxygen.

[0033] In one embodiment of the system of the invention, the seed reactor comprises a means for regulating temperature.

[0034] In one embodiment of the system of the invention, the seed reactor comprises a gas inlet and a gas outlet and a means for regulating the gas flow.

[0035] In one embodiment of the system of the invention, the at least one production reactor comprises a means for regulating pH.

[0036] In one embodiment of the system of the invention, the at least one production reactor comprises a means for regulating dissolved oxygen.

[0037] In one embodiment of the system of the invention, the at least one production reactor comprises a means for regulating temperature.

[0038] In one embodiment of the system of the invention, the at least one production reactor comprises a gas inlet and a gas outlet and a means for regulating the gas flow.

[0039] In one embodiment of the system of the invention, the at least one production reactor comprises a biomass sensor.

[0040] In one embodiment of the system of the invention, the system comprises a first feed container containing a first feed medium comprising a carbon source, wherein the first feed container is operably connected to an inlet of the seed reactor, wherein the system preferably comprises means for regulating feed flow from the first feed container to the seed reactor.

[0041] In one embodiment of the system of the invention, the system comprises second feed container containing a second feed medium comprising a carbon source, wherein the second

feed container is operably connected to an inlet of the at least one production reactor, wherein the system preferably comprises means for regulating feed flow from the second feed container to the at least one production reactor.

[0042] In one embodiment of the system of the invention, an outlet of the seed reactor and an outlet of a second feed reactor are connected to a mixing chamber, wherein an outlet of the mixing chamber is connected to an inlet of the at least one production reactor.

[0043] In one embodiment of the system of the invention, the seed reactor is a stirred tank reactor or plug flow reactor.

[0044] In one embodiment of the system of the invention, the at least one production reactor is a stirred tank reactor or a plug flow reactor.

[0045] In one embodiment of the system of the invention, the seed reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L.

[0046] In one embodiment of the system of the invention, the at least one production reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L.

[0047] In one embodiment of the system of the invention, the volume ratio of the seed reactor to the at least one production reactor is from about 1:10 to about 2:1, from about 1:5 to about 2:1, from about 1:2 to about to about 2:1, from about 1.5:1 to about 1:1.5, or about 1:1.

[0048] In one embodiment of the system of the invention, the at least one production reactor comprises a culture medium comprising cells with a biomass concentration from about 10 to about 90 g/L cell dry weight, preferably from about 20 to about 80 g/L cell dry weight, preferably from about 30 to about 70 g/L cell dry weight, preferably from about 35 to about 60 g/L cell dry weight.

[0049] The present invention further relates to a continuous fermentation process for the production of a nucleotide of interest or a protein of interest by a bacterial host cell, wherein the bacterial host cell comprise under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell, comprising

(a) culturing said bacterial host cell in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in a seed reactor;

(b) transferring at least an amount of the bacterial host cells obtained in (a) from said seed reactor to a production reactor; and

(c) culturing said bacterial host cells in said production reactor in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells;

wherein the seed reactor and production reactor is configured as an independent continuous fermentor and wherein the seed reactor and production reactor are connected with each other.

[0050] In one embodiment of the continuous fermentation process of the invention, (a) is for biomass production.

[0051] In one embodiment of the continuous fermentation process of the invention, in (c) growth of said bacterial host cells is inhibited by culturing said bacterial host cells in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, while said nucleotide of interest or said protein of interest is produced.

[0052] In one embodiment of the continuous fermentation process of the invention, the bacterial host cell comprises the nucleotide of interest or a nucleotide encoding the protein of interest.

[0053] In one embodiment of the continuous fermentation process of the invention, said nucleotide of interest encodes one or more proteins of interest.

[0054] In one embodiment of the continuous fermentation process of the invention, said nucleotide is a nucleic acid molecule.

[0055] In one embodiment of the continuous fermentation process of the invention, said nucleic acid molecule is a plasmid, minichromosome, or RNA.

[0056] In one embodiment of the continuous fermentation process of the invention, said nucleotide of interest or said nucleotide encoding the protein of interest is under the control of a second inducible promoter or under the control of a constitutive promoter.

[0057] In one embodiment of the continuous fermentation process of the invention, the promoter of said heterologous nucleotide sequence of is recognized by a RNA polymerase which is heterologous for said bacterial host cell, said heterologous RNA polymerase is encoded by a nucleotide sequence comprised by said bacterial host cell.

[0058] In one embodiment of the continuous fermentation process of the invention, said heterologous RNA polymerase is bacteriophage T3 RNA polymerase, T7 bacteriophage RNA polymerase, engineered orthogonal T7 RNA polymerase, bacteriophage SP6 RNA polymerase or bacteriophage Xp10 RNA polymerase.

[0059] In one embodiment of the continuous fermentation process of the invention, said nucleotide sequence encoding said heterologous RNA polymerase is under the control of a third inducible promoter or under the control of a constitutive promoter.

[0060] In one embodiment of the continuous fermentation process of the invention, said first, second or third inducible promoter is regulated by arabinose, IPTG, tryptophan, xylose, lactose, rhamnose, phosphate, propionate, benzoic acid, phage lambda cI protein or heat.

[0061] In one embodiment of the continuous fermentation process of the invention, said first, second or third promoter are different.

[0062] In one embodiment of the continuous fermentation process of the invention, said bacterial host cell has a non-functional arabinose operon.

[0063] In one embodiment of the continuous fermentation process of the invention, said bacterial host cell is *E. coli*, preferably *E. coli* B-lineage.

[0064] In one embodiment of the continuous fermentation process of the invention, (a) further comprises culturing said bacterial host cells in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells in culture medium in a production reactor for biomass production.

[0065] In one embodiment of the continuous fermentation process of the invention, (c) further comprises inducing said first inducible promoter controlling said nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells.

[0066] In one embodiment of the continuous fermentation process of the invention, (b) or (c) comprises inducing said second inducible promoter controlling said nucleotide of interest or said nucleotide encoding the protein of interest.

[0067] In one embodiment of the continuous fermentation process of the invention, (a), (b) or (c) comprises inducing said third inducible promoter controlling said nucleotide sequence encoding said heterologous RNA polymerase.

[0068] In one embodiment of the continuous fermentation process of the invention, the continuous fermentation process further comprises (d) harvesting the product resulting from the production of said nucleotide sequence of interest.

[0069] In one embodiment of the continuous fermentation process of the invention, the seed reactor does not comprise or does not essentially comprise an inducer for the first inducible promoter.

[0070] In one embodiment of the continuous fermentation process of the invention, at least one of the at least one production reactor comprises an inducer for the first inducible promoter.

[0071] In one embodiment of the continuous fermentation process of the invention, at least one of the at least one of the at least one production reactor comprises an inducer for the second inducible promoter.

[0072] In one embodiment of the continuous fermentation process of the invention, the first inducible promoter and the second inducible promoter can be induced by the same inducer.

[0073] In one embodiment of the continuous fermentation process of the invention, pH is regulated in the seed reactor.

[0074] In one embodiment of the continuous fermentation process of the invention, dissolved oxygen is regulated in the seed reactor.

[0075] In one embodiment of the continuous fermentation process of the invention, temperature is regulated in the seed reactor.

[0076] In one embodiment of the continuous fermentation process of the invention, gas flow is regulated in the seed reactor.

[0077] In one embodiment of the continuous fermentation process of the invention, the biomass concentration is regulated in the seed reactor by feed inflow and/or biomass outflow.

[0078] In one embodiment of the continuous fermentation process of the invention, pH is regulated in the at least one production reactor.

[0079] In one embodiment of the continuous fermentation process of the invention, dissolved oxygen is regulated in the at least one production reactor.

[0080] In one embodiment of the continuous fermentation process of the invention, temperature is regulated in the at least one production reactor.

[0081] In one embodiment of the continuous fermentation process of the invention, gas flow is regulated in the at least one production reactor.

[0082] In one embodiment of the continuous fermentation process of the invention, the at least one production reactor comprises a biomass sensor.

[0083] In one embodiment of the continuous fermentation process of the invention, the biomass concentration is regulated in the at least one production reactor by feed inflow, biomass inflow, and/or biomass outflow.

[0084] In one embodiment of the continuous fermentation process of the invention, the mean residence time of biomass in the at least one production reactor is from about 5 h to about 24 h, preferably from about 7 h to about 20 h, preferably from about 10 h to about 15 h.

[0085] In one embodiment of the continuous fermentation process of the invention, the continuous fermentation process further comprises a first feed container containing a first feed medium comprising a carbon source, wherein the first feed container is operably connected to an inlet of the seed reactor, wherein feed flow from the first feed container to the seed reactor is preferably regulated.

[0086] In one embodiment of the continuous fermentation process of the invention, the continuous fermentation process comprises a second feed container containing a second feed medium comprising a carbon source, wherein the second feed container is operably connected to an inlet of the at least one production reactor, wherein feed flow from the second feed container to the at least one production reactor is preferably regulated.

[0087] In one embodiment of the continuous fermentation process of the invention, an outlet of the seed reactor and an outlet of a second feed reactor are connected to a mixing chamber, wherein an outlet of the mixing chamber is connected to an inlet of the at least one production reactor.

[0088] In one embodiment of the continuous fermentation process of the invention, the seed reactor is a stirred tank reactor or a plug flow reactor.

[0089] In one embodiment of the continuous fermentation process of the invention, the at least one production reactor is a stirred tank reactor or a plug flow reactor.

[0090] In one embodiment of the continuous fermentation process of the invention, the seed reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L.

[0091] In one embodiment of the continuous fermentation process of the invention, the at least one production reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L.

[0092] In one embodiment of the continuous fermentation process of the invention, the volume ratio of the seed reactor to the at least one production reactor is from about 1:10 to about 2:1, from about 1:5 to about 2:1, from about 1:2 to about to about 2:1, from about 1.5:1 to about 1:1.5, or about 1:1.

[0093] In one embodiment of the continuous fermentation process of the invention, the at least one production reactor comprises a culture medium comprising cells with a biomass concentration from about 10 to about 90 g/L cell dry weight, preferably from about 20 to about 80 g/L cell dry weight, preferably from about 30 to about 70 g/L cell dry weight, preferably from about 35 to about 60 g/L cell dry weight.

[0094] In one embodiment of the continuous fermentation process of the invention, the bacterial host cells in the at least one production reactor is genetically stable for at least about 5 days, preferably at least about 7 days, preferably at least about 10 days.

[0095] In one embodiment of the continuous fermentation process of the invention, the process is operated for at least about 5 days, preferably at least about 7 days, preferably at least about 10 days.

Brief description of the drawings

Figure 1: Cell dry mass concentration [CDM, in g/L] for the seed reactor (1 stage) for biomass concentration (uninduced, without addition of IPTG and L-arabinose) in Example 1. Empty squares (□) shows BL21(DE3)pET30a<GFP> whereas empty triangle (Δ) shows enGenes-X-press pET30a<GFP>. CDM was followed for a period of 196 h, corresponding to 8 days. Abrupt population collapse is indicated by a blue arrow. Here, a drop in CDM is observable for BL21(DE3)pET30a<GFP> after 150 h in chemostat mode, not observable for enGenes-X-press pET30a<GFP>, thereby improving stability and productivity of the continuous production process unexpectedly even in at the seed stage reactor.

Figure 2: Cell dry mass concentration [CDM, in g/L] and productivity for total GFP [g/h] for the production reactor (2nd stage) (induced, with addition of IPTG and L-arabinose in case of X-press) in Example 1. Empty squares (□) shows BL21(DE3)pET30a<GFP> whereas empty triangle (Δ) shows enGenes-X-press pET30a<GFP>. Dashed line with filled square (■) shows BL21(DE3)pET30a<GFP> whereas dashed line with filled triangle (▲) shows enGenes-X-press pET30a<GFP>. CDM and total volumetric yield were followed for a period of 196 h, corresponding to 8 days. enGenes-X-press pET30a<GFP> shows a 20-fold increase in total GFP productivity(0.6 g/L/h vs. 0.03 g/L/h) after 196 h operated in 2-stage chemostat mode in comparison to BL21(DE3)pET30a<GFP>.

Figure 3: Schematic overview of the 2 stage process of the invention. $F1_{IN}$ is indicative of the feed medium flow into the seed vessel with a volume V1 and to achieve a biomass concentration of X1; $F1_{OUT}$ is indicative of the volume stream from the seed vessel to the production vessel, to achieve the volume V2 and the biomass concentration X2; $F2_{IN}$ is indicative of the feed medium flow, containing growth medium and inducer (IPTG and L-arabinose); $F2_{OUT}$ is indicative of the outflow of the production vessel containing the product enriched-biomass (in the case of Example 1, GFP) or the product enriched cell free supernatant (in the case of Example 2, SpA protein from *Staphylococcus aureus*).

Figure 4: FACS histogram for fluorescence readings shown for BL21(DE3)pET30a<GFP> in the seed reator (1st stage) compared to enGenes-X-press pET30a<GFP> for different time points (48h, 148h, 196h) in Example 1. Cells were gated in GFP positive (GFP +) and GFP negative (GFP -) cells with a fluorescent intensity of $1x10^3$ arbitrary units. The table below gives the absolute number (Number), the percentage (%Gated), the mean fluorescence intensity (X-AMean) and half peak coefficient of variation (HP X-CV) of cells in the corresponding gate (O+/-, I+/-, B +/-) for the different timepoints (48h, 148h, 196h). enGenes-X-

press shows a more uniform population fluorescence distribution compared to BL21(DE3), in which the major cell population shows low level fluorescence after 196h.

Figure 5: FACS histogram for fluorescence readings shown for BL21(DE3)pET30a<GFP> in the production reactor (2nd stage) compared to enGenes-X-press pET30a<GFP> for different time points (48h, 148h, 196h) in Example 1. Cells were gated in GFP positive (GFP +) and GFP negative (GFP -) cells with a fluorescent intensity of $1x10^3$ arbitrary units. The table below gives the absolute number (Number), the percentage (%Gated), the mean fluorescence intensity (X-AMean) and half peak coefficient of variation (HP X-CV) of cells in the corresponding gate (O+/-, I+/-, B +/-) for the different time points (48h, 148h, 196h). BL21(DE3) shows a clearly disappearing productive population after 196 h (25% GFP + for BL21(DE3) vs. 59% GFP+ cells for enGenes-X-press).

Figure 6: Cell dry mass concentration [CDM, in g/L] for the seed reactor for biomass production (uninduced, without addition of IPTG and L-arabinose) in Example 2. Empty squares (□) shows BL21(DE3)pET30a<SpA> whereas empty triangle (Δ) shows enGenes-X-press pET30a<SpA>. CDM was followed for a period of 309 h, corresponding to more than 12 days. Abrupt population collapse is indicated by a grey arrow. Here, a drop in CDM is observable for BL21(DE3)pET30a<SpA> after 150 h in chemostat mode, but not observable for enGenes-X-press pET30a<SpA>, thereby showing an improved (genetic) stability and productivity of continuous production. Overall process was terminated for BL21(DE3) after population collapse in seed reactor.

Figure 7: Cell dry mass concentration [CDM, in g/L] and total volumetric yield for secreted SpA [g/L] for the production reactor (2nd stage) (induced, with addition of IPTG and L-arabinose in the case of X-press) in Example 2. Dashed line with filled square (□) shows BL21(DE3)pET30a<SpA> whereas dashed line with filled triangle (▲) shows enGenes-X-press pET30a<SpA>. CDM and total volumetric yield were followed for a period of 309 h, corresponding to 8 days. enGenes-X-press pET30a<SpA> shows a constant productivity of 0.4 g/L/h of secreted SpA yield over a time span of 309 h operated in 2-stage chemostat mode whereas the process for BL21(DE3)pET30a<SpA> had to terminated after 168 h due to abrupt population collapse in seed reactor 1.

Figure 8: Cell dry mass concentration [CDM, in g/L], total produced lycopene [mg] and space time yield (STY) for the seed (phase 1) and production reactor (phase 2) (induced, with addition of IPTG and L-arabinose in the case of X-press) for BL21(DE3) pET30a<crtE-crtB-crtI>cer and enGenes-X-press pET30a<crtE-crtB-crtI>cer are shown. Process lasted for 310 h, or 13 days (enGenes-X-press), respectively 142 h, or 6 days for BL21(DE3). enGenes-X-press pET30a<crtE-crtB-crtI>cer shows a constant increase in produced lycopene over a time span of 310 h operated in 2-stage chemostat mode whereas the process for BL21(DE3)

pET30a<crtE-crtB-crtI>cer had to terminated after 142 h due to abrupt population collapse in production reactor (phase 2).

Figure 9: Double stranded DNA (dsDNA) per g cell dry mass (CDM) for the seed (phase 1) and production reactor (phase 2) (induced, with addition of IPTG and L-arabinose in the case of X-press) for BL21(DE3) pET30a<crtE-crtB-crtI>cer (B) and enGenes-X-press pET30a<crtE-crtB-crtI>cer (X) are shown. enGenes-X-press pET30a<crtE-crtB-crtI>cer (X) shows a constant amount of dsDNA in the fermentation supernatant (phase 1 and 2) during 2-stage chemostat mode whereas the process for BL21(DE3) pET30a<crtE-crtB-crtI>cer (B) had to terminated after 142 h due to abrupt population collapse in production reactor (phase 2) after 70 h.

Figure 10: Schematic representation of the pET30a<crtE-crtB-crtI>cer plasmid used in Example 3.

Detailed Description

[0096] The present inventors successfully developed a system and a process for continuous production of proteins and nucleotides, such as plasmids. The present invention is based on the uncoupling of the growth ("growth-decoupling") of the host cell from the production of a protein or nucleotide of interest in a two stage process. In the inventive system and process of the invention, the growth, i.e. the propagation of the host cells, is spatially separated from the production of the nucleotide or protein of interest. In one bioreactor, the seed reactor, the host cells are kept under conditions that are ideal for their propagation. No production of the nucleotide or protein of interest is induced. Host cells from the seed reactor are continuously transferred into a second bioreactor, the production reactor. The growth of the host cell is inhibited and the production of the protein or nucleotide of interest is induced in the production reactor. This concept is referred to herein as "growth decoupling. This allows a continuous production. A fraction of the host cells or the supernatant/culture medium of the production reaction is continuously removed from the production reactor and the protein or nucleotide of interest can be harvested. The fraction that is removed from the production reactor will be preferably continuously replaced by fresh host cells from the seed reactor. As shown in Examples 1 and 2, this process can be employed for longer periods of times such as about 12 days as shown in Example 2. This technology therefore allows the continuous production of proteins or nucleotides of interest in rather small reactors with still high yield/productivity and therefore improved S-T-Y (space-time-yield) of proteins or nucleotides of interest.

[0097] The double burden of a host cell caused by its proliferation and simultaneous expression of a heterologous protein reduces the yield of a protein of interest. In fact, the proliferation of the host cell during the production of a protein of interest poses an overload to the host cell resulting in a conflict in distribution of cellular resources. Thereby, several unwanted side effects like generation of toxic by-products, reduced oxygen transfer and induction of a stress response

or plasmid loss ("genetic instability") are provoked, eventually resulting in a reorientation of the cellular metabolism constraining transcription and translation and potentially cell death. Given that the cellular synthesis capacity is the basis of heterologous protein expression, one has to take the capacity of a host cell into account. In order to reduce or abolish the unwanted side effects of heterologous protein expression, the present inventors have developed an expression system that uncouples the production of the protein of interest from the proliferation of the host cell, thereby considerably reducing the burden on the host cell and increasing the yield of a protein of interest - see also WO 2016/174195, incorporated hereby by reference in its entirety.

[0098] More particularly, the present inventors employ phage proteins that inhibit growth of the bacterial host cells by designing a host cell comprising a phage protein inhibiting growth of the bacterial host cell under the control of an inducible promoter and apply these host cells in a two stage process. This allows switching OFF the host cell's proliferation in the production reactor and allows high yield production in the production reactor. By applying a seed reactor, a continuous flow of fresh host cells into the production reactor is established. This allows a continuous production of the protein or nucleotide of interest. Separation of growth and production further surprisingly improves genetic stability at both seed and production stage reactor. Rai et al. (2018), Biotechnology and Bioengineering, 116:693-703, describe a phenomenon which they refer to as "abrupt population collapse in chemostat fermentation" once minimal medium is employed. They observed a significant drop in biomass concentration in chemostat mode after ~150 h. This phenomenon may be due to genetic instability (e.g. loss of plasmid encoding the protein or nucleic acid of interest or mutations preventing the expression of the protein or nucleic acid of interest) was also observed by the present inventors when using bacterial host cells without an inducible protein from a phage, which inhibits growth of said bacterial host cell (see Examples 1 and 2). Thus, a person skilled in the art willing to develop a continuous fermentation process with a "standard" bacterial host cell, e.g., *E. coli*, does not have a reasonable expectation of success.

[0099] However, this abrupt population collapse is surprisingly not taking place in a bacterial host cell, wherein the bacterial host cell comprises under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell such as a modified BL21(DE3) strain, having the Gp2 protein integrated into its genome under control of an L-arabinose inducible promoter _(c.f. the enGenes-X-press strain disclosed herein). Using the bacterial host cell of the invention, a continuous production process in form a two stage production regime could by performed for extended periods of time, i.e. at least 12 days as described in experiment 1 and 2. The continuous fermentation process of the invention results in elevated continuous product yields and improved productivity. Thus, the system and method of the present invention surprisingly also overcomes the problem of

abrupt population collapse. The bacterial host cells of the invention can therefore be described more genetically stable in comparison to standard bacterial host cells.

[0100] Furthermore, oxygen consumption, nutritional requirements and metabolic heat development are reduced, a stress response in the production reactor is circumvented and therefore sufficient (internal) microbial metabolic resources for the production of the protein or nucleotide of interest are available. An additional problem of heterologous protein expression is the incorporation of the protein of interest in inclusion bodies that results in a decreased solubility and thereby yield. This effect can be avoided by reducing cellular proliferation and induction temperature as shown by Vernet et al. (2010, Protein Expression and Purification, Vol. 77, Issue 1: 104-111) and thus by the present growth decoupled production system.

[0101] Phage proteins which inhibit cell growth have been found by the present inventors to be useful in uncoupling growth of a host cell and production of a protein of interest of said host cell. In fact, the phage protein ideally brings the host cell to halt, while an expression system that is insensitive to said phage protein can ideally fully exploit the protein production machinery of the halted host cell. For example, bacteriophage T7 uses its proteins gp0.7 and Gp2 to shut off *E.coli* RNA polymerase after infection. Immediately after infection early viral class I genes of bacteriophage T7, under control of bacterial promoters, are expressed, such as T7 RNA polymerase which is highly specific for viral genes under control of the T7 promoter. Among the class I genes is Gp0.7, which phosphorylates inter alia *E. coli* RNA polymerase resulting in transcription termination of early genes and in switching from host to viral RNA polymerase. Subsequently, the viral gene Gp2 is expressed, binding to and further inhibiting the beta subunit of the host RNA polymerase. Together Gp0.7 and/or Gp2 inhibit *E.coli* RNA polymerase and thereby cellular proliferation, resulting in a take-over of the bacterial protein synthesis machinery for viral purposes. Inhibition of E. coli RNA polymerase by Gp2 was shown by Studier and Moffat (1986, J. Mol. Biol., 189, 113-130), whereas they missed to disclose an impact on cellular proliferation, yet provide any incentive to apply the host cell shut-off in a two stage production process as described herein.

