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(71) Applicant: **BASF SE** [DE/DE]; Carl-Bosch-Strasse 38, 67056 Ludwigshafen am Rhein (DE).

(72) Inventors: **JAITZIG, Jennifer**; 112 East 123rd Street, Apt 3, New York, 10035 (US). **KUMAR, Mukesh**; 6 Granada Crescent Apt # 6, Westchester, White Plains, 10603 (US). **BLANKSCHIEN, Matthew David**; 200 Beacon Hill Drive, APT# 15-H1, Westchester, New York State, Dobbs Ferry, 10522 (US). **RATANI, Shakir Siraj**; 162 Union Ave, Westchester, Tarrytown, 10591 (US). **WANG, Qingzhao**; 16 prospect avenue, Ardsley, 10502 (US).

(74) Agent: **BASF IP ASSOCIATION**; BASF SE, ZRX/C6, 67056 Ludwigshafen (DE).

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(54) Title: RECOMBINANT MICROORGANISM FOR IMPROVED PRODUCTION OF ALANINE

(57) Abstract: The present invention relates to a recombinant nucleic acid molecule, a recombinant micro-organism, to a method for producing alanine and to the use of the recombinant nucleic acid molecule or the recombinant microorganism for the fermentative production of alanine.

RECOMBINANT MICROORGANISM FOR IMPROVED PRODUCTION OF ALANINE

Field of the Invention

The present invention relates to a recombinant nucleic acid molecule, a recombinant microorganism, to a method for producing alanine and to the use of the recombinant nucleic acid molecule or the recombinant microorganism for the fermentative production of alanine.

Description of the Invention

Amino acids are organic compounds with a carboxy-group and an amino-group. The most important amino acids are the alpha-amino acids where the amino group is located next to the carboxy-group. Proteins are based on alpha-amino acids.

Alanine has drawn considerable interest because it has been used as an additive in the food, feed and pharmaceutical industries. Moreover alanine is a raw material for the industrial production of alanine, N,N-bis(carboxymethyl)-, trisodium salt (MGDA, trade name Trilon M) which is a strong chelating agent, showing an excellent performance at dissolving organic and inorganic scale (WO94/29421, WO2012/150155). Trilon M grades are readily biodegradable according to standard OECD tests. Due to the superb ecological and toxicological profile, Trilon M grades are particularly suitable for use in products for end-consumers and the demand for such biodegradable complex builders is constantly rising.

Alanine can be produced by fermentation with Coryneform bacteria (Hermann, 2003: Industrial production of amino acids by Coryneform bacteria, J. of Biotechnol, 104, 155- 172.) or E.coli. (WO2007/120198, WO2008/119009).

Alanine production in E. coli is more efficient and widely used for industrial production of alanine as raw material for the chemical industry. As the demand of the chemical industry for alanine is increasing, there is a demand for improvement of productivity of fermentative production of alanine.

It is one object of the present invention to provide microorganisms which can be used in fermentative production of alanine with high yield and efficiency.

Detailed Description of the Invention

A contribution to achieving the above mentioned aim is provided by a recombinant microorganism of the family of Escherichia coli (E. coli) having, compared to a respective reference microorganism, an introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase in said microorganism compared to a respective control microorganism not

comprising said introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene.

Accordingly, one embodiment of the invention at hand is a recombinant microorganism comprising compared to a respective reference microorganism an introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase and having compared to a respective reference microorganism a higher yield and/or productivity of alanine in fermentative production.

The term “higher”, “increase” or “enhanced” e.g. in reference to expression and/or activity of an enzyme or to yield or productivity, means a significantly higher, increased or enhanced expression and/or activity or yield or productivity as compared to a reference or control microorganism.

The term “reduced, repressed or deleted expression and/or activity of an enzyme”, means a significantly reduced, repressed or deleted expression and/or activity and also encompasses an undetectable expression and/or activity of the respective enzymes in a reference or control microorganism.