[0102] Yet, apart from Gp0.7 and Gp2 further such phage proteins are available and have been used by the present inventors to show that their concept of using a phage protein for uncoupling growth of a host cell from its capacity of producing a nucleotide or protein of interest by using an expression system that is insensitive to such a phage protein. Further such phage proteins are, for example, Nun, Gp6, Gp8 or A*, Bacillus phage SPO1 GP40 SPO1 GP40, Staphylococcus phage G1 GP67, Thermus thermophilus phage P23-45 GP39, Enterobacteria phage PhiEco32 GP79, Xanthomonas oryzae bacteriophage Xp10 P7 protein, Enterobacteria phage T4 Alc protein, Enterobacteria phage T4Asia or Bacillus subtilis ykzG protein which are known in the art and are also described herein.

[0103] The present inventors adopted this functional principle in a two stage process in two separate bioreactors for the purpose of producing a nucleotide or protein of interest. A (bacterial) host cell suitable for the system or the process of the invention may comprise (i) a phage protein under control of an inducible promoter which inhibits growth of the bacterial host cell, (ii) a heterologous RNA polymerase absent in the bacterial host cell and (iii) a protein of interest under control of a promoter recognized by said heterologous RNA polymerase, thereby facilitating to inhibit the cellular proliferation and concentrate the host cells capacity on the production of the protein of interest.

[0104] As described herein, the inventors developed a new and inventive system suitable for the continuous production of proteins or nucleotides of interest applying a two stage process, in which microbial or bacterial growth is spatially separated.

[0105] Accordingly, the present invention provides a system for use in continuous production of a protein or nucleotide of interest by a bacterial host cell, wherein the bacterial host cell comprises under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell, comprising

(a) a seed reactor comprising said bacterial host cell in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, the seed reactor having at least one inlet and at least one outlet, and

(b) at least one production reactor comprising said bacterial host cells in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, each production reactor having at least one inlet and at least one outlet,

wherein an inlet of the production reactor is connected to an outlet of the seed reactor.

[0106] In a preferred embodiment, the present invention relates to a system for use in continuous production of a protein of interest by a bacterial host cell, wherein the bacterial host cell comprises under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell, comprising

(a) a seed reactor comprising said bacterial host cell in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, the seed reactor having at least one inlet and at least one outlet, and

(b) at least one production reactor comprising said bacterial host cells in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, each production reactor having at least one inlet and at least one outlet,

wherein an inlet of the production reactor is connected to an outlet of the seed reactor.

[0107] The present invention further relates to a system for use in continuous production of a heterologous nucleotide sequence of interest by microbial host cells which comprise said heterologous nucleotide sequence of interest, wherein growth of said microbial host cells can be decoupled from production of said heterologous nucleotide sequence of interest through induction of genetic inhibition of growth of said microbial host cells, comprising

(a) a seed reactor comprising said microbial host cells in an uninduced state with respect to the genetic inhibition of growth, the seed reactor having at least one inlet and at least one outlet, and

(b) at least one production reactor comprising said microbial host cells in an induced state with respect to the genetic inhibition of growth, thereby growth of said microbial host cells is genetically inhibited, while said nucleotide sequence of interest is produced, each production reactor having at least one inlet and at least one outlet,

wherein an inlet of the production reactor is connected to an outlet of the seed reactor.

[0108] Preferably, genetic inhibition of growth is accomplished by a protein from a phage which inhibits growth of said bacterial host cells, said protein from a phage is encoded by a nucleotide sequence which is under the control of a first inducible promoter and is comprised by said bacterial host cells. Preferably, genetic inhibition of growth is accomplished by repressing bacterial DNA replication [targeting dnaA and oriC] or blocking nucleotide synthesis [targeting pyrF or thyA] through CRISPR interference technology.

[0109] The system of the present may also be used in a process for the production of a nucleotide of interest or a protein of interest. I.e., the present invention not only relates to a system for the production of a protein or nucleotide of interest but also to the 2 stage process itself that can be carried out making use of the system of the invention. 2 stage in this context does not necessarily mean that the growth of the microbial or bacterial host cells and the production of the protein or nucleotide of interest are followed by each other but they preferably are carried out at the same time. In other words, the growth and production preferably take place simultaneously and not in two separate steps followed by each other.

[0110] Accordingly, the present invention relates to a continuous fermentation process for the production of a protein or nucleotide of interest by a bacterial host cell, wherein the bacterial host cell comprise under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell, comprising

(a) culturing said bacterial host cell in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in a seed reactor;

(b) transferring at least an amount of the bacterial host cells obtained in (a) from said seed reactor to a production reactor; and

(c) culturing said bacterial host cells in said production reactor in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells;

wherein the seed reactor and production reactor is configured as an independent continuous fermenter and wherein the seed reactor and production reactor are connected with each other.

[0111] In a preferred embodiment, the present invention relates to a continuous fermentation process for the production of a protein of interest by a bacterial host cell, wherein the bacterial host cell comprise under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell, comprising

(a) culturing said bacterial host cell in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in a seed reactor;

(b) transferring at least an amount of the bacterial host cells obtained in (a) from said seed reactor to a production reactor; and

(c) culturing said bacterial host cells in said production reactor in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells;

wherein the seed reactor and production reactor is configured as an independent continuous fermenter and wherein the seed reactor and production reactor are connected with each other.

[0112] "Continuous fermentation" relates to operating conditions, in which a liquid medium, such as a culture or nutrient medium, or a cells broth, is added to the fermenter, while culture fluid is released from the fermenter. The influx of the liquid medium is preferably constant at a certain rate or intermittently. The efflux of the culture fluid is preferably at the same rate so that the amount of liquid in the fermenter remains essentially constant. During continuous fermentation, biomass preferably remain essentially unchanged. In the steady state, the fermentation condition can be maintained, such as nutrient concentration, product concentration, constant pH, biomass according to the need. Reactors used for continuous fermentation can be stirred tank reactors or tubular reactors. A "continuous fermenter" as used herein is a fermenter that is suitable for continuous fermentation.

[0113] (Initial) initiation of the continuous process of the invention may be done as following. Both, the seed and the production reactor, may be started as a batch culture followed by fed-batch until the necessary biomass concentration is achieved. Thus, during the initiation or, in other words, starting of the process of the invention, the microbial or bacterial host cells preferably are (both in the seed reactor and the production reactor) in an uninduced state with respect to the nucleotide sequence encoding a protein form a phage which inhibits growth of

said microbial or bacterial host cells. When the required cell density is reached, the production in the production reactor can be induced by addition of the inducer – in other words: The production reactor now is in an induced state with respect to the nucleotide sequence encoding a protein form a phage which inhibits growth of said microbial or bacterial host cells. Additionally, the continuous process can be started by transferring microbial or bacterial host cells from the seed reactor to the production reactor, e.g. by starting a flow of culture medium into the seed reactor, thereby creating a flow from the seed reactor outlet to the production reactor inlet. Thus, in the continuous process of the invention, the cell growth is preferably inhibited in the production reactor.

[0114] As described herein, the seed reactor is for propagation of the host cells. Accordingly, in the continuous fermentation process of the invention, (a) preferably is for biomass production.

[0115] In the continuous fermentation process of the invention, in (c) growth of said bacterial host cells preferably is inhibited by culturing said bacterial host cells in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, while said nucleotide of interest or said protein of interest is produced.

[0116] In the process of the invention, (a) may further comprise culturing said bacterial host cells in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells in culture medium in a production reactor for biomass production.

[0117] In the process of the invention, (c) may further comprise inducing said first inducible promoter controlling said nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells.

[0118] In the process of the invention, (b) or (c) may comprise inducing said second inducible promoter controlling said nucleotide of interest or said nucleotide encoding the protein of interest.

[0119] In the process of the invention, (a), (b) or (c) may comprise inducing said third inducible promoter controlling said nucleotide sequence encoding said heterologous RNA polymerase.

[0120] The process of the invention may further comprise (d) harvesting the product resulting from the production of said nucleotide sequence of interest. The harvesting may be done continuously or a defined volume can be removed from the production reactor on a regular basis.

[0121] Since the host cells continuously are replaced by the volume flow from the seed reactor, one advantage of the present invention is that the production reactor comprises genetic stable host cells or, in other words, cell division is stopped in the production reactor and adaptive evolution (leading to genetic instability) is hindered. Additionally, microbial or bacterial host cells

preferably are replaced before they can lose their expression plasmids. Accordingly, the bacterial host cells in the at least one production reactor preferably are genetically stable for at least about 5 days, preferably at least about 7 days, preferably at least about 10 days, more preferably at least 13 days. The genetic stability may also be accompanied by the option to operate the process for a longer period of time, thereby enabling automation of the process. Thus, the process of the invention may be operated for at least about 5 days, preferably at least about 7 days, preferably at least about 10 days, more preferably at least 13 days. A further possibility to genetically stabilize a plasmid-based continuous production system can be the use of auxotrophic markers in combination with an auxotrophic host strain.

[0122] The present invention further relates to a continuous fermentation process for the production of a heterologous nucleotide sequence of interest by microbial host cells comprising said heterologous nucleotide sequence of interest, wherein growth of said microbial host cells can be decoupled from production of said heterologous nucleotide sequence of interest through induction of genetic inhibition of growth of said microbial host cells, comprising

(a) culturing said microbial host cells in an uninduced state with respect to the genetic inhibition of growth in culture medium in a seed reactor for biomass production;

(b) transferring at least an amount of said bacterial host cells from said seed reactor to culture medium of a production reactor; and

(c) culturing said bacterial host cells in said production reactor in an induced state with respect to the genetic inhibition of growth, thereby growth of said microbial host cells is genetically inhibited, while said nucleotide sequence of interest is produced;

wherein the seed reactor and production reactor is configured as an independent continuous fermentor and wherein the seed reactor and production reactor are directly connected with each other.

[0123] Preferably, genetic inhibition of growth is accomplished by a protein from a phage which inhibits growth of said bacterial host cells, said protein from a phage is encoded by a nucleotide sequence which is under the control of a first inducible promoter and is comprised by said bacterial host cells. Preferably, genetic inhibition of growth is accomplished by repressing bacterial DNA replication [e.g., targeting dnaA and oriC] or blocking nucleotide synthesis [e.g., targeting pyrF or thyA] through CRISPR interference technology.

[0124] The term “microbial host cell” as used herein relate to microscopically small organisms that are not visible to the naked eye as individuals, into which a nucleic acid comprising an expression cassette or vector has been introduced, i.e. which has been genetically-engineered. Most microorganisms are unicellular organisms, but they may also include small organisms (fungi, algae, yeast) of corresponding size. Microorganisms include, but are not limited to,

bacteria (e.g. lactic acid bacteria), many fungi including yeasts such as baker's yeast, microscopic algae (e.g. chlorella) and protozoa. Preferably, the microbial host cells are fungal host cells, yeast host cells or bacterial host cells. Most preferably, the microbial host cells are bacterial host cells.

[0125] The term "bacterial host cell", as used herein, is intended to refer to any prokaryotic cell, into which a nucleic acid comprising an expression cassette or vector has been introduced, i.e. which has been genetically-engineered. A preferred example of a prokaryotic host cell is E. coli. However, also *Pseudomonas species, Salmonella species, Bacillus species, Lactobacillus species, Corynebacterium species, Microbacterium species* or *Actinomycetes* species are envisaged. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. A recombinant host cell may be an isolated cell, preferably grown in culture. In a preferred embodiment or the present invention the bacterial host cell is *E. coli*. In a more preferred embodiment, the bacterial host cell is *E. coli* B-lineage such as *E. coli* BL21 or more preferably *E. coli* BL21(DE3). The *E.* coli may also be from the K-lineage such as *E. coli* K12 or W-lineage. In a preferred embodiment, the bacterial host cell is *E. coli* BL21(DE3)::TN7(PPΔAra), wherein “PP” relates to a protein from a phage as described herein. The bacterial host cell may thus comprise a knockout of araABCD and a protein from a phage preferably under the control of an inducible promoter and preferably integrated at the attTN7 site. The protein from a phage may however also be integrated at another suitable site. In a more preferred embodiment, the bacterial host cell is *E. coli* BL21(DE3)::TN7(Gp2ΔAra), wherein Gp2 preferably has the amino acid sequence shown in SEQ ID NO: 1 or a fragment thereof which inhibits bacterial host cell RNA polymerase or has an identity of 40% or more to the amino acid sequence shown in SEQ ID NO: 1 and which inhibits bacterial host cell RNA polymerase. In a preferred embodiment, Gp2 is SEQ ID NO: 1. *E. coli* BL21(DE3)::TN7(Gp2ΔAra), wherein Gp2 has the amino acid sequence shown in SEQ ID NO: 1 is also described herein as “enGenes-X-press”. The bacterial host cells described herein may be created as described in WO 2016/174195, hereby incorporated by reference.

[0126] A skilled artisan is aware of genetic engineering techniques known in the art in order to generate a bacterial host cell for use in the system or process of the invention. For example, various kits are available for genetic engineering of bacterial host cell for the integration of nucleic acids comprising nucleotide sequences into a bacterial genome, either randomly or targeted; see e.g. Zhang et al. (1998), Nature Genetics 20, 123-128 or Sharan et al. (2009), Nature Protocols 4(2), 206-223. A skilled artisan is further aware of techniques for the

transformation of bacterial host cell as well as with any other cloning technique which he can use for the generation of extrachromosomal elements such as plasmids, cosmids, bacmids, etc.

[0127] The term “growth“ of the host cell as used herein means an increase of cell number due to cell division.

[0128] A promoter sequence as used herein is a non-coding expression control sequence preferably inserted nearby the start of the coding sequence of the expression cassette and regulates its expression. Put into a simplistic yet basically correct way, it is the interplay of the promoter with various specialized proteins called transcription factors that determine whether or not a given coding sequence may be transcribed and eventually translated into the actual protein encoded by the gene. It will be recognized by a person skilled in the art that any compatible promoter can be used for recombinant expression in host cells. The promoter itself may be preceded by an upstream activating sequence, an enhancer sequence or combination thereof. These sequences are known in the art as being any DNA sequence exhibiting a strong transcriptional activity in a cell and being derived from a gene encoding an extracellular or intracellular protein. It will also be recognized by a person skilled in the art that termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

[0129] The term “inducible promoter” as used herein refers to a promoter that regulates the expression of an operably linked gene or functional RNA in response to the presence or absence of an endogenous or exogenous stimulus. Such stimuli can be but are not limited to chemical compounds or environmental signals such as temperature shifts. Examples for inducible promoters include, but are not limited to, pBAD, OR2-OR1-PR, pLtetO, pLlacO, PesR, pLac, lacUV, Tac promoter, pPrpB, pTetO, FixK2, pLtetO-1 or PcpcG2.

[0130] In one embodiment, the bacterial host cell comprises the nucleotide of interest or a nucleotide encoding the protein of interest. In a preferred embodiment, the bacterial host cell comprises a nucleotide encoding the protein of interest.

[0131] As outlined herein, the production of the nucleotide or protein of interest preferably takes place in the production reactor. Accordingly, the nucleotide of interest or the protein of interest preferably is produced in the at least one production reactor.

[0132] The system and the process of the present invention are both suitable for the production of a nucleotide of interest or a protein of interest; they thus cannot only be used for the production of proteins but also to produce nucleotides, such as plasmids, in high amounts.

[0133] The terms “nucleotide”, "nucleotide sequence" or "nucleic acid molecule" as used herein may be used interchangeably and refer to a polymeric form of nucleotides (i.e. polynucleotide) which are usually linked from one deoxyribose or ribose to another. The term "nucleotide sequence" preferably includes single and double stranded forms of DNA or RNA. A nucleic acid

molecule of this invention may include both sense and antisense strands of RNA (containing ribonucleotides), cDNA, genomic DNA, and synthetic forms and mixed polymers of the above. They may be modified chemically or biochemically or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those of skill in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), pendent moieties (e.g., polypeptides), intercalators (e.g., acridine, psoralen, etc.), chelators, alkylators, and modified linkages (e.g., alpha anomeric nucleic acids, etc.) Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

[0134] In this regard, a nucleic acid being an expression product is preferably a DNA such as a plasmid, whereas a nucleic acid to be introduced into a cell is preferably DNA, e.g. genomic DNA or cDNA.

[0135] The nucleic acid molecule preferably is a plasmid, minichromosome, or RNA.

[0136] The nucleic acid can be in any topological conformation. For instance, the nucleic acid can be single-stranded, double-stranded, triple-stranded, quadruplexed, partially double-stranded, branched, hairpinned, circular, or in a padlocked conformation.

[0137] The nucleotide may be a nucleic acid molecule.

[0138] The nucleotide of interest preferably is inducible, e.g. by specific environmental conditions. The nucleotide of interest may be capable of runaway replication, e.g. as described in Camps (2010), Rect Pat DNA Gene Seq, 4(1):58-73, hereby incorporated by reference. The induction of the nucleotide of interest, which is suited for runaway replication, in the production reactor may be done, e.g., by amino acid starvation in the production bioreactor.

[0139] A "polypeptide" refers to a molecule comprising a polymer of amino acids linked together by peptide bonds. Said term is not meant herein to refer to a specific length of the molecule and is therefore herein interchangeably used with the term “protein”. When used herein, the term “polypeptide” or “protein” also includes a “polypeptide of interest” or “protein of interest”, which is expressed by the expression cassettes or vectors or can be isolated from the host cells of the invention. A protein of interest also includes proteins which may potentially be harmful or even toxic for host cells.

[0140] Examples of a protein of interest are enzymes more preferably an amylolytic enzyme, a lipolytic enzyme, a proteolytic enzyme, a cellulytic enzyme, an oxidoreductase or a plant cell-wall degrading enzyme; and most preferably an enzyme having an activity selected from the group consisting of aminopeptidase, amylase, amyloglucosidase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, desoxyribonuclease, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannosidase, oxidase, pectinase, peroxidase, phytase, phenoloxidase, polyphenoloxidase, protease, ribonuclease, transferase, transglutaminase, and xylanase. Furthermore, a protein of interest may also be a growth factor, cytokine, receptors, receptor ligands, therapeutic proteins such as interferons, BMPs, GDF proteins, fibroblast growth factors, peptides such as protein inhibitors, membrane proteins, membrane-associated proteins, peptide/protein hormones, peptidic toxins, peptidic antitoxins, antibody or functional fragments thereof such as Fab or $F(ab)_2$ or derivatives of an antibody such as bispecific antibodies (for example, scFvs), chimeric antibodies, humanized antibodies, single domain antibodies such as Nanobodies or domain antibodies (dAbs) or an anticalin and others.

[0141] A "polypeptide" as used herein encompasses both naturally-occurring and non-naturally-occurring proteins, and fragments, mutants, derivatives and analogues thereof. Polypeptides may be a polypeptide homologous (native) or heterologous to the host cell. The polypeptide of interest may also encompass a polypeptide native to the host cell, which is encoded by a nucleic acid sequence, which expression is controlled by one or more control sequences foreign to the nucleic acid sequence encoding the polypeptide. Polypeptides may be of any length. Polypeptides include proteins and/or peptides of any activity or bioactivity. A “peptide” encompasses analogues and mimetics that mimic structural and thus biological function.

[0142] Polypeptides may further form dimers, trimers and higher oligomers, i.e. consisting of more than one polypeptide molecule. Polypeptide molecules forming such dimers, trimers etc. may be identical or non-identical. The corresponding higher order structures are consequently termed homo- or heterodimers, homo- or heterotrimers etc.

[0143] Further, a polypeptide may comprise a number of different domains each of which has one or more distinct activities.

[0144] The nucleic acid sequence encoding a protein of the present invention or protein of interest may be obtained from any prokaryotic, eukaryotic, or other source.

[0145] As described herein, a nucleotide sequence encoding a protein of the present invention or protein of interest is preferably regulated by a (second) promoter. Said (second) promoter is preferably specifically transcribed by an RNA polymerase that is heterologous for said host cell

and the expression of which may be inducible. However, said RNA polymerase may also be constitutively expressed.

[0146] The nucleotide of interest may encode more than one protein of interest to, e.g., encode a complete biosynthesis pathway or cluster. Accordingly, the nucleotide of interest may encode one or more proteins of interest.

[0147] In the two stage process and system of the invention, the growth of the microbial or bacterial host cells is inhibited in the production reactor. Thereby, the microbial or bacterial host cells concentrate on the production while growth that would disturb the production, is inhibited.

[0148] When "growth of a bacterial host cell" is inhibited as described herein, growth may be inhibited by inhibition of transcription, DNA-replication and/or cell division. Accordingly, it is preferred that a phage protein, particularly one or more of the phage proteins described herein, inhibits transcription, DNA-replication and/or cell division.

[0149] "Genetic inhibition" as used herein relates to inhibition of growth of the microbial or bacterial host cell by expressing a molecule, e.g. a protein or RNA, that interferes with the growth of the microbial or bacterial host cell. Such a protein may be a phage protein.

[0150] A "phage protein" when referred to herein is a protein from a (bacterio)phage. A phage infects bacteria and is able to replicate in said bacterium. When infecting a bacterium and replicating in said bacterium a phage may have one or more proteins that inhibit growth of said bacterium, e.g., by inhibiting transcription, DNA-replication and/or cell division in order to fully exploit the protein synthesis machinery of said bacterium.

[0151] Accordingly, the present invention can be put into practice with any phage protein that effects the inhibition of the growth of the bacterial host cell by causing, e.g. a host transcription shut-off. In this case the bacterial host cell comprises under the control of an inducible promoter a nucleotide sequence encoding a protein from a phage which causes a transcription shut-off of said bacterial host cell. The term "host transcription shut-off" as used herein relates to the inhibition of transcription of the bacterial host cell. Proteins that can be used to cause a host transcription shut-off are described herein, such as Gp2 (e.g. SEQ ID NO: 1), GP0.7 (e.g. SEQ ID NO: 3), Nun (e.g. SEQ ID NO: 2), Gp6 (e.g. SEQ ID NO: 4), Gp8 (e.g. SEQ ID NO: 5), A* (e.g. SEQ ID NO: 6), YkzG Epsilon-Subunit (e.g. SEQ ID NO: 7), preferably Gp2. However, further proteins that effect a host transcription shut-off may be used as well to put the present invention into practice. Such proteins are for example Bacillus phage SPO1 GP40 (e.g. SEQ ID NO: 8), Staphylococcus phage G1 GP67 (e.g. SEQ ID NO: 9), Thermus thermophilus phage P23-45 GP39 (e.g. SEQ ID NO: 10), Enterobacteria phage PhiEco32 GP79 (e.g. SEQ ID NO: 11), Xanthomonas oryzae bacteriophage Xp10 P7 protein (e.g. SEQ ID NO: 12), Enterobacteria phage T4 Alc protein (e.g. SEQ ID NO: 13), Enterobacteria phage T4 Asia

protein (e.g. SEQ ID NO: 14). Another example for a phage protein which inhibits growth of the bacterial host cell is T7 Gp5.7 (e.g. SEQ ID NO: 17).

[0152] Accordingly, the phage protein of the present invention is preferably

(i) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 1 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 1, the protein is preferably capable of inhibiting bacterial host cell RNA polymerase;

(ii) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 2 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 2, the protein is preferably capable of inhibiting bacterial host cell RNA polymerase;

(iii) a protein which phosphorylates bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 3 or a fragment thereof which phosphorylates bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 3, the protein is preferably capable of phosphorylating bacterial host cell RNA polymerase;

(iv) a protein which inhibits bacterial host cell DNA replication, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 4 or a fragment thereof which inhibits bacterial host cell DNA replication; or

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 4, the protein is preferably capable of inhibiting bacterial host cell DNA replication;

(v) a protein which inhibits bacterial host cell DNA replication, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 5 or a fragment thereof which inhibits bacterial host cell DNA replication; or

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 5, the protein is preferably capable of inhibiting bacterial host cell DNA replication; or

(vi) a protein which inhibits bacterial host cell DNA replication, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 6 or a fragment thereof which inhibits bacterial host cell DNA replication; or

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 6 , the protein is preferably capable of inhibiting bacterial host cell DNA replication;

(vii) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 7 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 7, the protein is preferably capable of inhibiting bacterial host cell RNA polymerase;

(viii) a protein which causes host transcription shut-off, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 8, 9, 10, 11, 12, 13, 14 or a fragment thereof which causes host transcription shut-off; or

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 8, 9, 10, 11, 12, 13 or 14, the protein is preferably capable of causing host transcription shut-off; or

(ix) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 17 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 17 and which inhibits bacterial host cell RNA polymerase.

[0153] In a further preferred embodiment of the present invention said nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell, said nucleotide sequence encoding said RNA polymerase, said nucleotide sequence encoding a protein of interest, is integrated into the genome of said host cell or is comprised by an extrachromosomal vector.

[0154] The term "vector" as used herein refers to a nucleic acid sequence into which an expression cassette comprising nucleotide of interest or a gene of the present invention or gene encoding the protein of interest may be inserted or cloned. Furthermore, the vector may encode an antibiotic resistance gene conferring selection of the host cell. Preferably, the vector is an expression vector.

[0155] The vector may be capable of autonomous replication in a host cell (e. g., vectors having an origin of replication which functions in the host cell). The vector may have a linear, circular, or supercoiled configuration and may be complexed with other vectors or other material for certain purposes.

[0156] Vectors used herein for expressing an expression cassette comprising a gene of the present invention or gene encoding the protein of interest usually contain transcriptional control elements suitable to drive transcription such as e.g. promoters, enhancers, polyadenylation signals, transcription pausing or termination signals as elements of an expression cassette. For proper expression of the polypeptides, suitable translational control elements are preferably included in the vector, such as e.g. preferably optimized 5' untranslated regions leading to ribosome binding sites (RBS) suitable for recruiting ribosomes and stop codons to terminate the translation process. In particular, the nucleotide sequence serving as the selectable marker genes as well as the nucleotide sequence encoding the protein of interest can be transcribed under the control of transcription elements present in appropriate promoters. The resultant transcripts of the selectable marker genes and that of the protein of interest harbour functional translation elements that facilitate substantial levels of protein expression (i.e. translation) and proper translation termination. In a preferred embodiment of the invention the selectable marker used is an auxotrophic marker gene used in a host strain containing an knock-out or deletion of said auxotrophic marker gene.

[0157] The vector may comprise a polylinker (multiple cloning site), i.e. a short segment of DNA that contains many restriction sites, a standard feature on many plasmids used for molecular cloning. Multiple cloning sites typically contain more than 5, 10, 15, 20, 25, or more than 25 restrictions sites. Restriction sites within an MCS are typically unique (i.e., they occur only once within that particular plasmid). MCSs are commonly used during procedures involving molecular cloning or sub cloning.

[0158] One type of vector is a plasmid, which refers to a circular double stranded DNA loop into which additional DNA segments may be introduced via ligation or by means of restriction-free cloning. Other vectors include cosmids, bacterial artificial chromosomes (BAC), yeast artificial chromosomes (YAC) or mini-chromosomes. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome.

[0159] The expression cassette comprising a gene of the present invention or gene encoding the protein of interest is inserted into the expression vector as a DNA construct. This DNA construct can be recombinantly made from a synthetic DNA molecule, a genomic DNA molecule, a cDNA molecule or a combination thereof. The DNA construct is preferably made by ligating the different fragments to one another according to standard techniques known in the art.

[0160] The expression cassette comprising a gene of the present invention or gene encoding the protein of interest may be part of the expression vector. Preferably, the expression vector is a DNA vector. The vector conveniently comprises sequences that facilitate the proper expression of the gene encoding the protein of interest and the antibiotic resistance gene.

These sequences typically comprise but are not limited to promoter sequences, transcription initiation sites, transcription termination sites, and polyadenylation functions as described herein.

[0161] The expression cassettes may comprise an enhancer and/or an intron. Usually, introns are placed at the 5' end of the open reading frame. Accordingly, an intron may be comprised in the expression cassette for expressing the polypeptide of interest in order to increase the expression rate. Said intron may be located between the promoter and or promoter/enhancer element and the 5' end of the open reading frame of the polypeptide to be expressed. Several suitable introns are known in the state of the art that can be used in conjunction with the present invention.

[0162] The expression cassette or vector according to the invention which is present in the host may either be integrated into the genome of the host or it may be maintained in some form extrachromosomally.

[0163] Furthermore, the expression cassettes may comprise an appropriate transcription termination site. This, as continued transcription from an upstream promoter through a second transcription unit may inhibit the function of the downstream promoter, a phenomenon known as promoter occlusion or transcriptional interference. This event has been described in both prokaryotes and eukaryotes. The proper placement of transcriptional termination signals between two transcription units can prevent promoter occlusion. Transcription termination sites are well characterized and their incorporation in expression vectors has been shown to have multiple beneficial effects on gene expression.

[0164] The terms " 5’ " and " 3’ “ used herein refer to a convention used to describe features of a nucleotide sequence related to either the position of genetic elements and/or the direction of events (5’ to 3’), such as e.g. transcription by RNA polymerase or translation by the ribosome which proceeds in 5' to 3' direction. Synonyms are upstream (5') and downstream (3'). Conventionally, nucleotide sequences, gene maps, vector cards and RNA sequences are drawn with 5' to 3' from left to right or the 5' to 3' direction is indicated with arrows, wherein the arrowhead points in the 3' direction. Accordingly, 5' (upstream) indicates genetic elements positioned towards the left hand side, and 3' (downstream) indicates genetic elements positioned towards the right hand side, when following this convention.

[0165] The term "expression" as used herein means the transcription of a nucleotide sequence. Said nucleotide sequence encodes preferably a protein. Accordingly, said term also includes the production of mRNA (as transcription product from a nucleotide sequence) and translation of this mRNA to produce the corresponding gene product, such as a polypeptide, or protein.

[0166] The gene of interest, i.e. the nucleotide of interest or the nucleotide of interest, may be integrated into the genome of the microbial or bacterial host cell, e.g. by homologous recombination at a pre-selected site. An exemplary method for the genome integration of the gene of interested is described in WO 2008/142028, incorporated herewith in its entirety.

[0167] The RNA polymerase is advantageously heterologous to the bacterial host cell which comprises a nucleotide sequence encoding said RNA polymerase. “Heterologous” means that the RNA polymerase is not naturally occurring in said bacterial host cell, i.e., said bacterial host cell does not naturally comprise said RNA polymerase, unless a nucleotide sequence encoding said RNA polymerase is introduced in said bacterial host cell in accordance with the teaching of the present invention by means and methods known in the art. The RNA polymerase is thus ideally insensitive to a phage protein which inhibits growth of said bacterial host cell.

[0168] Preferably, the RNA polymerase is bacteriophage T3 RNA polymerase, T7 bacteriophage RNA polymerase, engineered orthogonal T7 RNA polymerase, bacteriophage SP6 RNA polymerase or bacteriophage Xp10 RNA polymerase. Further RNA polymerases, such as engineered orthogonal T7 polymerases are described in Temme et al. (2012), Nucleic Acids Research 40(17), 8773-8781. Preferably, the RNA polymerase is the T7 bacteriophage RNA polymerase.

[0169] In a preferred embodiment of the present invention the nucleotide sequence encoding the RNA polymerase is under the control of an inducible or constitutive promoter. Examples of inducible promoters are described herein in the context of an inducible promoter which controls a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell. These inducible promoters are also preferred examples for an inducible promoter that controls the RNA polymerase as described herein below.

[0170] In general, a second nucleotide or protein of interest could be under the control of a promoter that is recognized by a second heterologous RNA polymerase that is different to the first heterologous RNA polymerase to allow e.g. different expression levels of two different nucleotides or proteins of interest.

[0171] Preferably, the inducible promoter which controls a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell is regulated by arabinose, IPTG, tryptophan, xylose, lactose, rhamnose, phosphate, propionate, benzoic acid, phage lambda cI protein or heat.

[0172] As regards inducible promoters that control a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell as or that control a nucleotide sequence encoding said RNA polymerase, it is preferred that the same inducible promoters are applied. Preferred examples are described herein. This preferred embodiment allows to

simultaneously induce expression of the phage protein and the RNA polymerase in order to uncouple growth and nucleic acid/protein production. However, of course, also different inducible promoters can be used in accordance with the teaching of the present invention. Accordingly, the first, second or third promoter may be different.

[0173] In a preferred embodiment of the bacterial host cell of the process or system of the invention, said host cell has a non-functional arabinose operon.

[0174] The systems and the processes of the present invention relate on the use of bioreactors, preferably at least two separate bioreactors. In one bioreactor, the seed reactor, the microbial or bacterial cells are cultured and expanded, wherein in the second bioreactor, the production reactor, the growth of the microbial or bacterial cell is inhibited and the production of the nucleotide or protein of interest is induced. The first and the second bioreactor preferably are coupled to allow the transfer of new microbial or bacterial host cells from the seed to the production reactor. Thus, each of the first and the second bioreactor has at least one inlet and at least one outlet.

[0175] "Bioreactor" or "reactor", including the "seed bioreactor" or the "production bioreactor", as used herein bioreactor refers to a reaction vessel for fermentation for production of cells and biosynthetic products, which may range in size from benchtop fermenters to industrial tanks. Bioreactors preferably allow automatic regulation of the flow of oxygen, culture medium and other nutrients, and maintaining the temperature and pH; they preferably minimize the potential for contamination and can produce a higher density of cells than can be produced in traditional cultures.

[0176] "Seed reactor" as used herein in the process and the system of the invention relates to the bioreactor that is used for propagation of the microbial or bacterial host cell. In the seed reactorthe microbial or bacterial host cell is in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said microbial or bacterial host cell. Otherwise, propagation in the seed reactor could not take place.

[0177] The term "uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell" relates to a microbial or bacterial host cell, in which the promoter of the gene encoding the protein from a phage which inhibits growth of the microbial or bacterial host cell has not been induced. As described herein, inhibition of the growth of the microbial or bacterial host cell is inhibited by the expression of a protein from a phage. Since the protein from a phage is under the control of an inducible promoter, the uninduced state within the context of the invention thus means that the inducible promoter has not been induced. Uninduced in this context may mean, e.g. that in case of a chemically inducible promoter the inducer is not or not in sufficient concentration present in the

culture medium, or that the effects of the inducer are impaired by other factors (e.g. the presence of glucose may suppress induction of a lac operon even in the inducer lactose is present) or in case of a temperature-sensitive promoter, the temperature for activation is not reached. This is preferred in the seed reactor. Accordingly, the microbial or bacterial host cell preferably is cultured in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in the seed reactor for biomass production.

[0178] Accordingly, the seed reactor preferably does not comprise or does not essentially comprise an inducer for the first inducible promoter. The first inducible promoter controls a nucleotide sequence encoding a protein from a phage which inhibits growth of said microbial or bacterial host cell. The seed reactor preferably is substantially free of an inducer for the first promoter, i.e. preferably in a concentration that does not induce a detectable expression of the protein from a phage which inhibits growth of said microbial or bacterial host cell ("substantially free").

[0179] "Production reactor" as used herein in the process and the system of the invention relates to the bioreactor that is used for the production of the nucleotide or protein of interest. In the production reactor, the growth of the host cells is inhibited by inducing the first promoter, and the production of the production of the nucleotide or protein of interest, which is under control of the second inducible promoter, is induced. Accordingly, the microbial or bacterial host cell is in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said microbial or bacterial host cell. Otherwise, production in the production reactor could not take place.

[0180] Accordingly, the microbial or bacterial host cell preferably is in an "induced state with respect to the nucleotide sequence encoding a protein from a phage" in the production reactor. Thus, in this case the inducible promoter is induced and the growth of the microbial or bacterial host cell is inhibited. Accordingly, the microbial or bacterial host cell is cultured in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in the production reactor for production of said nucleotide sequence of interest by said bacterial host cells.

[0181] Accordingly, the production reactor or the at one of the least one production reactor comprises an inducer for the first inducible promoter. The first inducible promoter controls a nucleotide sequence encoding a protein from a phage which inhibits growth of said microbial or bacterial host cell.

[0182] Since the nucleotide or protein of interest is under the control of the second inducible promoter, the production reactor preferably comprises and inducer for the second inducible promoter. Accordingly, the at least one of the at least one production reactors comprises an inducer for the second inducible promoter.

[0183] The inducer for the first and the second inducible promoter may be different. The inducer for the first and the second inducible promoter may be the same. Accordingly, the first inducible promoter and the second inducible promoter preferably can be induced by the same inducer. The first and the second inducible promoter may be the same.

[0184] Bioreactors often comprise a variety of sensors that monitor important parameters such as pH, dissolved oxygen or temperature and means for controlling these parameters.

[0185] Accordingly, the seed reactor preferably comprises a means for regulating pH. The at least one production reactor preferably comprises a means for regulating pH. Means for regulating the pH may include a pH probe, a device for recording the signal of the pH probe, and/or a device for adding acid or base to the culture medium, preferably base.

[0186] The seed reactor preferably comprises means for regulating dissolved oxygen. The at least one production reactor preferably comprises means for regulating dissolved oxygen. Means for regulating dissolved oxygen may comprise a pO_2 probe, a control system for regulating pO_2 by pressure, ventilation rate, addition of oxygen, control of stirring and/or the like.

[0187] The seed reactor preferably comprises means for regulating temperature. The at least one production reactor preferably comprises means for regulating temperature. Means for regulating the temperature may comprise a thermometer, a heating device, a cooling device, wherein the temperature control by the heating and/or the cooling device preferably is carried out by heating and/or cooling the mantle of the bioreactor.

[0188] The seed reactor preferably comprises a gas inlet and a gas outlet and a means for regulating the gas flow. The at least one production reactor preferably comprises a gas inlet and a gas outlet and a means for regulating the gas flow. The at least one production reactor preferably comprises a biomass sensor.

[0189] Accordingly, the production reactor preferably comprises a means for regulating pH. The at least one production reactor preferably comprises a means for regulating pH. Means for regulating the pH may include a pH probe, a device for recording the signal of the pH probe, and/or a device for adding acid or base to the culture medium, preferably base.

[0190] The production reactor preferably comprises means for regulating dissolved oxygen. The at least one production reactor preferably comprises means for regulating dissolved oxygen. Means for regulating dissolved oxygen may comprise a pO_2 probe, a control system for regulating pO_2 by pressure, ventilation rate, addition of oxygen, control of stirring and/or the like.

[0191] The production reactor preferably comprises means for regulating temperature. The at least one production reactor preferably comprises means for regulating temperature. Means for regulating the temperature may comprise a thermometer, a heating device, a cooling device,

wherein the temperature control by the heating and/or the cooling device preferably is carried out by heating and/or cooling the mantle of the bioreactor.

[0192] The production reactor preferably comprises a gas inlet and a gas outlet and a means for regulating the gas flow. The at least one production reactor preferably comprises a gas inlet and a gas outlet and a means for regulating the gas flow. The at least one production reactor preferably comprises a biomass sensor.

[0193] The biomass concentration may be regulated in the seed reactor by feed inflow and/or biomass outflow. The biomass concentration may be regulated in the at least one production reactor by feed inflow, biomass inflow, and/or biomass outflow.

[0194] The gas flow may be regulated in the at least one production reactor. The gas flow may be regulated in the at least one seed reactor.

[0195] The seed and the production reactor(s) are linked or coupled to allow the transfer of microbial or bacterial host cells from the seed reactor(s) to the production reactor(s). The flow of the culture medium comprising the host cells from the seed reactor to the production reactor preferably is controlled. This control can be done, e.g., by applying a chemostat or a turbidostat. Accordingly, the system of the present invention preferably further comprises (c) means for operating the seed and production reactors as linked chemostats or turbidostats. Preferably, the seed reactor outflow serves as inflow to the production reactor.

[0196] A “chemostat” (from chemical environment is static) as used herein relates to a bioreactor to which fresh medium, preferably comprising the microbial or bacterial host cells grown in the seed reactor, is continuously added (to the production reactor), while culture liquid containing left over nutrients, metabolic end products and the microbial or bacterial host cells, preferably comprising the nucleotide or protein of interest, are continuously removed at the same rate to keep the culture volume constant. One of the most important features of chemostats is that the microbial or bacterial host cells can be kept in a physiological steady state under constant environmental conditions. In this steady state, all culture parameters preferably remain constant (culture volume, dissolved oxygen concentration, nutrient and product concentrations, pH, cell density, etc.) as well as the number of the microbial or bacterial host cells (in the production reactor). In addition, environmental conditions can be controlled.

[0197] A “turbidostat” as used herein relates to a continuous microbiological culture device, similar to a chemostat, which has feedback between the turbidity of the culture vessel and the dilution rate. A turbidostat dynamically adjusts the flow rate (and therefore the dilution rate) to make the turbidity constant. At steady state, operation of both the chemostat and turbidostat are identical. While most turbidostats use a spectrophotometer/turbidimeter to measure the optical density for control purposes, there exist other methods, such as dielectric permittivity.

[0198] The system of the invention preferably comprises a first feed container containing a first feed medium comprising a carbon source, wherein the first feed container is operably connected to an inlet of the seed reactor, wherein the system preferably comprises means for regulating feed flow from the first feed container to the seed reactor.

[0199] "Carbon source" as used herein carbon source refers to the molecules used by an organism as the source of carbon for building its biomass. A carbon source can be an organic compound or an inorganic compound. Heterotrophs needs organic compounds as source of carbon and source of energy, while autotrophs can use inorganic compounds as carbon source and an abiotic sources of energy, as light (photoautotrophs) or inorganic chemical energy (chemolithotrophs). Examples for carbon sources include, but are not limited to, glucose, maltose, lactose, galactose, fructose, sorbitol, mannose, arabinose, xylose, ribose, glycerol, pyruvate, oxaloacetate, succinate, fumarate, malate or the like.

[0200] The "feed flow" describes the amount of culture medium, preferably comprising the microbial or bacterial host cell, that is transferred to the production reactor in a distinct period of time and may be expressed as volume per time, e.g. liters per minute. The feed flow may be regulated e.g. by an adjustable or controllable pump.

[0201] In one embodiment of the system of the invention, the system comprises a second feed container containing a second feed medium comprising a carbon source, wherein the second feed container is operably connected to an inlet of the at least one production reactor, wherein the system preferably comprises means for regulating feed flow from the second feed container to the at least one production reactor. The second feed may comprise the inducer.

[0202] The system of the present invention is not limited to a single seed reactor. Several seed reactors may be employed. Thus, the system of the invention may comprise at least one seed reactor, two seed reactors, three seed reactors, four seed reactors, five seed reactors or at least five seed reactors.

[0203] In one embodiment an outlet of the seed reactor and an outlet of a second feed reactor are connected to a mixing chamber, wherein an outlet of the mixing chamber is connected to an inlet of the at least one production reactor. This configuration has the advantage that the microbial or bacterial host cells can be contacted and/or homogenously mixed with the inducer prior to the introduction into the production reactor.

[0204] Just like a multitude of seed reactors may be used, also a plurality of production reactors can be used. Thus, the system or the process of the invention may comprise at least one bioreactor, at least two bioreactors, two bioreactors, at least three bioreactors, three bioreactors, at least four bioreactors, four bioreactors or at least five bioreactors.

[0205] The bioreactors of the present invention, i.e. the seed and/or the production reactor, may be any bioreactor suitable for the purposes of the present invention. In one embodiment, the seed reactor is a stirred tank reactor. In one embodiment, the seed reactor is a plug flow reactor. In one embodiment, the production reactor is a stirred tank reactor. In one embodiment, the production reactor is a plug flow reactor.

[0206] In one embodiment the outlet of the production reactor is connected to a cell retention system (e.g. cross-flow filtration system or a disc (stack) centrifuge) that preferably allows continuous separation of the cell free supernatant (culture medium) from the bacterial host cells (in case the retentate comprising the bacterial host cells is recirculated into the production reactor).

[0207] The size or the volume of the bioreactors may differ. The seed reactor preferably has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L. The at least one production reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L. The seed reactor preferably has a volume of up to 50 m^3, up to 45 m^3, up to 40 m^3, up to 35 m^3, up to 30 m^3, up to 25 m^3, up to 20 m^3, up to 15 m^3, up to 10 m^3, up to 5 m^3, up to 2.5 m^3 or up to 1 m^3. The production reactor preferably has a volume of up to 50 m^3, up to 45 m^3, up to 40 m^3, up to 35 m^3, up to 30 m^3, up to 25 m^3, up to 20 m^3, up to 15 m^3, up to 10 m^3, up to 5 m^3, up to 2.5 m^3 or up to 1 m^3.

[0208] The volume ratio of the seed reactor to the at least one production reactor may be from about 1:10 to about 2:1, from about 1:5 to about 2:1, from about 1:2 to about to about 2:1, from about 1.5:1 to about 1:1.5, or about 1:1.

[0209] In a preferred embodiment, the at least one production reactor comprises a culture medium comprising cells with a biomass concentration from about 10 to about 90 g/L cell dry weight, preferably from about 20 to about 80 g/L cell dry weight, preferably from about 30 to about 70 g/L cell dry weight, preferably from about 35 to about 60 g/L cell dry weight.