The term “reference microorganism” as used herein means a control microorganism to which the recombinant microorganism is compared. This reference microorganism has substantially the same genotype as the recombinant microorganism with the exception of the difference to be analyzed. Preferably the reference microorganism is the strain from which the recombinant microorganism is originated. For example, a gene has been introduced into a wild type microorganism, thus creating a recombinant microorganism, in this case the wild type would be a suitable reference microorganism for this recombinant microorganism. It is also possible, that into a recombinant microorganism A a further mutation is introduced, thereby creating a recombinant microorganism B. The recombinant microorganism A would then be the suitable reference microorganism for recombinant microorganism B. In the event, the performance of a recombinant microorganism and the respective reference microorganism shall be compared both microorganisms are grown under substantially identical conditions.

It is obvious for the skilled person that a microorganism having an increased yield and/or productivity of alanine can also be used for the production of other metabolites that are closely related to alanine, for example metabolites that are intermediates in the alanine pathway, that share common intermediates with the alanine pathway or that are metabolites which use alanine as intermediate in their pathway. The microorganisms of the invention can also be easily adapted for having an increased yield and/or productivity of such related metabolites by increasing or introducing certain enzyme activities or by knocking out or decreasing certain enzyme activities.

Such metabolites are for example pyruvate, succinate, aspartate, malate, lactate, valine and leucine.

For example, in order to use the recombinant microorganism of the invention to produce succinate, the genes ldh, pfl, pta and adhE have to be knocked out and a PEP carboxylase gene and/or a pyruvate carboxylase gene have to be introduced in the genome of the microorganism of the invention. The respective pathway and necessary mutations are described for example in Zhang et al. (2009), PNAS (106) pp20180-20185.

Accordingly, another embodiment of the invention at hand is a recombinant microorganism comprising compared to a respective reference microorganism an introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase and having compared to a respective reference microorganism a higher yield and/or productivity of pyruvate, succinate, aspartate, malate, lactate, valine and/or leucine in fermentative production.

Furthermore another embodiment of the invention at hand is a recombinant microorganism comprising compared to a respective reference microorganism an introduced, increased or enhanced activity and/or expression of an asd and a gdhA gene and having compared to a respective reference microorganism a higher yield and/or productivity of alanine in fermentative production.

In some embodiments, the microorganism is a prokaryotic cell. Suitable prokaryotic cells include Gram-positive, Gram negative and Gram-variable bacterial cells, preferably Gram-negative.

Thus, microorganisms that can be used in the present invention include, but are not limited to, Gluconobacter oxydans, Gluconobacter asaii, Achromobacter delmarvae, Achromobacter viscosus, Achromobacter lacticum, Agrobacterium tumefaciens, Agrobacterium radiobacter, Alcaligenes faecalis, Arthrobacter citreus, Arthrobacter tumescens, Arthrobacter paraffineus, Arthrobacter hydrocarboglutamicus, Arthrobacter oxydans, Aureobacterium saperdae, Azotobacter indicus, Brevibacterium ammoniagenes, Brevibacterium divaricatum, Brevibacterium lactofermentum, Brevibacterium flavum, Brevibacterium globosum, Brevibacterium fuscum, Brevibacterium ketoglutamicum, Brevibacterium helcolum, Brevibacterium pusillum, Brevibacterium testaceum, Brevibacterium roseum, Brevibacterium immariophilium, Brevibacterium linens, Brevibacterium protopharmiae, Corynebacterium acetophilum, Corynebacterium glutamicum, Corynebacterium callunae, Corynebacterium acetoacidophilum, Corynebacterium acetoglutamicum, Enterobacter aerogenes, Erwinia amylovora, Erwinia carotovora, Erwinia herbicola, Erwinia chrysanthemi, Flavobacterium peregrinum, Flavobacterium fucatum, Flavobacterium aurantinum, Flavobacterium rhenanum, Flavobacterium sewanense, Flavobacterium breve, Flavobacterium meningosepticum, Micrococcus