[0210] A large number of suitable methods exist in the art to produce polypeptides in host cells of the invention. Conveniently, the produced protein is harvested from the culture medium, lysates of the cultured host cell or from isolated (biological) membranes by established techniques. For example, an expression cassette comprising, inter alia, the nucleotide sequence encoding the protein of interest can be synthesized by PCR and inserted into the expression vector. Subsequently, a cell may be transformed with the expression vector. Thereafter, the cell is cultured to produce/express the desired protein(s), which is/are isolated and purified. For example, the product may be recovered from the host cell and/or culture

medium by conventional procedures including, but not limited to, cell lysis, breaking up host cells, centrifugation, filtration, ultra-filtration, extraction, evaporation, spray drying or precipitation. Purification may be performed by a variety of procedures known in the art including, but not limited to, chromatography (e.g. ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g. ammonium sulfate precipitation) or extraction.

[0211] "Isolating the protein of interest" refers to the separation of the protein of interest produced during or after expression of the vector introduced. In the case of proteins or peptides as expression products, said proteins or peptides, apart from the sequence necessary and sufficient for the protein to be functional, may comprise additional N- or C- terminal amino acid sequences. Such proteins are referred to as fusion proteins.

[0212] When a polypeptide of interest is expressed in a host cell of the invention, it may be necessary to modify the nucleotide sequence encoding said polypeptide by adapting the codon usage of said nucleotide sequence to meet the frequency of the preferred codon usage of said host cell. As used herein, "frequency of preferred codon usage" refers to the preference exhibited by the host cell of the invention in usage of nucleotide codons to specify a given amino acid. To determine the frequency of usage of a particular codon in a gene, the number of occurrences of that codon in the gene is divided by the total number of occurrences of all codons specifying the same amino acid in the gene. Similarly, the frequency of preferred codon usage exhibited by a host cell can be calculated by averaging frequency of preferred codon usage in a large number of genes expressed by the host cell. It is preferable that this analysis be limited to genes that are highly expressed by the host cell. The percent deviation of the frequency of preferred codon usage for a synthetic gene from that employed by a host cell is calculated first by determining the percent deviation of the frequency of usage of a single codon from that of the host cell followed by obtaining the average deviation over all codons. As defined herein, this calculation includes unique codons (i.e., ATG and TGG).

[0213] A tag may be used to allow identification and/or purification of the protein of interest. Accordingly, it is preferred that a protein of interest comprises a tag. Hence, a nucleotide sequence encoding a protein of interest preferably also encodes a tag which is advantageously genetically fused in frame to the nucleotide sequence encoding said protein of interest. Said tag may be at the C-or N-terminus of said protein of interest. Examples of tags that may be used in accordance with the invention include, but are not limited to, HAT, FLAG, c-myc, hemagglutinin antigen, His (e.g., 6xHis) tags, flag-tag, strep-tag, strepII-tag, TAP-tag, chitin binding domain (CBD), maltose-binding protein, immunoglobulin A (IgA), His-6-tag, glutathione-S-transferase (GST) tag, intein and streptavidin binding protein (SBP) tag.

[0214] A leader peptide tag may be used to target the protein of interest to the periplasmic space or to the extracellular space. Examples of leader peptide sequences in accordance with the invention are, but are not limited to, pelB (e.g. positions 1-19 of UniProt entry P14005, version 1 of the sequence), ompA (e.g. positions 1-21 of UniProt entry P0A910, version 1 of the sequence), torA (e.g. positions 1-39 of UniProt entry P33225, version 2 of the sequence), dsbA (e.g. positions 1-19 of UniProt entry P0AEG4, version 1 of the sequence), enterotoxin STII (e.g. positions 1-23 of UniProt entry P22542, version 1 of the sequence) and phoA (e.g. positions 1-21 of UniProt entry P00634, version 1 of the sequence).

[0215] The system and process of the present invention is also suitable for the production of a nucleotide of interest such as a plasmid. Instead of overexpressing the protein of interest, a nucleotide of interest is expressed in this embodiment. The nucleotide of interest may be a plasmid. In comparison its expression is not necessary to be induced but may be due to an intrinsic factor of the plasmid.

[0216] Thus, preferably the nucleic acid of interest is a plasmid, more preferably a plasmid with a high plasmid copy numbers. “Plasmid copy number” describes the average number of plasmid copies per cell. A “high” plasmid copy number describes plasmids with a plasmid copy number of at least 100, at least 200, at least 500, at least 1000 or even at least 5000.

[0217] After leaving the production reactor through the outlet, the microbial or bacterial host cells may be stored or directly processed. An exemplary process is described in WO 2004/085643, which is hereby incorporated by reference. However, isolation of nucleic acids such as plasmid DNA is well-known to a person skilled in the art. In general, the production of the nucleic acid of the invention includes the growth of the microbial/bacterial culture making use of the system or process of the invention, which is optionally followed by harvesting and lysis of the microbial/bacterial host cells and optionally the purification of plasmid DNA.

[0218] The first step of the processing usually is disintegrating the microbial/bacterial host cells by lysis, preferably alkaline lysis. The lysis reaction, in a preferred embodiment of the present invention, is performed according to methods known in the art, using an alkaline lysis solution that contains a detergent. A typical lysing solution consists of NaOH (0,2 M) and SDS (1%), but also other alkaline solutions and other detergents can be used (see e.g. WO 97/29190).

[0219] The lysing is followed by neutralization of the alkaline solution. Also this step may, in principle, be performed according to methods known per se, preferably according to methods that are gentle and can be run in a continuous and automated mode. In a preferred embodiment, in the neutralization step the lysed cell solution is mixed with the neutralizing solution. Typically a buffered solution with acidic pH and high salt concentration is used for

neutralization. Preferable this solution consists of 3 M potassium acetate (KAc) at pH 5.5. But also other neutralizing salts can be used or added.

[0220] The plasmid DNA may then be precipitated by adding an aqueous solution comprising about 70% ethanol. By centrifugation, the nucleic acids may be pelleted and washed by aspirating the supernatant, resuspending the nucleic acid comprising pellet in the aqueous solution comprising about 70% ethanol followed by pelleting by centrifugation again. After washing, the pelleted nucleic acid may be dried.

[0221] Alternatively, or additionally to this process, also a nucleic-acid binding resin in form of a column may be used. Before capturing/purification by means of a resin, it may be necessary to adjust the parameters of the solution (like salt composition, conductivity, pH- value) to ensure binding of the desired biomolecule to the chromatographic support, usually a resin (this step is, in the meaning of the present invention, termed "conditioning step"). The simplest conditioning procedure is dilution of the cleared lysate with water or low salt buffer, especially in case the chromatographic resin in the subsequent capture step is achieved by anion exchange chromatography (WO 97/29190 Al). Furthermore, in particular when hydrophobic interaction chromatography is used as first purification step, a high concentration salt solution may be added and the possibly resulting precipitate (which is present if a certain salt concentration in the solution is exceeded) separated by filtration or centrifugation (WO 02/04027). In the case ammonium sulfate is used in high concentrations, this treatment reduces the RNA content (WO 98/11208).

[0222] For capturing and purification several steps are applied to obtain a highly purified biomolecule which meets the requirements for pharmaceuticals. As for the previous steps, enzymes, detergents and organic solvents should be avoided. Isolation and purification are performed according to methods known in the art, in particular by a combination of different chromatographic techniques (anion exchange chromatography AIEC, hydrophobic interaction chromatography HIC, size exclusion chromatography (SEC), ultra(dia)filtration, filtration or precipitation and extraction. A method that may advantageously be used, in particular for obtaining pDNA for therapeutic applications, comprises a combination of two steps that are based on different chromatographic principles, in which either of the two steps is selected from hydrophobic interaction chromatography (HIC), polar interaction chromatography (PIC) and anion exchange chromatography (AIEC) and in which at least in one of the two steps, preferably in both steps, the chromatographic support is a porous monolithic bed, preferably a rigid methacrylate-based monolith in the form of a monolithic column. Suitable monolithic columns are commercially available under the trademark CLM® from BIA Separations). This purification process may advantageously be performed with a chromatographic support in the form of a single monolithic bed comprising a tube-in-a-tube system, the outer and inner tube carrying different functional moieties. In such a system one of the monolithic tubes represents the

support for the chromatographic principle of one step and the other tube represents the support for the chromatographic principle of the other step. Preferably, the capturing/purification step can be operated in a batch wise mode or in a quasi-continuous or continuous mode, employing technologies such as annular chromatography, carousel chromatography or a simulated moving.

[0223] The system of the present invention is not only suitable for protein production but can also be used for the continuous production of a compound of interest (see Example 3). This method may be used to convert a precursor compound to the compound of interest. Alternatively or additionally, the method can also be used to produce a compound of interest based on the nutrients already provided in the medium. Accordingly, the present invention also relates to a method for the (continuous) production of a compound of interest, comprising culturing the bacterial host cell as defined herein in the system of the invention and optionally adding a compound that is to be converted and/or used by said bacterial host cell for the production of said compound of interest to the seed and/or production reactor.

[0224] The term “compound of interest” as used herein may be but is not limited to precurors or building block molecules for plastics such as conversion of bicyclo[3.2.0]-hept-2-en-6-one to lactones, alcohols, such as conversion of prochiral carbonyl compounds to chiral, conversion of ferulic acid to coniferyl aldehyde to coniferyl alcohol, or conversion of eugenol to ferulic acid to coniferyl alcohol to vanillin.

[0225] In one embodiment, the compound of interest is lycopene. Here, the host cell can be genetically modified to further comprise enzymes of a lycopene producing pathway. In one illustrative example for such a lycopene producing pathway, the bacterial host cell is genetically modified to comprise the gene(s) *crtI, and optionally crtB, and/or crtE*. Preferably, said genes are from *C. glutamicum*, more preferably *crtE* comprises or consists of SEQ ID NO: 18 or a functional homolog thereof, *crtB* comprises or consists of SEQ ID NO: 19 or a functional homolog thereof and *crtI* comprises or consists of SEQ ID NO: 20 or a functional homolog thereof. A “functional homolog” is a nucleic acid having at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity or at least 99% sequence identity. The protein encoded by a functional homolog preferably has an activity, which has at least 80%, at least 85%, at least 90%, at least 95% or at least 99% of the activity of the protein being expressed by SEQ ID NO: 18, 19 or 20, respectively. In case the bacterial host cell comprises any one or all of SEQ ID NO: 18, 19 or 20 or a functional homolog thereof, it is preferably not necessary to add a compound to be converted.

[0226] The system of the present invention can also be used to degrade plastic materials such as polyesters. Polyester is a category of polymers that contain the ester functional group in every repeat unit of their main chain. Polyesters include naturally occurring chemicals, such as in the cutin of plant cuticles, as well as synthetics such as polybutyrate. An illustrative example

of a polymer is Polyethylene terephthalate (PET). Accordingly, the compound to be converted may be polyester such as Polyethylene terephthalate (PET) and the compound of interest can be the corresponding polyester monomer such as mono-2-hydroxyethyl terephthalate (MHET). To enable the bacterial host cell to convert PET into its monomer, it may be genetically modified to comprise a PETase. PETases are an esterase class of enzymes that catalyze the hydrolysis of polyethylene terephthalate (PET) plastic to monomeric mono-2-hydroxyethyl terephthalate (MHET). Illustrative examples include lipases, esterases, and cutinases. PETase exhibits shared qualities with both lipases and cutinases in that it possesses an α/β-hydrolase fold; although, the active-site cleft observed in PETase is more open than in cutinases. The *Ideonella sakaiensis* PETase is similar to dienelactone hydrolase.

[0227] The present invention also relates to the following items:

1. System for use in continuous production of a protein of interest or a nucleotide of interest by a bacterial host cell, wherein the bacterial host cell comprises under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell, comprising

(a) a seed reactor comprising said bacterial host cell in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, the seed reactor having at least one inlet and at least one outlet, and

(b) at least one production reactor comprising said bacterial host cells in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, each production reactor having at least one inlet and at least one outlet,

wherein an inlet of the production reactor is connected to an outlet of the seed reactor.

2. The system of item 1, wherein the bacterial host cell comprises a nucleotide encoding the protein of interest or the nucleotide of interest.

3. The system of item 1 or 2 wherein the protein of interest or the nucleotide of interest is produced in at least one production reactor.

4. The system of any one of the preceding items, wherein said nucleotide of interest encodes one or more proteins of interest.

5. The system of any one of the preceding items, wherein said nucleotide is a nucleic acid molecule.

6. The system of item 5, wherein said nucleic acid molecule is a plasmid, minichromosome, or RNA.

7. The system of any one of the preceding items, wherein said nucleotide of interest or said nucleotide encoding the protein of interest is under the control of a second inducible promoter or under the control of a constitutive promoter.

8. The system of any one of the preceding items, wherein the promoter of said heterologous nucleotide of interest is recognized by an RNA polymerase which is heterologous

for said bacterial host cell, said heterologous RNA polymerase is encoded by a nucleotide sequence comprised by said bacterial host cell.

9. The system of item 8, wherein said RNA polymerase is bacteriophage T3 RNA polymerase, T7 bacteriophage RNA polymerase, engineered orthogonal T7 RNA polymerase, bacteriophage SP6 RNA polymerase or bacteriophage Xp10 RNA polymerase.

10. The system of item 8 or 9, wherein said nucleotide sequence encoding said RNA polymerase is under the control of a third inducible promoter or under the control of a constitutive promoter.

11. The system of any one of the preceding items, wherein said first, second or third inducible promoter is regulated by arabinose, IPTG, tryptophan, xylose, lactose, rhamnose, phosphate, propionate, benzoic acid, phage lambda cI protein or heat.

12. The system of item 11, wherein said first, second or third promoter are different.

13. The system any one of the preceding items, wherein said bacterial host cell has a non-functional arabinose operon.

14. The system of any one of the preceding items, wherein said bacterial host cell is *E. coli*, preferably *E. coli* B-lineage.

15. The system of any one of the preceding items, wherein growth is inhibited by inhibiting transcription, DNA-replication and/or cell division.

16. The system of any one of the preceding items, wherein said phage protein is

(i) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 1 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 1 and which inhibits bacterial host cell RNA polymerase;

(ii) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 2 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 2 and which inhibits bacterial host cell RNA polymerase;

(iii) a protein which phosphorylates bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 3 or a fragment thereof which phosphorylates bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 3 and which phosphorylates bacterial host cell RNA polymerase;

(iv) a protein which inhibits bacterial host cell DNA replication, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 4 or a fragment thereof which inhibits bacterial host cell DNA replication; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 4 and which inhibits bacterial host cell DNA replication;

(v) a protein which inhibits bacterial host cell DNA replication, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 5 or a fragment thereof which inhibits bacterial host cell DNA replication; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 5 and which inhibits bacterial host cell DNA replication; or

(vi) a protein which inhibits bacterial host cell DNA replication, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 6 or a fragment thereof which inhibits bacterial host cell DNA replication; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 6 and which inhibits bacterial host cell DNA replication;

(vii) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 7 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 7 and which inhibits bacterial host cell RNA polymerase;

(viii) a protein which causes host transcription shut-off, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 8, 9, 10, 11, 12, 13, 14 or a fragment thereof which causes host transcription shut-off;

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 8, 9, 10, 11, 12, 13 or 14 and which causes host transcription shut-off; or

(ix) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 17 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 17 and which inhibits bacterial host cell RNA polymerase.

17. The system of any one of the preceding items, wherein the bacterial host cell is cultured in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in the seed reactor for biomass production.

18. The system of any one of the preceding items, wherein the bacterial host cell is cultured in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in the production reactor for production of said nucleotide sequence of interest by said bacterial host cells.

19. The system of any one of the preceding items, further comprising

(c) a means for operating the seed and production reactors as linked chemostats or turbidostats.

20. The system of any one of the preceding items, whereby the seed reactor outflow serves as inflow to the production reactor.

21. The system of any one of the preceding items, wherein the seed reactor does not comprise or does not essentially comprise an inducer for the first inducible promoter.

22. The system of any one of the preceding items, wherein at least one of the at least one production reactor comprises an inducer for the first inducible promoter.

23. The system of any one of the preceding items, wherein at least one of the at least one production reactor comprises an inducer for the second inducible promoter.

24. The system of any one of the preceding items, wherein the first inducible promoter and the second inducible promoter can be induced by the same inducer.

25. The system of any one of the preceding items, wherein the seed reactor comprises a means for regulating pH.

26. The system of any one of the preceding items, wherein the seed reactor comprises a means for regulating dissolved oxygen.

27. The system of any one of the preceding items, wherein the seed reactor comprises a means for regulating temperature.

28. The system of any one of the preceding items, wherein the seed reactor comprises a gas inlet and a gas outlet and a means for regulating the gas flow.

29. The system of any one of the preceding items, wherein the at least one production reactor comprises a means for regulating pH.

30. The system of any one of the preceding items, wherein the at least one production reactor comprises a means for regulating dissolved oxygen.

31. The system of any one of the preceding items, wherein the at least one production reactor comprises a means for regulating temperature.

32. The system of any one of the preceding items, wherein the at least one production reactor comprises a gas inlet and a gas outlet and a means for regulating the gas flow.

33. The system of any one of the preceding items, wherein the at least one production reactor comprises a biomass sensor.

34. The system of any one of the preceding items comprising a first feed container containing a first feed medium comprising a carbon source, wherein the first feed container is operably connected to an inlet of the seed reactor, wherein the system preferably comprises means for regulating feed flow from the first feed container to the seed reactor.

35. The system of any one of the preceding items comprising a second feed container containing a second feed medium comprising a carbon source, wherein the second feed container is operably connected to an inlet of the at least one production reactor, wherein the system preferably comprises means for regulating feed flow from the second feed container to the at least one production reactor.

36. The system of any one of the preceding items, wherein an outlet of the seed reactor and an outlet of a second feed reactor are connected to a mixing chamber, wherein an outlet of the mixing chamber is connected to an inlet of the at least one production reactor.

37. The system of any one of the preceding items, wherein the seed reactor is a stirred tank reactor or plug flow reactor.

38. The system of any one of the preceding items, wherein the at least one production reactor is a stirred tank reactor or a plug flow reactor.

39. The system of any one of the preceding items, wherein the seed reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L.

40. The system of any one of the preceding items, wherein the at least one production reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L.

41. The system of any one of the preceding items, wherein the volume ratio of the seed reactor to the at least one production reactor is from about 1:10 to about 2:1, from about 1:5 to about 2:1, from about 1:2 to about to about 2:1, from about 1.5:1 to about 1:1.5, or about 1:1.

42. The system of any one of the preceding items, wherein the at least one production reactor comprises a culture medium comprising cells with a biomass concentration from about 10 to about 90 g/L cell dry weight, preferably from about 20 to about 80 g/L cell dry weight, preferably from about 30 to about 70 g/L cell dry weight, preferably from about 35 to about 60 g/L cell dry weight.

43. A continuous fermentation process for the production of a protein of interest or a nucleotide of interest by a bacterial host cell, wherein the bacterial host cell comprise under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell, comprising

(a) culturing said bacterial host cell in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in a seed reactor;

(b) transferring at least an amount of the bacterial host cells obtained in (a) from said seed reactor to a production reactor; and

(c) culturing said bacterial host cells in said production reactor in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells;

wherein the seed reactor and production reactor is configured as an independent continuous fermentor and wherein the seed reactor and production reactor are connected with each other.

44. The process of item 43, wherein (a) is for biomass production.

45. The process of item 43 or 44, wherein in (c) growth of said bacterial host cells is inhibited by culturing said bacterial host cells in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, while said nucleotide of interest or said protein of interest is produced.

46. The process of any one of items 43 to 45, wherein the bacterial host cell comprises the nucleotide of interest or a nucleotide encoding the protein of interest.

47. The process of any one of items 43 to 46, wherein said nucleotide of interest encodes one or more proteins of interest.

48. The process of any one of items 43 to 47, wherein said nucleotide is a nucleic acid molecule.

49. The process of item 48, wherein said nucleic acid molecule is a plasmid, minichromosome, or RNA.

50. The process of any one of items 43 to 49, wherein said nucleotide of interest or said nucleotide encoding the protein of interest is under the control of a second inducible promoter or under the control of a constitutive promoter.

51. The process of any one items 43 to 50, wherein the promoter of said heterologous nucleotide sequence of is recognized by an RNA polymerase which is heterologous for said bacterial host cell, said heterologous RNA polymerase is encoded by a nucleotide sequence comprised by said bacterial host cell.

52. The process of item 51, wherein said heterologous RNA polymerase is bacteriophage T3 RNA polymerase, T7 bacteriophage RNA polymerase, engineered orthogonal T7 RNA polymerase, bacteriophage SP6 RNA polymerase or bacteriophage Xp10 RNA polymerase.

53. The process of item 51 or 52, wherein said nucleotide sequence encoding said heterologous RNA polymerase is under the control of a third inducible promoter or under the control of a constitutive promoter.

54. The process of any one of items 43 to 53, wherein said first, second or third inducible promoter is regulated by arabinose, IPTG, tryptophan, xylose, lactose, rhamnose, phosphate, propionate, benzoic acid, phage lambda cI protein or heat.

55. The process of item 54, whereby said first, second or third promoter are different.

56. The process of any one of items 43 to 55, wherein said bacterial host cell has a non-functional arabinose operon.

57. The process of any one of items 43 to 56, wherein said bacterial host cell is *E. coli*, preferably *E. coli* B-lineage.

58. The process of any one of items 43 to 57, wherein (a) further comprises culturing said bacterial host cells in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells in culture medium in a production reactor for biomass production.

59. The process of any one of items 43 to 58, wherein (c) further comprises inducing said first inducible promoter controlling said nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells.

60. The process of any one of items 43 to 59, wherein (b) or (c) comprises inducing said second inducible promoter controlling said nucleotide of interest or said nucleotide encoding the protein of interest.

61. The process of any one of items 43 to 60, wherein (a), (b) or (c) comprises inducing said third inducible promoter controlling said nucleotide sequence encoding said heterologous RNA polymerase.

62. The process of any one of items 43 to 61, further comprising (d) harvesting the product resulting from the production of said nucleotide sequence of interest.

63. The process of any one of items 43 to 62, wherein the seed reactor does not comprise or does not essentially comprise an inducer for the first inducible promoter.

64. The process of any one of items 43 to 63, wherein at least one of the at least one production reactor comprises an inducer for the first inducible promoter.

65. The process of any one of items 43 to 64, wherein at least one of the at least one of the at least one production reactor comprises an inducer for the second inducible promoter.

66. The process of any one of items 43 to 65, wherein the first inducible promoter and the second inducible promoter can be induced by the same inducer.

67. The process of any one of items 43 to 66, wherein pH is regulated in the seed reactor.

68. The process of any one of items 43 to 67, wherein dissolved oxygen is regulated in the seed reactor.

69. The process of any one of items 43 to 68, wherein temperature is regulated in the seed reactor.

70. The process of any one of items 43 to 69, wherein gas flow is regulated in the seed reactor.

71. The process of any one of items 43 to 70, wherein the biomass concentration is regulated in the seed reactor by feed inflow and/or biomass outflow.

72. The process of any one of items 43 to 71, wherein pH is regulated in the at least one production reactor.

73. The process of any one of items 43 to 72, wherein dissolved oxygen is regulated in the at least one production reactor.

74. The process of any one of items 43 to 73, wherein temperature is regulated in the at least one production reactor.

75. The process of any one of items 43 to 74, wherein gas flow is regulated in the at least one production reactor.

76. The process of any one of items 43 to 75, wherein the at least one production reactor comprises a biomass sensor.

77. The process of any one of items 43 to 76, wherein the biomass concentration is regulated in the at least one production reactor by feed inflow, biomass inflow, and/or biomass outflow.

78. The process of any one of items 43 to 77, wherein the mean residence time of biomass in the at least one production reactor is from about 5 h to about 24 h, preferably from about 7 h to about 20 h, preferably from about 10 h to about 15 h.

79. The process of any one of items 43 to 78, comprising a first feed container containing a first feed medium comprising a carbon source, wherein the first feed container is operably connected to an inlet of the seed reactor, wherein feed flow from the first feed container to the seed reactor is preferably regulated.

80. The process of any one of items 43 to 79, comprising a second feed container containing a second feed medium comprising a carbon source, wherein the second feed container is operably connected to an inlet of the at least one production reactor, wherein feed flow from the second feed container to the at least one production reactor is preferably regulated.

81. The process of any one of items 43 to 80, wherein an outlet of the seed reactor and an outlet of a second feed reactor are connected to a mixing chamber, wherein an outlet of the mixing chamber is connected to an inlet of the at least one production reactor.

82. The process of any one of items 43 to 81, wherein the seed reactor is a stirred tank reactor or a plug flow reactor.

83. The process of any one of items 43 to 82, wherein the at least one production reactor is a stirred tank reactor or a plug flow reactor.

84. The process of any one of items 43 to 83, wherein the seed reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L.

85. The process of any one of items 43 to 84, wherein the at least one production reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L.

86. The process of any one of items 43 to 85, wherein the volume ratio of the seed reactor to the at least one production reactor is from about 1:10 to about 2:1, from about 1:5 to about 2:1, from about 1:2 to about to about 2:1, from about 1.5:1 to about 1:1.5, or about 1:1.

87. The process of any one of items 43 to 86, wherein the at least one production reactor comprises a culture medium comprising cells with a biomass concentration from about 10 to about 90 g/L cell dry weight, preferably from about 20 to about 80 g/L cell dry weight, preferably from about 30 to about 70 g/L cell dry weight, preferably from about 35 to about 60 g/L cell dry weight.

88. The process of any one of items 43 to 87, wherein the bacterial host cells in the at least one production reactor is genetically stable for at least about 5 days, preferably at least about 7 days, preferably at least about 10 days.

89. The process of any one of items 43 to 88, wherein the process is operated for at least about 5 days, preferably at least about 7 days, preferably at least about 10 days.

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[0228] It must be noted that as used herein, the singular forms “a”, “an”, and “the”, include plural references unless the context clearly indicates otherwise. Thus, for example, reference to “an expression cassette” includes one or more of the expression cassettes disclosed herein and reference to “the method” includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

[0229] All publications and patents cited in this disclosure are incorporated by reference in their entirety. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0230] Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the present invention.

[0231] Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not

the exclusion of any other integer or step or group of integer or step. When used herein the term “comprising” can be substituted with the term “containing” or sometimes when used herein with the term “having”.

[0232] When used herein “consisting of" excludes any element, step, or ingredient not specified in the claim element. When used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim. In each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms.

[0233] The term "about" or "approximately" as used herein means within 20%, preferably within 10%, and more preferably within 5% of a given value or range. It includes also the concrete number, e.g., about 20 includes 20.

[0234] Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The methods and techniques of the present invention are generally performed according to conventional methods well-known in the art. Generally, nomenclatures used in connection with techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art.

[0235] The methods and techniques of the present invention are generally performed according to conventional methods well-known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, e. g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y. (2001); Ausubel et al., Current Protocols in Molecular Biology, J, Greene Publishing Associates (1992, and Supplements to 2002); Handbook of Biochemistry: Section A Proteins, Vol I 1976 CRC Press; Handbook of Biochemistry: Section A Proteins, Vol II 1976 CRC Press. The nomenclatures used in connection with, and the laboratory procedures and techniques of, molecular and cellular biology, protein biochemistry, enzymology and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art.

Examples

[0236] The following Examples illustrate the invention, but are not to be construed as limiting the scope of the invention.

Example 1: Continuous fermentation process for Green fluorescent protein (GFP).

[0237] To test the ability of the enGenes-X-press strain for continuous growth decoupled recombinant protein production the fluorescent model protein GFP was employed. The two stage continuous fermentation experiments were carried out using the host *E. coli* strain BL21(DE3) as a reference strain, i.e. a strain that does not comprise under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell. The model protein GFP is expressed from the pET30a plasmid in the reference strain. The reference strain is thus called BL21(DE3)pET30a<GFP> herein. The enGenes-X-press strain is used as an example of bacterial host cell that comprises under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell as required by the invention. The difference between enGenes-X-press and an *E. coli* BL21(DE3) is the following: araABCD is knocked out, Gp2 is integrated at the attTn7 site and under the control of the arabinose-inducible promoter of araB. The genotype of enGenes-X-press can therefore be described as BL21(DE3)::TN7(Gp2ΔAra) For protein production, enGenes-X-press has been transformed with a pET30a plasmid comprising GFP (SEQ ID NO: 15), leading to a genotype of BL21(DE3)::TN7(Gp2ΔAra)pET30<GFP> or, in short, enGenes-X-press pET30a<GFP>. enGenes-X-press pET30a<GFP> was generated according to WO 2016/174195, hereby incorporated by reference. As shown in FIG. 1 and 2, the use of a enGenes-X-press instead of “standard” BL21(DE3) in a in a continuous fermentation process of the invention provides for a significant increase in productivity (space-time-yield) in comparison to “standard” continuous fermentation processes known in prior art.

[0238] The design of this 2 stage chemostat cultivation processes with a seed and a production bioreactor is shown in FIG. 3. In both bioreactors a batch process followed by a fed-batch phase was conducted. The batch volume was set to 600 mL and the batch medium used allowed for production of 5 g CDM, the feed volume was set to 460 mL with medium designed to produce another 27 g of CDM which corresponds to a final CDM concentration of 30 g/L. In a next step the seed bioreactor was shifted to chemostat mode with a dilution rate of 0.05 h^{-1}. The medium used as continuous feed to reactor ($F1_{in}$) provide nutrients to maintain a CDM concentration of 40 g/L. The outflow from the seed bioreactor ($F1_{out}$) represents one of the feeds to the production bioreactor. To provide the carbon source required to maintain metabolic activity of the induced system in the production reactor, a second medium feed with a flow rate of 11.31 mL/h and a glucose concentration of 400 g/L was introduced. To operate the production reactor at the same dilution rate of 0.05 h^{-1} the outflow $F1_{out}$ was started at the timepoint when working volume of 1260 mL was reached in the production bioreactor. Temperature was stabilized at 30°C. The culture in the production vessel was induced by either adding 20 µmol

IPTG/g CDM (BL21/DE3) strain) or 20 µmol IPTG/g CDM and 100 mM L-arabinose (enGenes-X-press strain) to the medium fed to the production reactor. GFP, accumulated in soluble and insoluble form was quantified via ELISA (soluble form) and SDS PAGE for the insoluble fraction according to Reischer et al. (2004) Journal of Biotechnology, 108(2):115-125, incorporated hereby by reference. Cell dry mass CDM was determined by centrifugation of 10 mL of cell suspension. The minimal medium used for cultivations contained 3 g KH_2PO_4 and 6 g $K_2HPO_4 \cdot 3\ H_2O$ per litre; these concentrations provided the required buffer capacity and served as sources of P and K. The other components were added in relation to the theoretical grams of CDM to be produced (calculated for 5 g in batch-phase and 136 g CDM in feed-phase, based on the constant glucose yield coefficient YX/S of 0.3 g/g): 0.25 g sodium citrate (trisodium salt·2 H_2O; ACROS organics), 0.10 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 50 µL trace element solution, and 3 g glucose·H_2O. The trace element solution was prepared in 5 N HCl and included 40 g/L $FeSO_4 \cdot 7H_2O$, 10 g/L $MnSO_4 \cdot H_2O$, 10 g/L $AlCl_3 \cdot 6H_2O$, 4 g/L $CoCl_2$ (Fluka), 2 g/L $ZnSO_4 \cdot 7H_2O$, 2 g/L $Na_2MoO_2 \cdot 2H_2O$, 1 g/L $CuCl_2 \cdot 2H_2O$, and 0.5 g/L H_3BO_3. We also added 4 mg $CuCl_2 \cdot 2H_2O$ and 3.2 mg $ZnSO_4 \cdot 7H_2O$ per g CDM. To accelerate initial growth of the population, the complex component yeast extract (0.15 g per g theoretical CDM) was added to the minimal medium to obtain the batch medium.

[0239] The inventors observed an abrupt population collapse in the seed reactor with BL21(DE3)pET30a<GFP> indicated by a significant decrease in CDM after 148 h process time (from 40 g/L to 20 g/L CDM, Figure 1) whereas that collapse was surprisingly not observable for enGenes-X-press pET30a<GFP> cells (engineered to allow growth decoupling of protein production and cell growth using the T7 phage derived Gp2 protein that is under the control of an arabinose promoter, integrated into the E. coli genome at the attTn7 site, generated according to WO 2016/174195, hereby incorporated by reference). GFP productivity in the 2nd stage production reactor was more constant for the X-press strain (range between 0.3 g/h and 0.6 g/h) whereas for the reference BL21(DE3) the inventors observed a steady decline in total GFP yields (from 0.5 g/h to 0.03 g/h, Figure 2). Even though the CDM of the reference strain BL21(DE3) in the seed reactors seems to recover at 200 h, the productivity of BL21(DE3) remains low with a continuous decreasing trend which is in contrast to the enGenes-X-press cells. This drop in GFP productivity in BL21(DE3) can be explained on the population level by doing FACS analysis, measuring the fluorescence intensity of individual cells at 488 nm extinction. The results in FIG. 4 show FACS histograms of BL21(DE3)pET30a<GFP> strain (left hand side) and X-press pET30a<GFP> (right hand side) in the seed stage reactor and clearly shows that the GFP positive population nearly disappears after the drop in CDM (from 148h to 196h). In contrast to this observation the population of X-press cells stays more uniform, able to maintain a high level of GFP positive cells, even in the seed reactor stage. This development at the seed reactor stage for biomass production is then also reflected at the production stage reactor (FIG. 5) where the peak for GFP positive cells begins to shift for

BL21(DE3)pET30a<GFP> and nearly disappears at the end of process (196 h). This is also in good agreement with the low GFP productivity for BL21(DE3) at the end of the process, overall indicating a lower population stability of BL21(DE3) strains compared to growth-decoupled enGenes-X-press cells. The growth-decoupled protein production in the production reactor stage of the invention prevents or reduces manifestation of faster growing, plasmid-free, non-producing cells and thereby stabilizes the production system due to the lack of genetic changes being the consequence of adaptive evolution. Adaptive evolution is not at play in a population of non-dividing cells, a phenomenon also known as genetic instability.

[0240] The growth-decoupled enGenes-X-press strain produced about 90 g of GFP over the course of 196 h. The reference *E. coli* strain BL21(DE3) produced less than 5 g of GFP over the course of their respective cultivations, with a steadily declining productivity. In addition to producing lower overall peak levels of test protein, the reference *E. coli* strain BL21(DE3) was only capable of sustaining peak levels of production for a few days, whereas the enGenes-X-press strain sustained high expression levels throughout the entire fermentation. Importantly, the enGenes-X-press fermentations were not subject to culture collapse in the seed reactor nor the production reactor (culture collapse was always observed at the seed reactor level for the reference strain), indicating the enhanced genetic stability of the X-press strain itself.

[0241] The described growth-decoupled 2-stage continuous production system is capable of producing at least 20 times more product than the same 2-stage cultivation system (using a typical *E. coli* strain) in non-growth decoupled mode. The used E. coli enGenes-X-press strain that allows growth-decoupled production in the 2nd stage production bioreactor showed improved genetic stability and population stability unanticipated in the 1st stage seed bioreactor. The improved genetic stability of the fermentation organism improves the longevity and did not show signs of population collapse in the observed timespan. This unique growth-decoupled 2-stage fermentation process results in a highly productive stable platform for making proteins and other fermentation products at high levels relative to current strains used, not applicable for genetic decoupling of recombinant product formation.

Example 2: Continuous fermentation process for SpA (Protein A) protein from Staphylococcus aureus.

[0242] This example is essentially carried out as described in example 1, while using SpA having a periplasmic leader sequence instead of GFP as model protein. Accordingly, the reference strain is *E. coli* BL21(DE3)pET30a<pelbB-SpA> and the growth-decoupled bacterial host cell of described herein is enGenes-X-press pET30<pelB-SpA>. The nucleic acid sequence of pelB-SpA used in Example 2 is shown in SEQ ID NO: 16. Comparison of cell dry mass and productivity profiles of each of these strains, shown in FIG. 6 and 7, shows that

growth decoupled protein production in a 2 stage process is significantly improved in comparison to commonly used BL21(DE3) production strains.

[0243] In these experiments all strains were transformed with the same pET30a<pelbB-SpA> plasmid with kanamycin as selection marker. All strains were tested in the 2-stage system configured as shown in FIG. 3 already described in example 1. Operation conditions were identical to the experiments described in example 1. SpA concentrations were determined using an HPLC IMAC column and a His-tagged Protein A standard directly from cell free culture supernatants. Cell dry mass CDM was determined by centrifugation of 10 mL of cell suspension.

[0244] In this experiment the inventors also observed an abrupt population collapse in the seed reactor of BL21(DE3)pET30a<pelB-SpA> cells indicated by a significant decrease in CDM after 168 h (from 46 g/L to 7 g/L CDM) whereas that collapse was not observable for enGenes-X-press pET30a<pelB-SpA> cells (Figure 6). SpA productivity in the production reactor was again constant for X-press strain at a high level ranging from 0.3 to 0.5 g/h whereas for BL21(DE3) the inventors observed a peak of 0.6 g/h after 75 h in the production phase followed by a steady decline in total SpA yields for the reference strain. This maximum SpA productivity is in coincidence with the population collapse in the seed reactor and led to breakdown of the production system since the low biomass concentrations in the seed reactor could no longer sustain the targeted biomass concentration in the production bioreactor. The reduced biomass in the production reactor will inevitably also lead to a reduced productivity.

[0245] The growth-decoupled enGenes-X-press strain produced about 120 g of SpA within the 309 h in chemostat mode (Fig. 7). The reference strain *E. coli* BL21(DE3) was only capable of sustaining peak levels of production for a few days, whereas the enGenes-X-press strain sustained peak expression throughout the entire fermentation duration. Importantly, the enGenes-X-press fermentations were again not subject to culture collapse in the seed reactor nor the production reactor (culture collapse was always observed at the seed reactor level), confirming the high stability of this system in continuous cultivation mode.

Example 3: Continuous fermentation process for Lycopene production using lycopene pathway genes from Corynebacterium glutamicum

[0246] This example is essentially carried out as described in example 1, while using a pET-derived plasmid pET30a<crtE-crtB-crtI>cer encoding genes from *Corynebacterium glutamicum MB001* [crtE (locus tag: cgp_0723, EC-number: 2.5.1.29, SEQ ID NO: 18); crtB (locus tag: cgp_0721, EC-number: 2.5.1., SEQ ID NO: 19); crtI (locus tag: cgp_0720, EC-number: 1.3.99., SEQ ID NO: 20)] having a. polycistronic arrangement (controlled by one T7

promoter/terminator) and three RBS (ribosomal binding sites, one for each gene). Accordingly, the reference strain is *E. coli* BL21(DE3)pET30a<crtE-crtB-crtI>cer and the growth-decoupled bacterial host cell of described herein is enGenes-X-press pET30a<crtE-crtB-crtI>cer. Comparison of cell dry mass and productivity profiles of each of these strains, shown in Figure 8, shows that growth decoupled protein production in a 2-stage process is significantly improved in comparison to commonly used BL21(DE3) production strains.

[0247] In these experiments all strains were transformed with the same pET30a<crtE-crtB-crtI>cer plasmid with kanamycin as selection marker. All strains were tested in the 2-stage system configured as shown in FIG. 3 already described in example 1. Experiment were performed with a dilution rate of 0.1 h-1, working volume for phase 1 was 700 mL and for phase 2 1000 mL. Lycopene concentrations were determined by linear regression analysis using spectrophotometry and a lycopene standard. Lycopene has been extracted from the biomass using acetone (100%) and was measured at wavelength 460 nm using an Ultrospec 500 pro (Amersham Bioscience, UK) spectrometer. Cell dry mass (CDM) was gravimetrically determined by centrifugation of 10 mL of cell suspension. dsDNA concentration was measured using the Qubit Flurometer (Thermo Fisher Scientific) and the corresponding Qubit™ dsDNA BR Assay Kit following the manufacturer's instructions. The determined concentration in the cell free supernatant has then been normalized to cell dry mass (see Fig. 9).

[0248] In this experiment the inventors observed an decline in lycopene production after 10 doublings post induction (70 h) with BL21(DE3)pET30a<crtE-crtB-crtI>cer also indicated by a significant decrease in CDM after 70 h (from 54.0 g/L to 24.6 g/L CDM). This decrease in lycopene production and cell dry mass concentration was not observable for enGenes-X-press pET30a<crtE-crtB-crtI>cer cells (Figure 8). Lycopene production in the production reactor was steadily increasing again for X-press strain with a final amount of 334 mg lycopene produced at the one litre scale after 310 h post induction whereas for BL21(DE3) the inventors observed a peak of 141 mg after 84 h in the production phase with already declining biomass yields. Therefore, the process was terminated after 142 h because the reduced biomass in the production reactor will inevitably also lead to a reduced productivity.

[0249] Importantly, the enGenes-X-press fermentations were again not subject to culture collapse in the seed reactor nor the production reactor. In the case of example one culture collapse or cell lysis was observed in the production rather than the seed reactor (as shown for example 1 and 2). Again, this confirms the improved stability of the enGenes-X-press strain also for the production of metabolites.

Claims

1. System for use in continuous production of a protein of interest or a polynucleotide of interest by a bacterial host cell, wherein the bacterial host cell comprises under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell, comprising

(a) a seed reactor comprising said bacterial host cell in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, the seed reactor having at least one inlet and at least one outlet, and

(b) at least one production reactor comprising said bacterial host cells in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, each production reactor having at least one inlet and at least one outlet,

wherein an inlet of the production reactor is connected to an outlet of the seed reactor.

2. The system of claim 1, wherein the bacterial host cell comprises a nucleotide encoding the protein of interest or the polynucleotide of interest.

3. The system of claim 1 or 2 wherein the protein of interest or the polynucleotide of interest is produced in at least one production reactor.

4. The system of any one of the preceding claims, wherein said polynucleotide of interest encodes one or more proteins of interest.

5. The system of any one of the preceding claims, wherein said nucleotide is a nucleic acid molecule.

6. The system of claim 5, wherein said nucleic acid molecule is a plasmid, minichromosome, or RNA.

7. The system of any one of the preceding claims, wherein said polynucleotide of interest or said nucleotide encoding the protein of interest is under the control of a second inducible promoter or under the control of a constitutive promoter.

8. The system of any one of the preceding claims, wherein the promoter of said heterologous polynucleotide of interest is recognized by an RNA polymerase which is

heterologous for said bacterial host cell, said heterologous RNA polymerase is encoded by a nucleotide sequence comprised by said bacterial host cell.

9. The system of claim 8, wherein said RNA polymerase is bacteriophage T3 RNA polymerase, T7 bacteriophage RNA polymerase, engineered orthogonal T7 RNA polymerase, bacteriophage SP6 RNA polymerase or bacteriophage Xp10 RNA polymerase.

10. The system of claim 8 or 9, wherein said nucleotide sequence encoding said RNA polymerase is under the control of a third inducible promoter or under the control of a constitutive promoter.

11. The system of any one of the preceding claims, wherein said first, second or third inducible promoter is regulated by arabinose, IPTG, tryptophan, xylose, lactose, rhamnose, phosphate, propionate, benzoic acid, phage lambda cI protein or heat.

12. The system of claim 11, wherein said first, second or third promoter are different.

13. The system any one of the preceding claims, wherein said bacterial host cell has a non-functional arabinose operon.

14. The system of any one of the preceding claims, wherein said bacterial host cell is *E. coli*, preferably *E. coli* B-lineage.

15. The system of any one of the preceding claims, wherein growth is inhibited by inhibiting transcription, DNA-replication and/or cell division.

16. The system of any one of the preceding claims, wherein said phage protein is

(i) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 1 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 1 and which inhibits bacterial host cell RNA polymerase;

(ii) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 2 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 2 and which inhibits bacterial host cell RNA polymerase;

(iii) a protein which phosphorylates bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 3 or a fragment thereof which phosphorylates bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 3 and which phosphorylates bacterial host cell RNA polymerase;

(iv) a protein which inhibits bacterial host cell DNA replication, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 4 or a fragment thereof which inhibits bacterial host cell DNA replication; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 4 and which inhibits bacterial host cell DNA replication;

(v) a protein which inhibits bacterial host cell DNA replication, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 5 or a fragment thereof which inhibits bacterial host cell DNA replication; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 5 and which inhibits bacterial host cell DNA replication; or

(vi) a protein which inhibits bacterial host cell DNA replication, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 6 or a fragment thereof which inhibits bacterial host cell DNA replication; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 6 and which inhibits bacterial host cell DNA replication;

(vii) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 7 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 7 and which inhibits bacterial host cell RNA polymerase;

(viii) a protein which causes host transcription shut-off, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 8, 9, 10, 11, 12, 13, 14 or a fragment thereof which causes host transcription shut-off;

(b) a protein having an amino acid sequence which has an identity of 40% or more, such as 50%, 60%, 70%, 80% or 90% to the amino acid sequence shown in Seq Id No: 8, 9, 10, 11, 12, 13 or 14 and which causes host transcription shut-off; or

(ix) a protein which inhibits bacterial host cell RNA polymerase, wherein said protein is

(a) a protein having the amino acid sequence shown in Seq Id No: 17 or a fragment thereof which inhibits bacterial host cell RNA polymerase; or

(b) a protein having an amino acid sequence which has an identity of 40% or more to the amino acid sequence shown in Seq Id No: 17 and which inhibits bacterial host cell RNA polymerase.

17. The system of any one of the preceding claims, wherein the bacterial host cell is cultured in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in the seed reactor for biomass production.