sp. CCM825, Morganella morganii, Nocardia opaca, Nocardia rugosa, Planococcus eucinatus, Proteus rettgeri, Propionibacterium shermanii, Pseudomonas synxantha, Pseudomonas azotoformans, Pseudomonas fluorescens, Pseudomonas ovalis, Pseudomonas stutzeri, Pseudomonas acidovolans, Pseudomonas mucidolens, Pseudomonas testosteroni, Pseudomonas aeruginosa, Rhodococcus erythropolis, Rhodococcus rhodochrous, Rhodococcus sp. ATCC 15592, Rhodococcus sp. ATCC 19070, Sporosarcina ureae, Staphylococcus aureus, Vibrio metschnikovii, Vibrio tyrogenes, Actinomadura madurae, Actinomyces violaceochromogenes, Kitasatosporia parulosa, Streptomyces avermitilis, Streptomyces coelicolor, Streptomyces flavelus, Streptomyces griseolus, Streptomyces lividans, Streptomyces olivaceus, Streptomyces tanashiensis, Streptomyces virginiae, Streptomyces antibioticus, Streptomyces cacaoi, Streptomyces lavendulae, Streptomyces viridochromogenes, Aeromonas salmonicida, Bacillus pumilus, Bacillus circulans, Bacillus thiaminolyticus, Escherichia freundii, Microbacterium ammoniaphilum, Serratia marcescens, Salmonella typhimurium, Salmonella schottmulleri, Xanthomonas citri and so forth.

In some embodiments, the microorganism is a eukaryotic cell. Suitable eukaryotic cells include yeast cells, as for example Saccharomyces spec, such as Saccharomyces cerevisiae, Hansenula spec, such as Hansenula polymorpha, Schizosaccharomyces spec, such as Schizosaccharomyces pombe, Kluyveromyces spec, such as Kluyveromyces lactis and Kluyveromyces marxianus, Yarrowia spec, such as Yarrowia lipolytica, Pichia spec, such as Pichia methanolica, Pichia stipites and Pichia pastoris, Zygosaccharomyces spec, such as Zygosaccharomyces rouxii and Zygosaccharomyces bailii, Candida spec, such as Candida boidinii, Candida utilis, Candida freyschussii, Candida glabrata and Candida sonorensis, Schwanniomyces spec, such as Schwanniomyces occidentalis, Arxula spec, such as Arxula adeninivorans, Ogataea spec such as Ogataea minuta, Klebsiella spec, such as Klebsiella pneumonia.

Numerous bacterial industrial strains are especially suitable for use in the methods disclosed herein. In some embodiments, the microorganism is a species of the genus Corynebacterium, e.g. C. acetophilum, C. glutamicum, C. callunae, C. acetoacidophilum, C. acetoglutamicum. In some embodiments, the microorganism is a species of the genus Bacillus, e.g., B. thuringiensis, B. anthracis, B. megaterium, B. subtilis, B. lentils, B. circulans, B. pumilus, B. lautus, B.coagulans, B. brevis, B. firmus, B. alkaophius, B. licheniformis, B. clausii, B. stearothermophilus, B. halodurans, B. subtilis, B. pumilus, and B. amyloliquefaciens. In some embodiments, the microorganism is a species of the genus Erwinia, e.g., E. uredovora, E. carotovora, E. ananas, E. herbicola, E. punctata and E. terreus. In some embodiments, the microorganism is a species of the genus Escherichia, e.g., E. coli. In other embodiments the microorganism is a species of the genus Pantoea, e.g., P. citrea or P. agglomerans. In still other embodiments, the microorganism is a species of the genus Streptomyces, e.g., S. ambofaciens, S. achromogenes, S. avermitilis, S. coelicolor, S. aureofaciens, S. aureus, S. fungicidicus, S. griseus or S. lividans. In further embodiments, the microorganism is a species of the genus Zymomonas, e.g., Z. mobilis or Z. lipolytica. In further

embodiments, the microorganism is a species of the genus Rhodococcus, e.g. R opacus.

Preferably the microorganism is selected from the family of Enterobacteriaceae, preferably of the genus Escherichia, for example Escherichia coli (E. coli), preferably the strain E.coli W, which corresponds to DSMZ 1116, which corresponds to ATCC9637.

In addition to the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase, the recombinant microorganism of the invention may further comprise (a) a reduced, repressed or deleted activity and/or expression of a pflB gene encoding a pyruvate formate lyase I, wherein the reduction, repression or deletion of the activity and/or expression of the pflB gene is determined compared to a respective reference microorganism.

In addition to the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase, the recombinant microorganism of the invention may further comprise (b) a reduced, repressed or deleted activity and/or expression of a adhE gene encoding a bifunctional acetaldehyde-CoA dehydrogenase/iron-dependent alcohol dehydrogenase/pyruvate-formate lyase deactivase), wherein the reduction, repression or deletion of the activity and/or expression of the adhE gene is determined compared to a respective reference microorganism.

In addition to the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase, the recombinant microorganism of the invention may further comprise (c) a reduced, repressed or deleted activity and/or expression of a ldhA gene encoding a NAD-dependent fermentative D-lactate dehydrogenase, wherein the reduction, repression or deletion of the activity and/or expression of the ldhA gene is determined compared to a respective reference microorganism.

In addition to the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase, the recombinant microorganism of the invention may further comprise (d) a reduced, repressed or deleted activity and/or expression of a pta gene encoding a phosphate acetyltransferase and/or a reduced, repressed or deleted activity and/or expression of an ackA gene encoding an acetate kinase A and propionate kinase 2, wherein the reduction, repression or deletion of the activity and/or expression of the pta gene and/or the ackA gene is determined compared to a respective reference microorganism.

In addition to the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase, the recombinant microorganism of the invention may further comprise (e) a

reduced, repressed or deleted activity and/or expression of a frdA gene encoding a fumarate reductase, wherein the reduction, repression or deletion of the activity and/or expression of the frdA gene is determined compared to a respective reference microorganism.

In addition to the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase, the recombinant microorganism of the invention may further comprise (f) an introduced, increased or enhanced activity and/or expression of an alaD gene encoding an alanine dehydrogenase, wherein the increase or enhancement of the activity and/or expression of the alaD gene is determined compared to a respective reference microorganism.

In addition to the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase, the recombinant microorganism of the invention may further comprise (g) a reduced, repressed or deleted activity and/or expression of a dadX gene encoding a alanine racemase, wherein the reduction, repression or deletion of the activity and/or expression of the dadX gene is determined compared to a respective reference microorganism.

Preferably, the recombinant microorganism of the invention comprising the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase is additionally having at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, most preferably all of the features selected from the group of

(a) a reduced, repressed or deleted activity and/or expression of a pflB gene encoding a pyruvate formate lyase I and
(b) a reduced, repressed or deleted activity and/or expression of a adhE gene encoding a bifunctional acetaldehyde-CoA dehydrogenase/iron-dependent alcohol dehydrogenase/pyruvate-formate lyase deactivase) and
(c) a reduced, repressed or deleted activity and/or expression of a ldhA gene encoding a NAD-dependent fermentative D-lactate dehydrogenase and
(d) a reduced, repressed or deleted activity and/or expression of a pta gene encoding a phosphate acetyltransferase and/or a reduced, repressed or deleted activity and/or expression of an ackA gene encoding an acetate kinase A and propionate kinase 2 and
(e) a reduced, repressed or deleted activity and/or expression of a frdA gene encoding a fumarate reductase and
(f) an introduced, increased or enhanced activity and/or expression of an alaD gene encoding an alanine dehydrogenase,
(g) a reduced, repressed or deleted activity and/or expression of a dadX gene encoding an alanine racemase

wherein the reduction, repression, deletion, introduction, increase or enhancement of the activity and/or expression of a gene is determined compared to a respective reference microorganism.

The alaD gene may be derived from any organism or may be a synthetic gene designed by man, for example having codon usage optimized for expression in the recombinant microorganism of the invention or being optimized for enzyme activity, e.g. having improved Vmax or Km. Preferably the alaD gene is derived from a microorganism of one of the the geni Bacillus, Geobacillus, Paenibacillus, Halobacillus, Brevibacillus. In a more preferred embodiment the alaD gene is derived from a microorganism of the genus Geobacillus. In a most preferred embodiment, the alaD gene is derived from *Geobacillus stearothermophilus*.