18. The system of any one of the preceding claims, wherein the bacterial host cell is cultured in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in the production reactor for production of said nucleotide sequence of interest by said bacterial host cells.

19. The system of any one of the preceding claims, further comprising

(c) a means for operating the seed and production reactors as linked chemostats or turbidostats.

20. The system of any one of the preceding claims, whereby the seed reactor outflow serves as inflow to the production reactor.

21. The system of any one of the preceding claims, wherein the seed reactor does not comprise or does not essentially comprise an inducer for the first inducible promoter.

22. The system of any one of the preceding claims, wherein at least one of the at least one production reactor comprises an inducer for the first inducible promoter.

23. The system of any one of the preceding claims, wherein at least one of the at least one production reactor comprises an inducer for the second inducible promoter.

24. The system of any one of the preceding claims, wherein the first inducible promoter and the second inducible promoter can be induced by the same inducer.

25. The system of any one of the preceding claims, wherein the seed reactor comprises a means for regulating pH.

26. The system of any one of the preceding claims, wherein the seed reactor comprises a means for regulating dissolved oxygen.

27. The system of any one of the preceding claims, wherein the seed reactor comprises a means for regulating temperature.

28. The system of any one of the preceding claims, wherein the seed reactor comprises a gas inlet and a gas outlet and a means for regulating the gas flow.

29. The system of any one of the preceding claims, wherein the at least one production reactor comprises a means for regulating pH.

30. The system of any one of the preceding claims, wherein the at least one production reactor comprises a means for regulating dissolved oxygen.

31. The system of any one of the preceding claims, wherein the at least one production reactor comprises a means for regulating temperature.

32. The system of any one of the preceding claims, wherein the at least one production reactor comprises a gas inlet and a gas outlet and a means for regulating the gas flow.

33. The system of any one of the preceding claims, wherein the at least one production reactor comprises a biomass sensor.

34. The system of any one of the preceding claims comprising a first feed container containing a first feed medium comprising a carbon source, wherein the first feed container is operably connected to an inlet of the seed reactor, wherein the system preferably comprises means for regulating feed flow from the first feed container to the seed reactor.

35. The system of any one of the preceding claims comprising a second feed container containing a second feed medium comprising a carbon source, wherein the second feed

container is operably connected to an inlet of the at least one production reactor, wherein the system preferably comprises means for regulating feed flow from the second feed container to the at least one production reactor.

36. The system of any one of the preceding claims, wherein an outlet of the seed reactor and an outlet of a second feed reactor are connected to a mixing chamber, wherein an outlet of the mixing chamber is connected to an inlet of the at least one production reactor.

37. The system of any one of the preceding claims, wherein the seed reactor is a stirred tank reactor or plug flow reactor.

38. The system of any one of the preceding claims, wherein the at least one production reactor is a stirred tank reactor or a plug flow reactor.

39. The system of any one of the preceding claims, wherein the seed reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L.

40. The system of any one of the preceding claims, wherein the at least one production reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L.

41. The system of any one of the preceding claims, wherein the volume ratio of the seed reactor to the at least one production reactor is from about 1:10 to about 2:1, from about 1:5 to about 2:1, from about 1:2 to about to about 2:1, from about 1.5:1 to about 1:1.5, or about 1:1.

42. The system of any one of the preceding claims, wherein the at least one production reactor comprises a culture medium comprising cells with a biomass concentration from about 10 to about 90 g/L cell dry weight, preferably from about 20 to about 80 g/L cell dry weight, preferably from about 30 to about 70 g/L cell dry weight, preferably from about 35 to about 60 g/L cell dry weight.

43. A continuous fermentation process for the production of a protein of interest or a polynucleotide of interest by a bacterial host cell, wherein the bacterial host cell comprise

under the control of a first inducible promoter a nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell, comprising

(a) culturing said bacterial host cell in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cell in a seed reactor;

(b) transferring at least an amount of the bacterial host cells obtained in (a) from said seed reactor to a production reactor; and

(c) culturing said bacterial host cells in said production reactor in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells;

wherein the seed reactor and production reactor is configured as an independent continuous fermentor and wherein the seed reactor and production reactor are connected with each other.

44. The process of claim 43, wherein (a) is for biomass production.

45. The process of claim 43 or 44, wherein in (c) growth of said bacterial host cells is inhibited by culturing said bacterial host cells in an induced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells, while said polynucleotide of interest or said protein of interest is produced.

46. The process of any one of claims 43 to 45, wherein the bacterial host cell comprises the polynucleotide of interest or a nucleotide encoding the protein of interest.

47. The process of any one of claims 43 to 46, wherein said polynucleotide of interest encodes one or more proteins of interest.

48. The process of any one of claims 43 to 47, wherein said nucleotide is a nucleic acid molecule.

49. The process of claim 48, wherein said nucleic acid molecule is a plasmid, minichromosome, or RNA.

50. The process of any one of claims 43 to 49, wherein said polynucleotide of interest or said nucleotide encoding the protein of interest is under the control of a second inducible promoter or under the control of a constitutive promoter.

51. The process of any one claims 43 to 50, wherein the promoter of said heterologous nucleotide sequence of is recognized by an RNA polymerase which is heterologous for said bacterial host cell, said heterologous RNA polymerase is encoded by a nucleotide sequence comprised by said bacterial host cell.

52. The process of claim 51, wherein said heterologous RNA polymerase is bacteriophage T3 RNA polymerase, T7 bacteriophage RNA polymerase, engineered orthogonal T7 RNA polymerase, bacteriophage SP6 RNA polymerase or bacteriophage Xp10 RNA polymerase.

53. The process of claim 51 or 52, wherein said nucleotide sequence encoding said heterologous RNA polymerase is under the control of a third inducible promoter or under the control of a constitutive promoter.

54. The process of any one of claims 43 to 53, wherein said first, second or third inducible promoter is regulated by arabinose, IPTG, tryptophan, xylose, lactose, rhamnose, phosphate, propionate, benzoic acid, phage lambda cI protein or heat.

55. The process of claim 54, whereby said first, second or third promoter are different.

56. The process of any one of claims 43 to 55, wherein said bacterial host cell has a non-functional arabinose operon.

57. The process of any one of claims 43 to 56, wherein said bacterial host cell is *E. coli*, preferably *E. coli* B-lineage.

58. The process of any one of claims 43 to 57, wherein (a) further comprises culturing said bacterial host cells in an uninduced state with respect to the nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells in culture medium in a production reactor for biomass production.

59. The process of any one of claims 43 to 58, wherein (c) further comprises inducing said first inducible promoter controlling said nucleotide sequence encoding a protein from a phage which inhibits growth of said bacterial host cells.

60. The process of any one of claims 43 to 59, wherein (b) or (c) comprises inducing said second inducible promoter controlling said polynucleotide of interest or said nucleotide encoding the protein of interest.

61. The process of any one of claims 43 to 60, wherein (a), (b) or (c) comprises inducing said third inducible promoter controlling said nucleotide sequence encoding said heterologous RNA polymerase.

62. The process of any one of claims 43 to 61, further comprising (d) harvesting the product resulting from the production of said nucleotide sequence of interest.

63. The process of any one of claims 43 to 62, wherein the seed reactor does not comprise or does not essentially comprise an inducer for the first inducible promoter.

64. The process of any one of claims 43 to 63, wherein at least one of the at least one production reactor comprises an inducer for the first inducible promoter.

65. The process of any one of claims 43 to 64, wherein at least one of the at least one of the at least one production reactor comprises an inducer for the second inducible promoter.

66. The process of any one of claims 43 to 65, wherein the first inducible promoter and the second inducible promoter can be induced by the same inducer.

67. The process of any one of claims 43 to 66, wherein pH is regulated in the seed reactor.

68. The process of any one of claims 43 to 67, wherein dissolved oxygen is regulated in the seed reactor.

69. The process of any one of claims 43 to 68, wherein temperature is regulated in the seed reactor.

70. The process of any one of claims 43 to 69, wherein gas flow is regulated in the seed reactor.

71. The process of any one of claims 43 to 70, wherein the biomass concentration is regulated in the seed reactor by feed inflow and/or biomass outflow.

72. The process of any one of claims 43 to 71, wherein pH is regulated in the at least one production reactor.

73. The process of any one of claims 43 to 72, wherein dissolved oxygen is regulated in the at least one production reactor.

74. The process of any one of claims 43 to 73, wherein temperature is regulated in the at least one production reactor.

75. The process of any one of claims 43 to 74, wherein gas flow is regulated in the at least one production reactor.

76. The process of any one of claims 43 to 75, wherein the at least one production reactor comprises a biomass sensor.

77. The process of any one of claims 43 to 76, wherein the biomass concentration is regulated in the at least one production reactor by feed inflow, biomass inflow, and/or biomass outflow.

78. The process of any one of claims 43 to 77, wherein the mean residence time of biomass in the at least one production reactor is from about 5 h to about 24 h, preferably from about 7 h to about 20 h, preferably from about 10 h to about 15 h.

79. The process of any one of claims 43 to 78, comprising a first feed container containing a first feed medium comprising a carbon source, wherein the first feed container is operably connected to an inlet of the seed reactor, wherein feed flow from the first feed container to the seed reactor is preferably regulated.

80. The process of any one of claims 43 to 79, comprising a second feed container containing a second feed medium comprising a carbon source, wherein the second feed container is operably connected to an inlet of the at least one production reactor, wherein feed flow from the second feed container to the at least one production reactor is preferably regulated.

81. The process of any one of claims 43 to 80, wherein an outlet of the seed reactor and an outlet of a second feed reactor are connected to a mixing chamber, wherein an outlet of the mixing chamber is connected to an inlet of the at least one production reactor.

82. The process of any one of claims 43 to 81, wherein the seed reactor is a stirred tank reactor or a plug flow reactor.

83. The process of any one of claims 43 to 82, wherein the at least one production reactor is a stirred tank reactor or a plug flow reactor.

84. The process of any one of claims 43 to 83, wherein the seed reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L.

85. The process of any one of claims 43 to 84, wherein the at least one production reactor has a volume of at least about 0.25 L, at least about 0.5 L, at least about 1 L, at least about 5 L, at least about 10 L, at least about 25 L, at least about 50 L, at least about 100 L, at least about 250 L, at least about 500 L, or at least about 1000 L.

86. The process of any one of claims 43 to 85, wherein the volume ratio of the seed reactor to the at least one production reactor is from about 1:10 to about 2:1, from about 1:5 to about 2:1, from about 1:2 to about to about 2:1, from about 1.5:1 to about 1:1.5, or about 1:1.

87. The process of any one of claims 43 to 86, wherein the at least one production reactor comprises a culture medium comprising cells with a biomass concentration from about 10 to about 90 g/L cell dry weight, preferably from about 20 to about 80 g/L cell dry weight, preferably from about 30 to about 70 g/L cell dry weight, preferably from about 35 to about 60 g/L cell dry weight.

88. The process of any one of claims 43 to 87, wherein the bacterial host cells in the at least one production reactor is genetically stable for at least about 5 days, preferably at least about 7 days, preferably at least about 10 days.

89. The process of any one of claims 43 to 88, wherein the process is operated for at least about 5 days, preferably at least about 7 days, preferably at least about 10 days.

90. A method for the continuous production of a compound of interest, comprising culturing the bacterial host cell as defined in any of claims 1-89 in the system of any one of claims 1- 42 and optionally adding a compound that is to be converted and/or used by said bacterial host cell for the production of said compound of interest to the seed and/or production reactor.

91. The method of claim 90, wherein the compound of interest is lycopene.

92. The method of claim 90 or 91, wherein the bacterial host cell has been genetically modified to comprise SEQ ID NOs: 18-20.

Figure 1

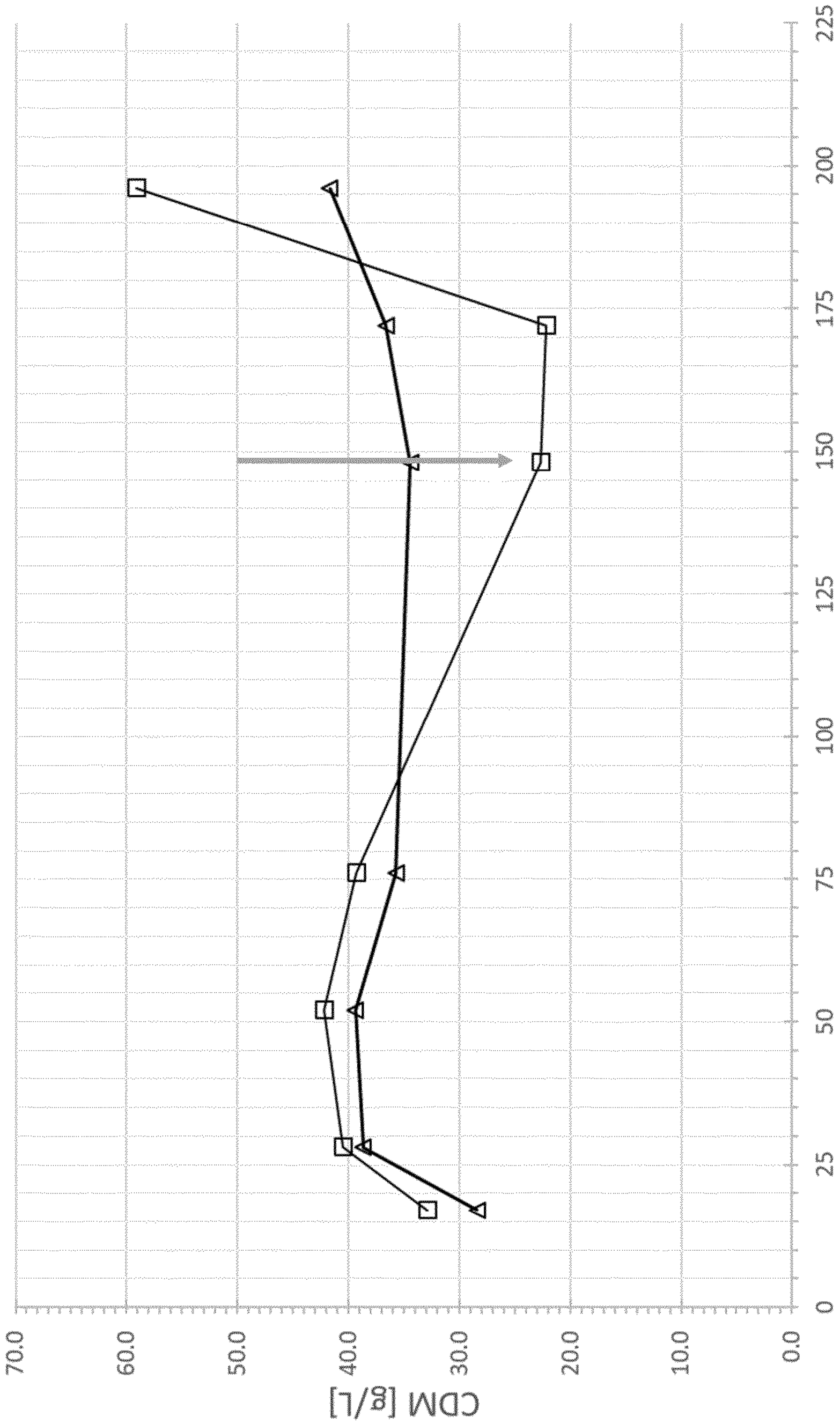

Seed reactor for biomass production / uninduced
Cell dry mass (CDM) BL21(DE3) vs. enGenes-X-press
CDM [g/L]
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
25
50
75
100
125
150
175
200
225
Process duration [h]
CDM BL unind
CDM Xpress unind