In a preferred embodiment the alaD gene has been codon optimized for the expression in the recombinant microorganism of the invention.

The microorganism of the invention may comprise further genetic modifications, such as mutations, knock-outs or enhanced or introduced enzyme activities that further improve yield and/or productivity of alanine, pyruvate, succinate, aspartate, malate, lactate, valine and/or leucine, preferably succinate or alanine, more preferably alanine. For example, the microorganism of the invention may further comprise an enhanced or increased expression and/or activity of the ygaW gene from E.coli or homologs or functional equivalents thereof which has recently been described to improve alanine productivity of a microorganism when overexpressed (WO2012/172822).

In a further example, the microorganism of the invention may in addition comprise any one of, any combination of or all of the genes that are specified and described in detail in the applications PCT/IB2014/064426 and PCT/IB2014/066686 and that are beneficial for production of alanine, pyruvate, succinate, aspartate, malate, lactate, valine and/or leucine, preferably succinate or alanine, more preferably alanine.

In one embodiment the asd gene encoding an aspartate-beta-semialdehyde dehydrogenase, is selected from the group of

(i) a nucleic acid molecule comprising a sequence of SEQ ID NO: 25, or

(ii) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 25, or

(iii) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 25 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(iv) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 26, or

(v) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 26,

wherein the polypeptide encoded by (ii), (iii) or (v) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 26.

In one embodiment the gdhA gene encoding a glutamate dehydrogenase, is selected from the group of

(i) a nucleic acid molecule comprising a sequence of SEQ ID NO: 37, or

(ii) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 37, or

(iii) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 37 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(iv) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 38, or

(v) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 38,

wherein the polypeptide encoded by (ii), (iii) or (v) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 38.

The recombinant microorganism of the invention comprising the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase may further comprise any one, two, three, four, five, six or all of the features as defined above under (a) to (g), wherein the pflB gene is selected from the group consisting of

(A) a nucleic acid molecule comprising a sequence of SEQ ID NO: 5, or

(B) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 5, or

(C) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 5 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(D) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 6, or

(E) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 6,

wherein the polypeptide encoded by (B), (C) or (E) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 6 and

wherein the adhE gene is selected from the group consisting of

(F) a nucleic acid molecule comprising a sequence of SEQ ID NO: 7, or

(G) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 7, or

(H) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 7 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(I) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 8, or

(J) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 8,

wherein the polypeptide encoded by (G), (H) or (J) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 8 and

wherein the ldhA gene is selected from the group consisting of

(K) a nucleic acid molecule comprising a sequence of SEQ ID NO: 9, or

(L) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 9, or

(M) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 9 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(N) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 10, or

(O) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 10,

wherein the polypeptide encoded by (L), (M) or (O) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 10 and

wherein the pta gene is selected from the group consisting of

(P1) a nucleic acid molecule comprising a sequence of SEQ ID NO: 11, or

(Q1) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 11, or

(R1) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 11 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(S1) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 12, or

(T1) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 12,

wherein the polypeptide encoded by (Q1), (R1) or (T1) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 12 and

wherein the ackA gene is selected from the group consisting of

(P2) a nucleic acid molecule comprising a sequence of SEQ ID NO: 32, or

(Q2) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 32, or

(R2) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 32 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(S2) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 33, or

(T2) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 33,

wherein the polypeptide encoded by (Q2), (R2) or (T2) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 33 and

wherein the frdA gene is selected from the group consisting of

(U) a nucleic acid molecule comprising a sequence of SEQ ID NO: 13, or

(V) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 13, or

(W) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 13 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(X) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 14, or

(Y) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 14,

wherein the polypeptide encoded by (V), (W) or (Y) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 14 and

wherein the alaD gene is selected from the group consisting of

(Z) a nucleic acid molecule comprising a sequence of SEQ ID NO: 1, or

(AA) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 1, or

(BB) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 1 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(CC) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 2, or

(DD) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 2,

wherein the polypeptide encoded by (AA), (BB) or (DD) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 2 and

wherein the dadX gene is selected from the group consisting of

(EE) a nucleic acid molecule comprising a sequence of SEQ ID NO: 15, or

(FF) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 15, or

(GG) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 15 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(HH) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 16, or

(II) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%,at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 16,

wherein the polypeptide encoded by (FF), (GG) or (II) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 16.

In a further example, the microorganism of the invention may in addition comprise any one of, any combination of or all of the genes that are specified and described in detail in the applications WO2015/044818 and PCT/IB2014/066686 and that are beneficial for production of alanine.

The recombinant microorganism of the invention comprising the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase may further comprise (h) a reduced, repressed or deleted activity and/or expression of a brnQ gene encoding a brnQ protein having a branched chain amino acid transporter activity, wherein the reduction, repression or deletion of the activity and/or expression of the brnQ gene is determined compared to a respective reference microorganism.

The recombinant microorganism of the invention comprising the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase may further comprise (i) a reduced, repressed or deleted activity and/or expression of a gcvB gene encoding a non-protein encoding RNA, wherein the reduction, repression or deletion of the activity and/or expression of the gcvB gene is determined compared to a respective reference microorganism.

The recombinant microorganism of the invention comprising the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase may further comprise (j) an increased or enhanced activity and/or expression of a zipA gene encoding a cell division protein involved in Z ring assembly, wherein the an increased or enhanced activity and/or expression of the zipA gene is determined compared to a respective reference microorganism.

The recombinant microorganism of the invention comprising the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase may further comprise (k) an increased or enhanced activity and/or expression of a lpd gene encoding a lipoamide dehydrogenase, wherein the an increased or enhanced activity and/or expression of the lpd gene is determined compared to a respective reference microorganism.

The recombinant microorganism of the invention comprising the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase may further comprise (l) a mutated lpxD gene encoding an UDP-3-O-(3-hydroxymyristoyl)-glucosamine N-acyltransferase protein, wherein lpxD gene is mutated compared to a respective reference microorganism.

The recombinant microorganism of the invention comprising the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase may further comprise (m) an increased or enhanced activity and/or expression of a gcvA gene encoding a DNA-binding protein, wherein the an increased or enhanced activity and/or expression of the gcvA gene is determined compared to a respective reference microorganism.

The recombinant microorganism of the invention comprising the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase may further comprise (n) an increased or enhanced activity and/or expression of an ygaW gene encoding an alanine transporter, wherein the an increased or enhanced activity and/or expression of the ygaW

gene is determined compared to a respective reference microorganism.

Preferably, the recombinant microorganism of the invention comprising the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase having the features as defined above under a) to g) is additionally having at least one, preferably at least two, more preferably at least three, more preferably at least four, more preferably at least five, even more preferably at least six, most preferably all of the features selected from the group of

(h) a reduced, repressed or deleted activity and/or expression of a brnQ gene encoding a brnQ protein having a branched chain amino acid transporter activity and
(i) a reduced, repressed or deleted activity and/or expression of a gcvB gene encoding a non-protein encoding RNA and
(j) an increased and/or enhanced activity and/or expression of a zipA gene encoding cell division protein involved in Z ring assembly and
(k) an increased and/or enhanced activity and/or expression of a lpd gene encoding a lipoamide dehydrogenase and
(l) a changed activity of a lpxD gene encoding encoding an UDP-3-O-(3-hydroxymyristoyl)-glucosamine N-acyltransferase protein and
(m) an increased and/or enhanced activity and/or expression of a gcvA gene encoding a DNA-binding protein and
(n) an increased and/or enhanced activity and/or expression of a ygaW gene encoding an alanine transporter,

wherein the reduction, repression, deletion, increase, enhancement or change of the activity and/or expression of a gene is determined compared to a respective reference microorganism.