Figure 2

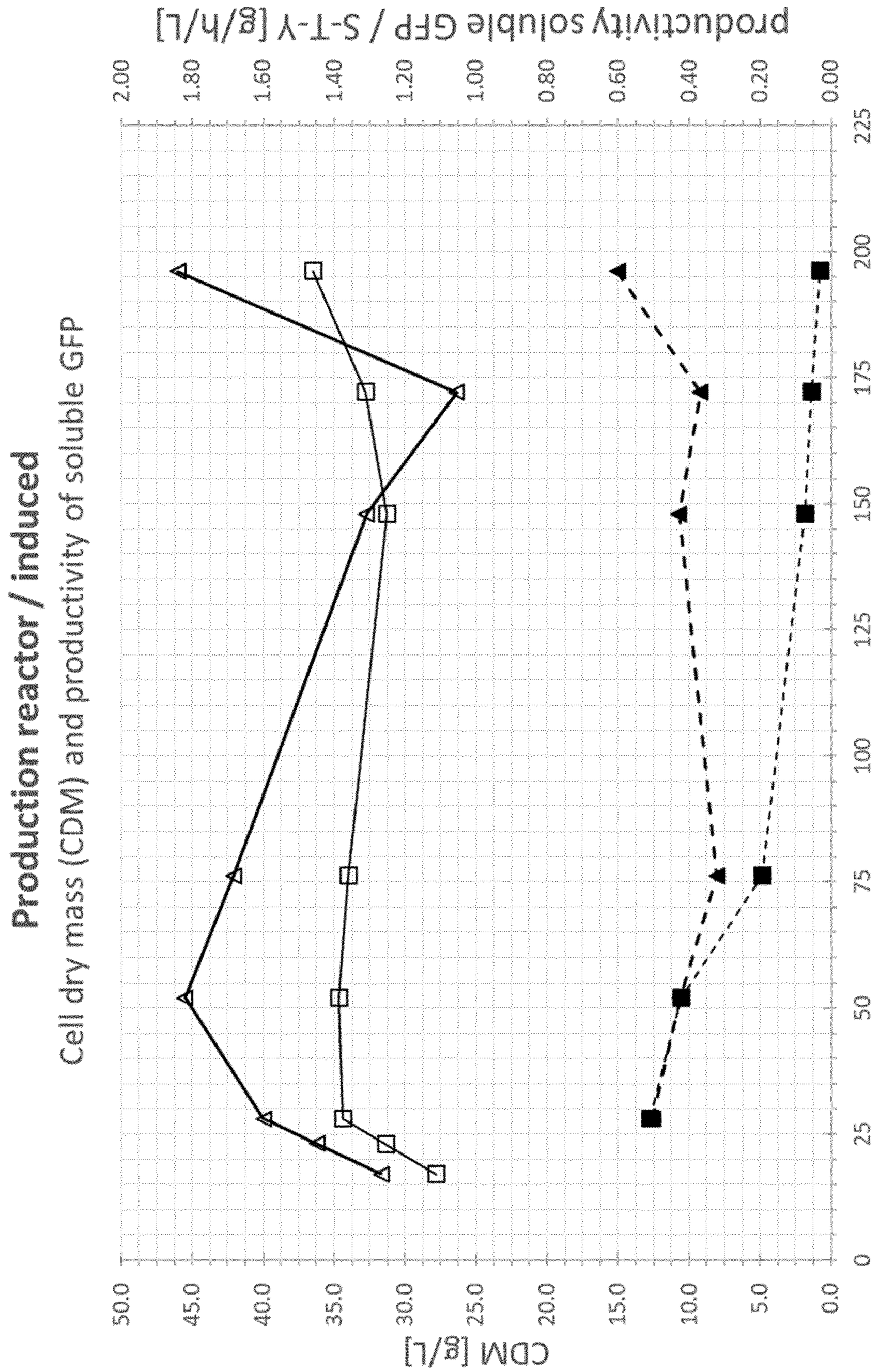
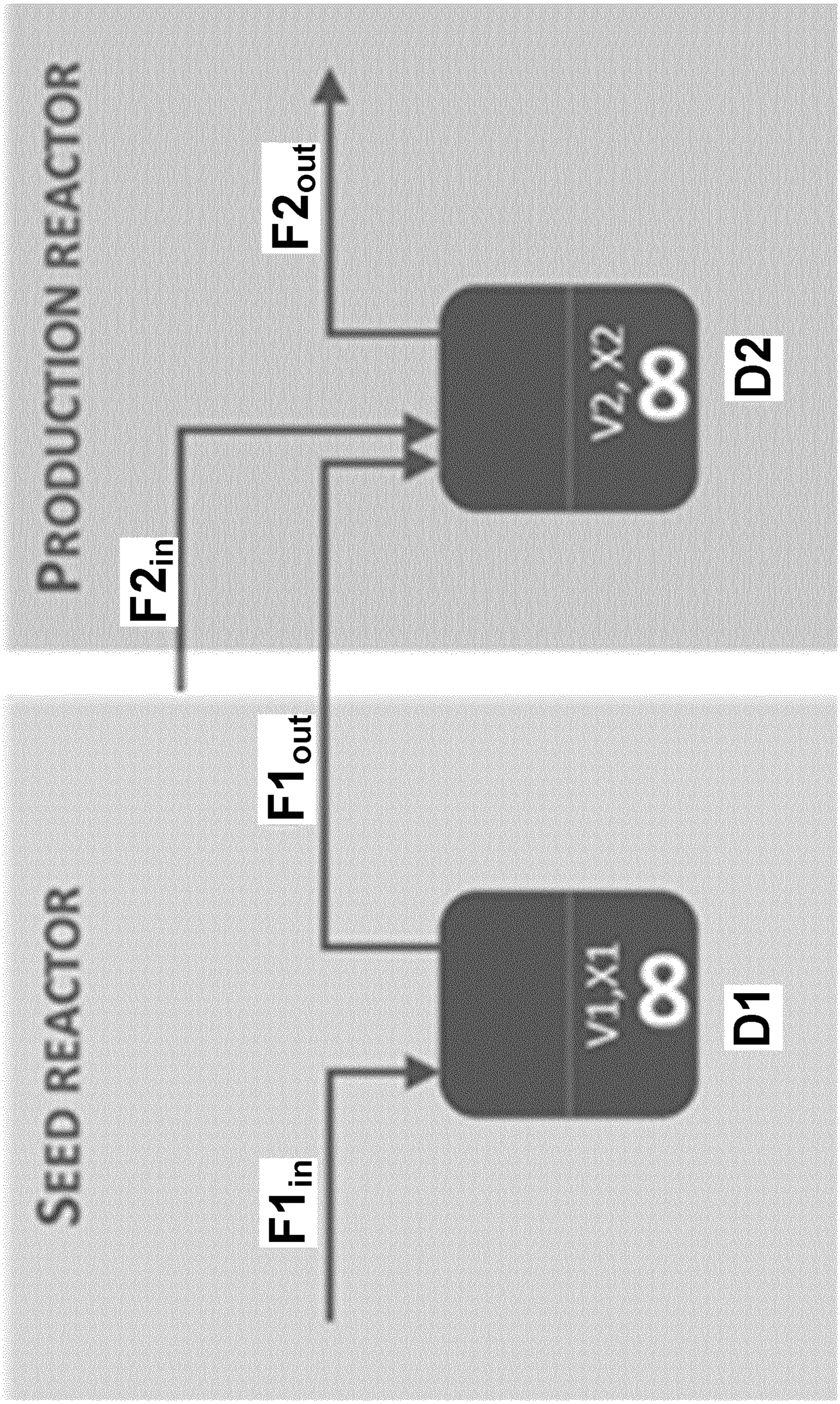
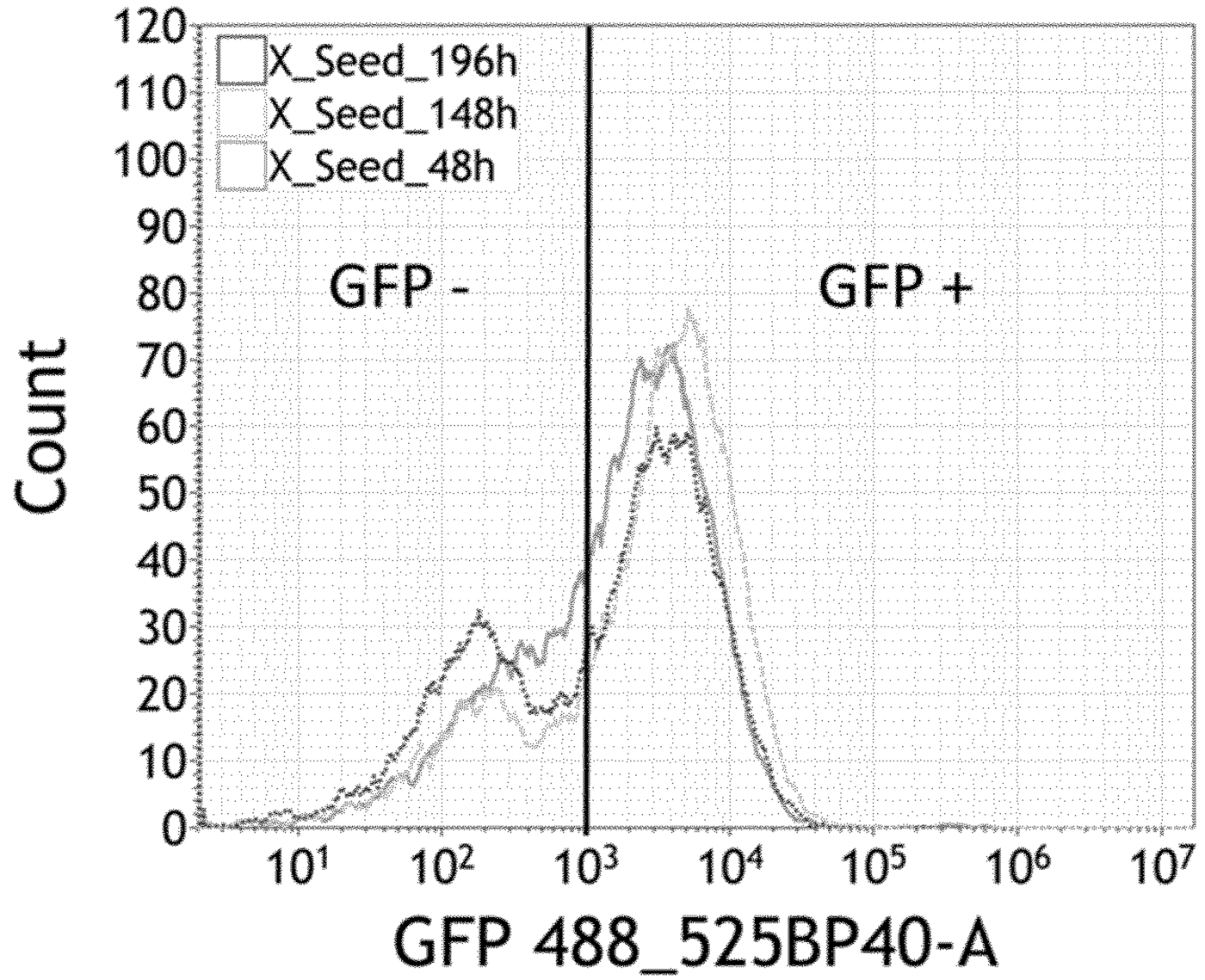
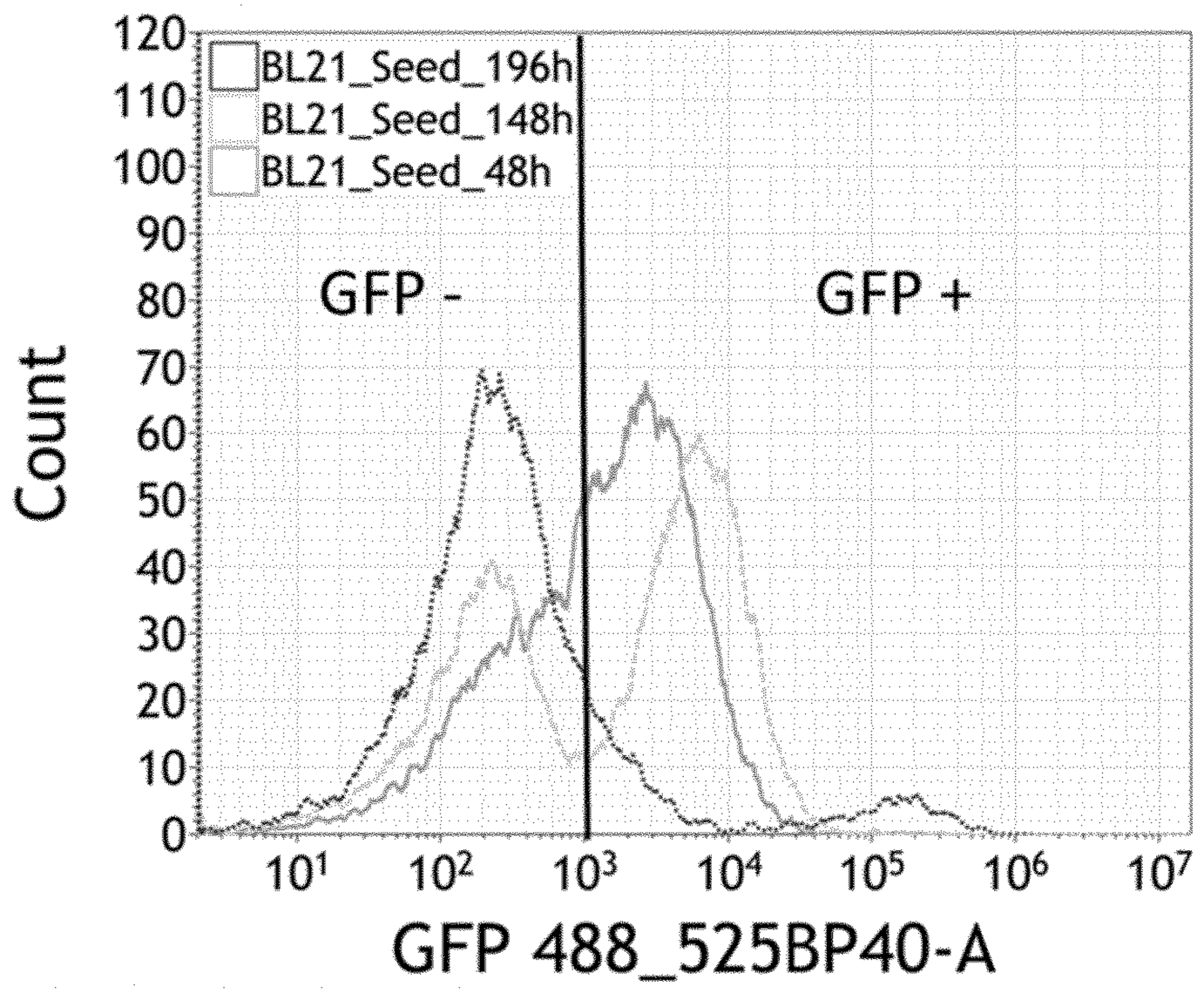

Production reactor / induced
Cell dry mass (CDM) and productivity of soluble GFP
CDM [g/L]
productivity soluble GFP / S-T-Y [g/h/L]
Process duration [h]
CDM BL21
CDM Xpress
productivity / S-T-Y BL21
productivity / S-T-Y Xpress

Figure 3


PRODUCTION REACTOR
F2out
v2x2
8
D2
F2in
SEED REACTOR
F1out
v1x1
8
D1
F1in





196 h

Gate	Number	%Gated	X-AMean	HP X-CV
O-	7,605	50.08	142.68	0.67
O+	7,581	49.92	5,535.17	61.26

Gate	Number	%Gated	X-AMean	HP X-CV
O-	13,458	87.63	151.83	0.67
O+	1,899	12.37	64,063.66	21.14

148 h

Gate	Number	%Gated	X-AMean	HP X-CV
I-	5,183	34.41	144.07	0.67
I+	9,879	65.59	6,487.49	58.19

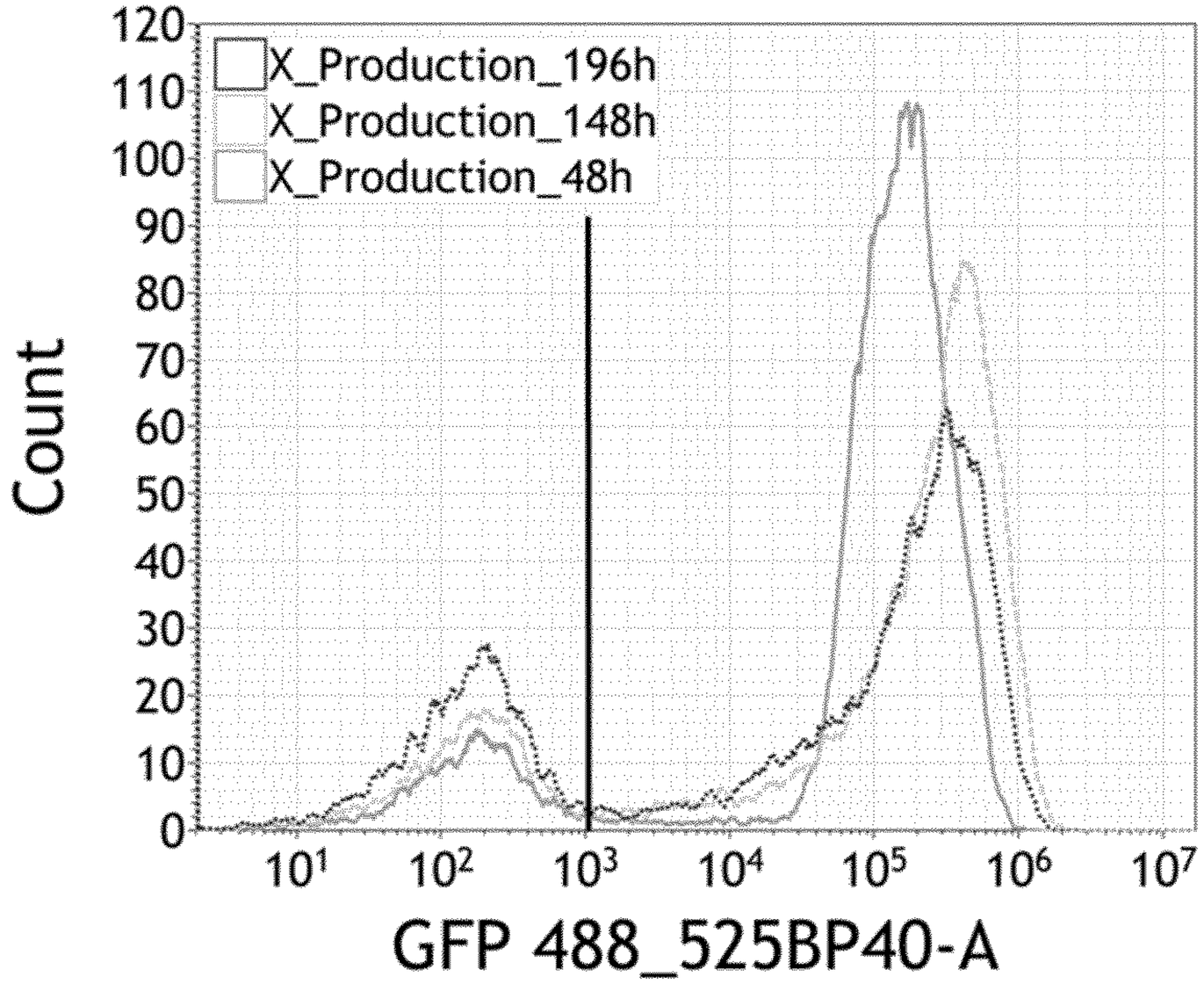
Gate	Number	%Gated	X-AMean	HP X-CV
J-	8,246	52.61	114.55	0.67
J+	7,428	47.39	8,008.30	70.09

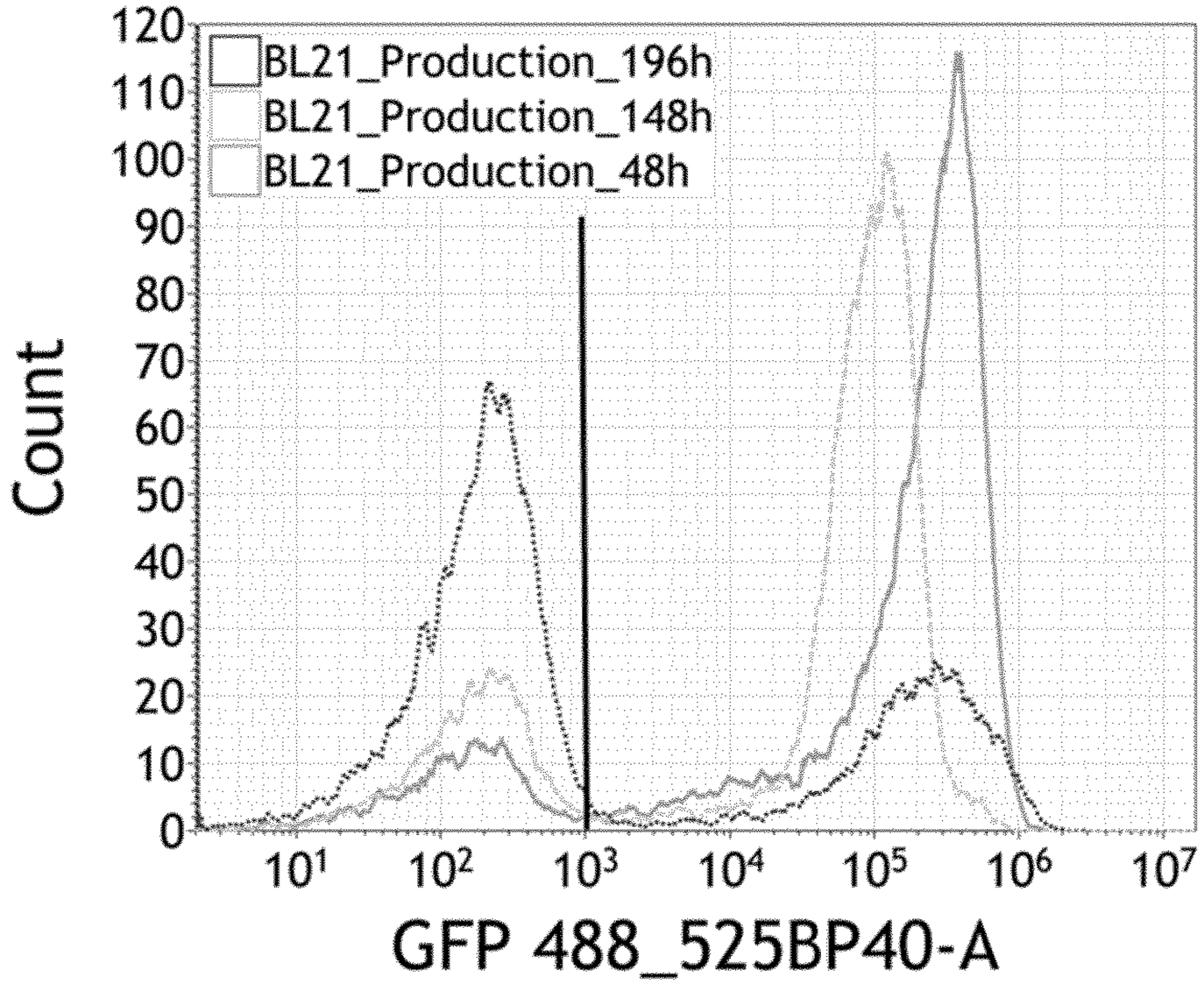
48 h

Gate	Number	%Gated	X-AMean	HP X-CV
B-	6,000	39.78	272.23	0.67
B+	9,083	60.22	5,980.36	37.03

Gate	Number	%Gated	X-AMean	HP X-CV
B-	5,043	33.41	99.69	0.67
B+	10,051	66.59	3,960.27	65.54

Figure 4





196 h

Gate	Number	%Gated	X-AMean	HP X-CV
O-	6,227	40.89	53.04	0.67
O+	9,002	59.11	284,044.54	54.60

Gate	Number	%Gated	X-AMean	HP X-CV
P-	11,545	74.27	123.46	0.67
P+	4,000	25.73	284,971.24	4.95

148 h

Gate	Number	%Gated	X-AMean	HP X-CV
I-	4,229	28.09	60.57	0.67
I+	10,828	71.91	361,384.17	45.46

Gate	Number	%Gated	X-AMean	HP X-CV
J-	4,826	32.04	87.81	0.67
J+	10,237	67.96	113,305.80	40.36

48 h

Gate	Number	%Gated	X-AMean	HP X-CV
B-	2,920	19.31	80.12	0.67
B+	12,201	80.69	188,138.45	57.42

Gate	Number	%Gated	X-AMean	HP X-CV
B-	3,370	22.42	47.07	0.67
B+	11,659	77.58	283,280.48	38.27

Figure 5

Figure 6

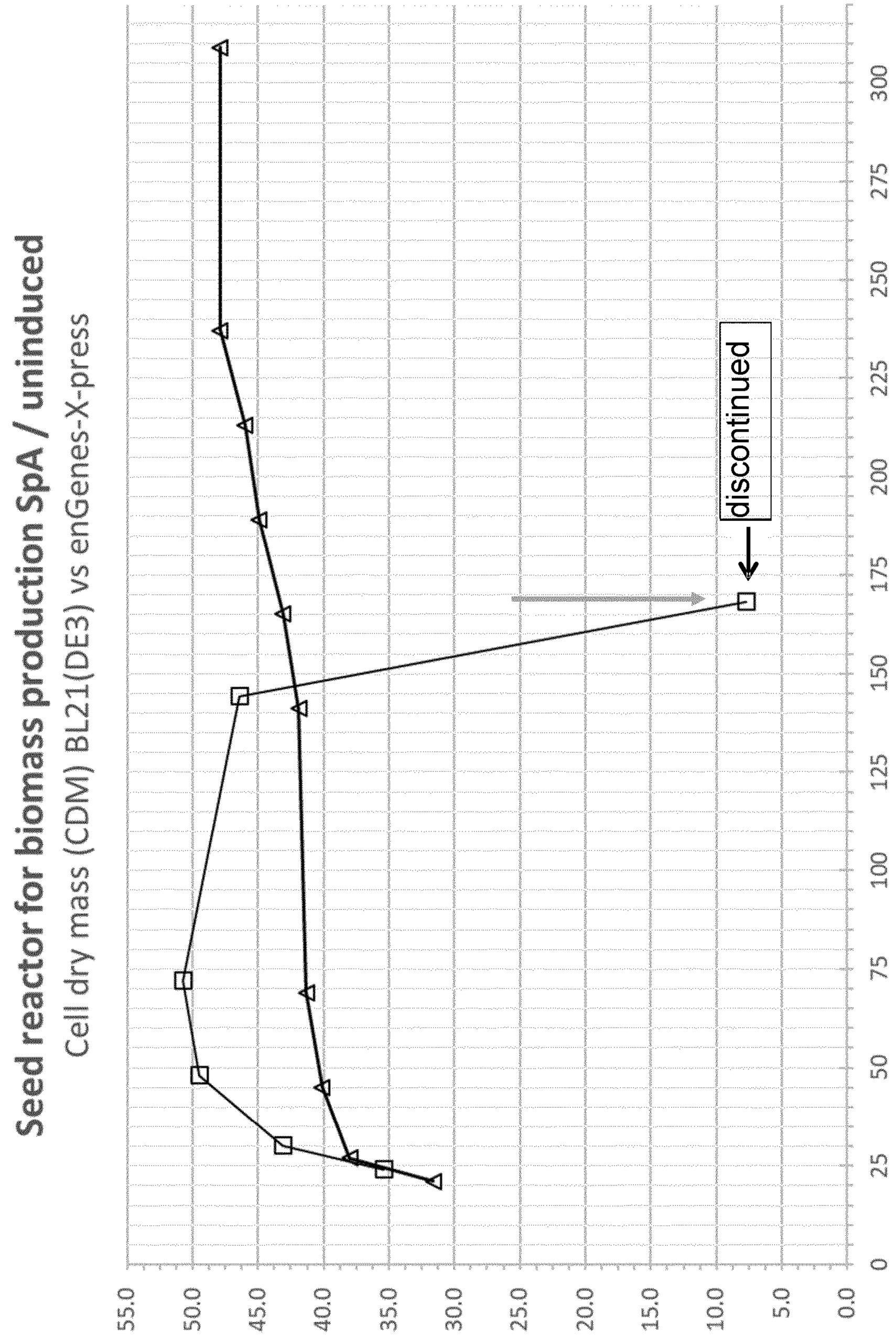

Seed reactor for biomass production SpA / uninduced
Cell dry mass (CDM) BL21(DE3) vs enGenes-X-press
discontinued
Feed duration [h]
CDM [g/L]
CDM BL21 unind
CDM Xpress unind
0
25
50
75
100
125
150
175
200
225
250
275
300
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
55.0

Figure 7

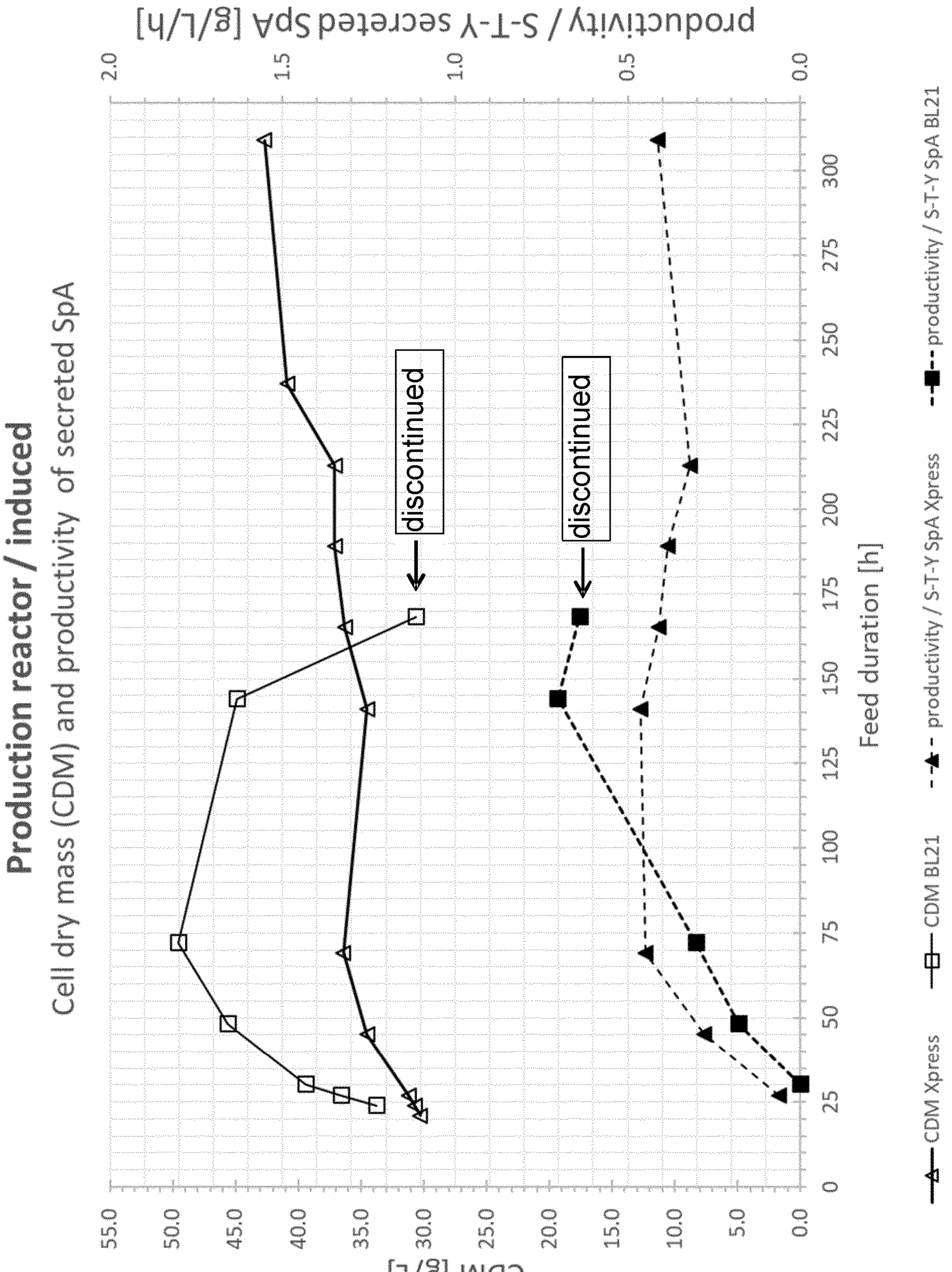

Production reactor / induced
Cell dry mass (CDM) and productivity of secreted SpA
CDM [g/L]
Feed duration [h]
productivity / S-T-Y secreted SpA [g/L/h]
discontinued
discontinued
CDM Xpress
CDM BL21
productivity / S-T-Y SpA Xpress
productivity / S-T-Y SpA BL21

Figure 8

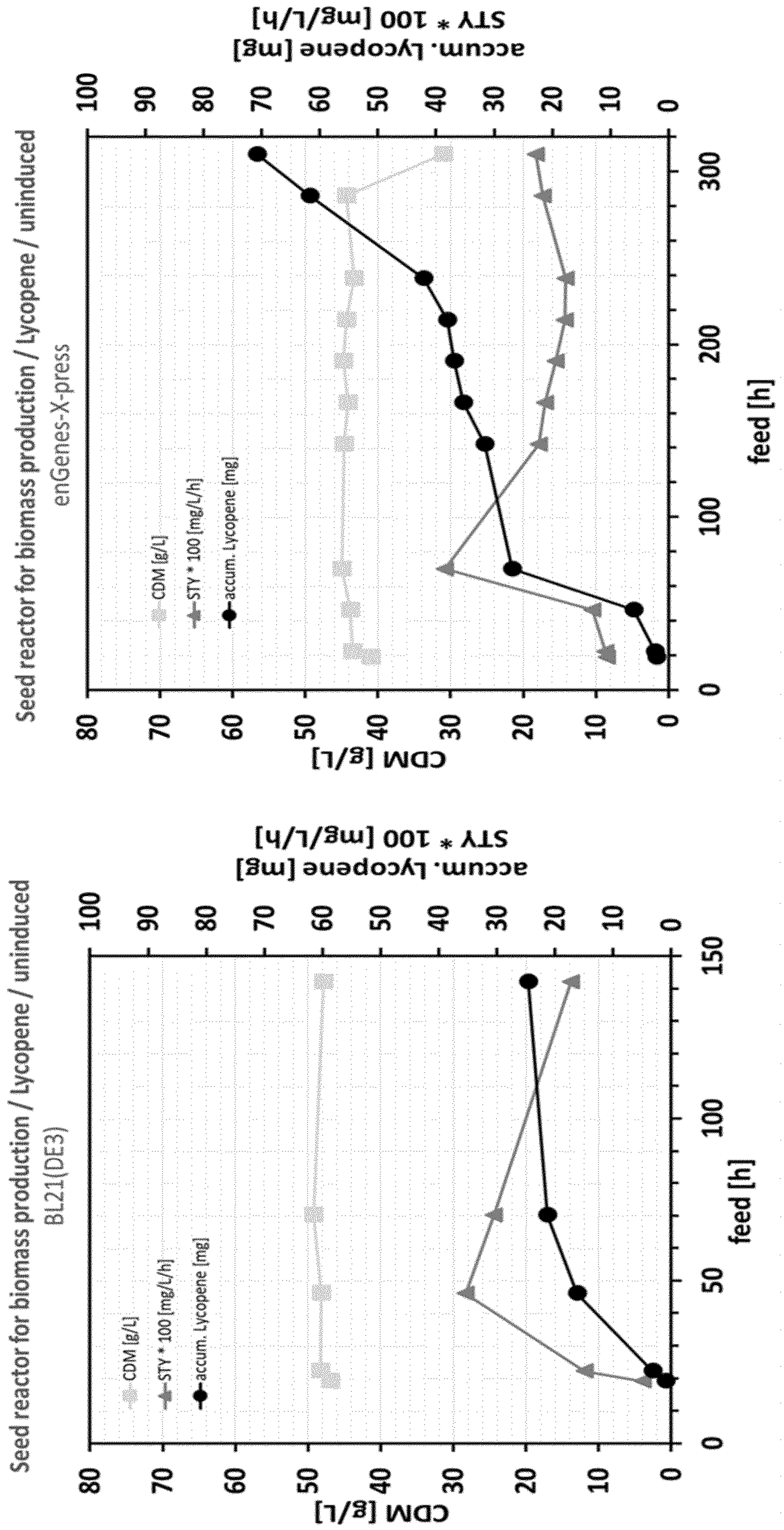

Seed reactor for biomass production / Lycopene / uninduced
enGenes-X-press
CDM [g/L]
STY * 100 [mg/L/h]
accum. Lycopene [mg]
feed [h]
Seed reactor for biomass production / Lycopene / uninduced
BL21(DE3)
CDM [g/L]
STY * 100 [mg/L/h]
accum. Lycopene [mg]
feed [h]

Figure 8 (continued)

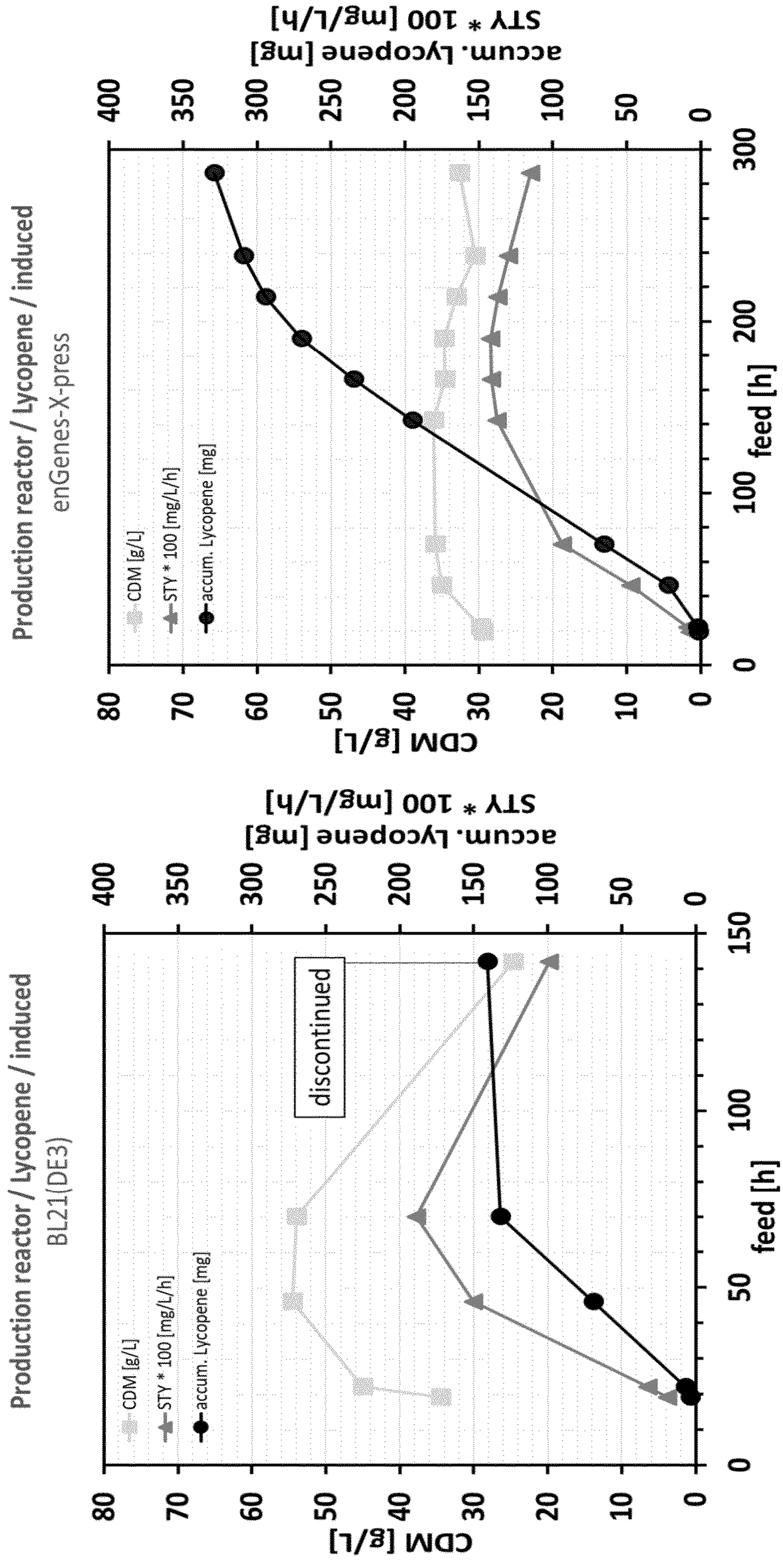


Figure 9

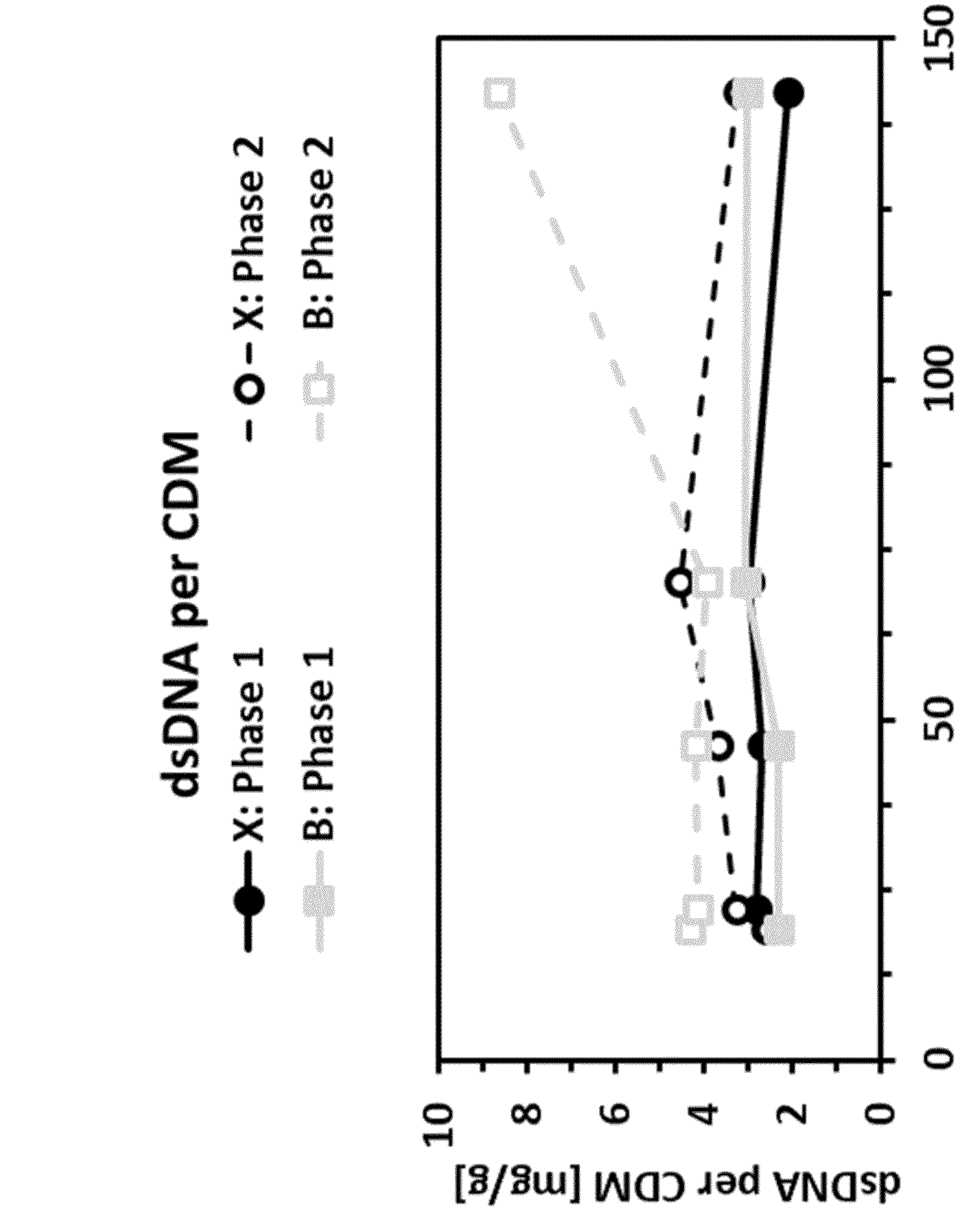
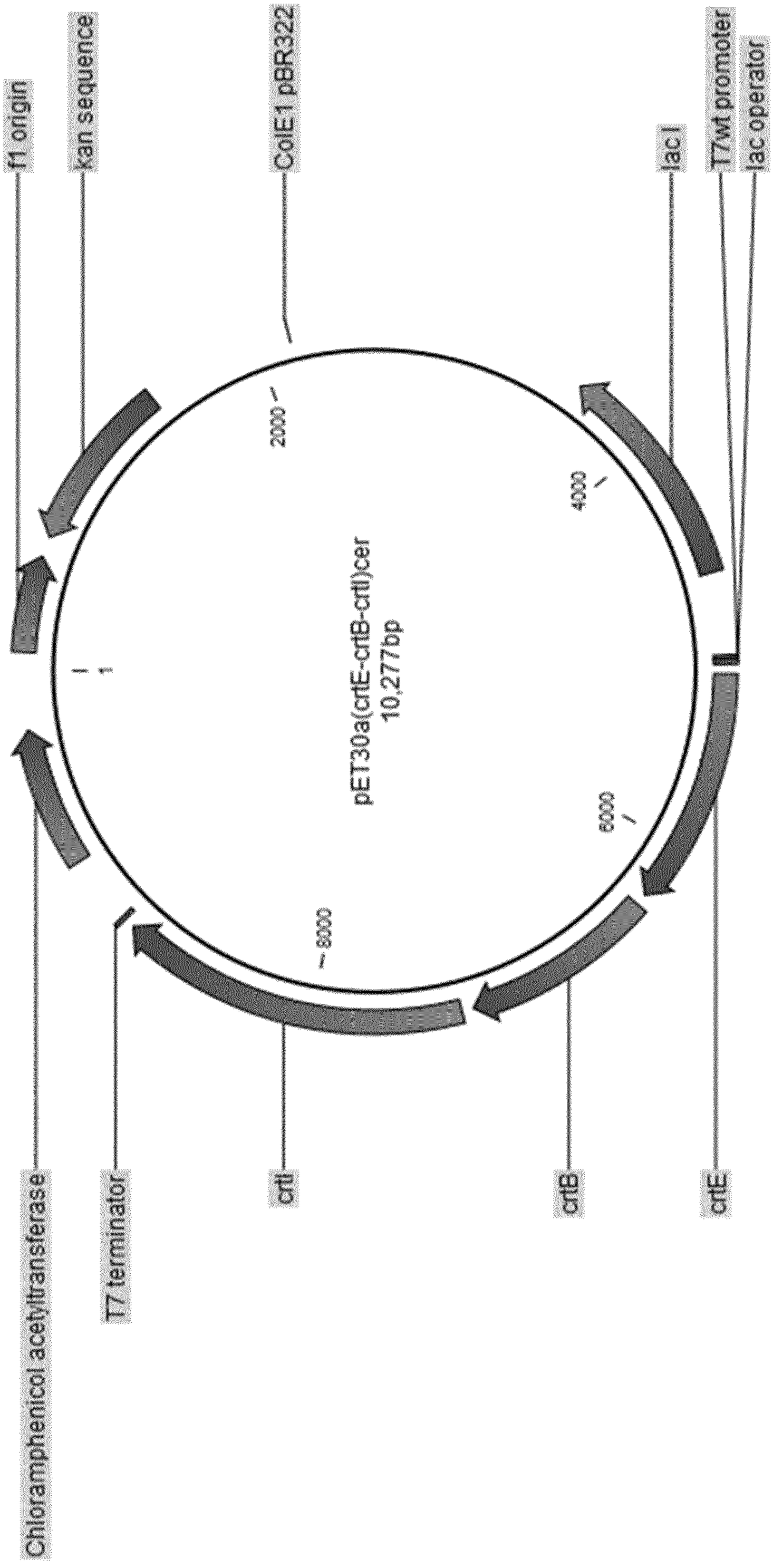

dsDNA per CDM [mg/g]
0
2
4
6
8
10
feed [h]
0
50
100
150
X: Phase 1
B: Phase 1
X: Phase 2
B: Phase 2

Figure 10


f1 origin
kan sequence
ColE1 pBR322
lacI
T7wt promoter
lac operator
2000
4000
6000
8000
1
pET30a(crtE-crtB-crtI)cer
10,277bp
Chloramphenicol acetyltransferase
T7 terminator
crtI
crtB
crtE

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/087354

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/63 C12P1/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2016/174195 A1 (UNIVERSITÄT FÜR BODENKULTUR WIEN [AT]) 3 November 2016 (2016-11-03) cited in the application paragraph [0010] - paragraph [0096]; figures 1-20; examples 1-7 -----	1-92
A	US 2018/282737 A1 (MAIRHOFER JUERGEN [AT]) 4 October 2018 (2018-10-04) paragraph [0010] - paragraph [0165]; examples 1-7 -----	1-92

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 April 2021	26/04/2021
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Westphal-Daniel, K

4

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/087354

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Moritz Wolf: "Development and Optimization of Mammalian Cell Perfusion Cultures for Continuous Biomanufacturing", , 1 January 2018 (2018-01-01), XP055718976, DOI: 10.3929/ethz-b-000308467 Retrieved from the Internet: URL:https://www.research-collection.ethz.ch/ds2/stream/?#/documents/5626123/page/1 Chapter 6 -----	1-92
A	NAVNEET RAI ET AL: "Population collapse and adaptive rescue during long-term chemostat fermentation", BIOTECHNOLOGY AND BIOENGINEERING, vol. 116, no. 3, 16 January 2019 (2019-01-16), pages 693-703, XP055718817, US ISSN: 0006-3592, DOI: 10.1002/bit.26898 the whole document -----	1-92
A	CÁMARA B ET AL: "T7 phage protein Gp2 inhibits the Escherichia coli RNA polymerase by antagonizing stable DNA strand separation near the transcription start site", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, US, vol. 107, no. 5, 2 February 2010 (2010-02-02), pages 2247-2252, XP002751044, ISSN: 0027-8424, DOI: 10.1073/PNAS.0907908107 [retrieved on 2010-01-19] the whole document -----	1-92
A	MARTIN LEMMERER ET AL: "Decoupling of recombinant protein production from Escherichia coli cell growth enhances functional expression of plant Leloir glycosyltransferases", BIOTECHNOLOGY AND BIOENGINEERING, vol. 116, no. 6, 5 February 2019 (2019-02-05), pages 1259-1268, XP055718897, ISSN: 0006-3592, DOI: 10.1002/bit.26934 the whole document ----- -/--	1-92

4

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/087354

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MATTHEW GAGNON ET AL: "Novel, linked bioreactor system for continuous production of biologics", BIOTECHNOLOGY AND BIOENGINEERING, vol. 116, no. 8, 23 May 2019 (2019-05-23), pages 1946-1958, XP055718967, ISSN: 0006-3592, DOI: 10.1002/bit.26985 the whole document -----	1-92
A	WO 2018/020012 A1 (UNIV DANMARKS TEKNISKE [DK]) 1 February 2018 (2018-02-01) page 2, line 23 - page 122, line 5 -----	1-92
T	PATRICK STARGARDT ET AL: "Bacteriophage Inspired Growth-Decoupled Recombinant Protein Production in Escherichia coli", ACS SYNTHETIC BIOLOGY, vol. 9, no. 6, 23 April 2020 (2020-04-23), pages 1336-1348, XP055718823, Washington, DC,USA ISSN: 2161-5063, DOI: 10.1021/acssynbio.0c00028 -----	1-92

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2020/087354

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016174195 A1	03-11-2016	AU 2016256579 A1	07-12-2017
		CA 2983894 A1	03-11-2016
		CN 107849573 A	27-03-2018
		DK 3289088 T3	29-06-2020
		EP 3289088 A1	07-03-2018
		ES 2806985 T3	19-02-2021
		JP 2018514231 A	07-06-2018
		KR 20170141240 A	22-12-2017
		SI 3289088 T1	31-08-2020
		WO 2016174195 A1	03-11-2016
US 2018282737 A1	04-10-2018	NONE	
WO 2018020012 A1	01-02-2018	EP 3519582 A1	07-08-2019
		US 2019367930 A1	05-12-2019
		WO 2018020012 A1	01-02-2018