The recombinant microorganism of the invention comprising the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase and comprising any one, two, three, four, five, six or all of the features as defined above under (a) to (g) and/or (A) to (II), may further comprise any one, two, three, four, five, six or all of the features as defined above under (h) to (n)

wherein the brnQ gene is selected from the group consisting of

(1) a nucleic acid molecule comprising a sequence of SEQ ID NO: 23, or
(2) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 23, or
(3) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 23 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(4) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 24, or

(5) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 24,

wherein the polypeptide encoded by (2), (3) or (5) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 23 and

wherein the gcvB gene is selected from the group consisting of

(6) a nucleic acid molecule comprising a sequence of SEQ ID NO: 31, or

(7) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 31, or

wherein the nucleic acid molecule under (7) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the nucleic acid molecule having SEQ ID NO: 31 and

wherein the zipA gene is selected from the group consisting of

(8) a nucleic acid molecule comprising a sequence of SEQ ID NO: 19, or

(9) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 19, or

(10) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 19 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(11) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 20, or

(12) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 20,

wherein the polypeptide encoded by (9), (10) or (12) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 20 and

wherein the lpd gene is selected from the group consisting of

(13) a nucleic acid molecule comprising a sequence of SEQ ID NO: 21, or

(14) a nucleic acid molecule having at least 80%, preferably at least 85% for example at

least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 21, or

(15) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 21 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(16) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 22, or

(17) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 22,

wherein the polypeptide encoded by (14), (15) or (17) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 22 and

wherein the lpxD gene is selected from the group consisting of

(23) a nucleic acid molecule comprising a sequence of SEQ ID NO: 27, or

(24) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 27, or

(25) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 27 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(26) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 28, or

(27) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 28,

wherein the polypeptide encoded by (24), (25) or (27) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 28 and

wherein the polypeptide encoded by (24) to (27) comprises at the position equivalent to position 15 of SEQ ID NO: 28 a threonine, and

wherein the gcvA gene is selected from the group consisting of

(28) a nucleic acid molecule comprising a sequence of SEQ ID NO: 29, or

(29) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a

nucleic acid molecule of SEQ ID NO: 29, or

(30) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 29 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(31) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 30, or

(32) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%, at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 30,

wherein the polypeptide encoded by (29), (30) or (32) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 30 and

wherein the ygaW gene is selected from the group consisting of

(33) a nucleic acid molecule comprising a sequence of SEQ ID NO: 17, or

(34) a nucleic acid molecule having at least 80%, preferably at least 85% for example at least 90%, more preferably at least 95% for example at least 96%, even more preferably at least 97% for example at least 98%, most preferably at least 99% identity to a nucleic acid molecule of SEQ ID NO: 17, or

(35) a nucleic acid molecule hybridizing to a nucleic acid molecule having SEQ ID NO: 17 under medium stringent conditions, more preferably under high stringent conditions, most preferably under very high stringent conditions, or

(36) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 18, or

(37) a nucleic acid molecule encoding a polypeptide having at least 60% preferably at least 70% for example at least 75%, more preferably at least 80% for example at least 85%, even more preferably at least 90% for example at least 95%, most preferably at least 96%,at least 97%, at least 98% or at least 99% homology to a polypeptide of SEQ ID NO: 18,

wherein the polypeptide encoded by (34), (35) or (37) is having at least 10%, 20% preferably at least 30% or 50%, more preferably at least 60% or 70%, even more preferably at least 75%, 80%, 85% or 90 %, most preferred at least 95% of the activity as the polypeptide having SEQ ID NO: 18 and

wherein the polypeptide encoded by (34) to (37) preferably comprises at the position equivalent to position 5 of SEQ ID NO: 18 preferably a histidine, asparagine, arginine or tyrosine.

Preferably the recombinant microorganism of the invention comprising the introduced, increased or enhanced activity and/or expression of an asd or a gdhA gene encoding an aspartate-beta-semialdehyde dehydrogenase or a glutamate dehydrogenase is comprising all of the features as defined above under (a) to (g) or (A) to (II) and (j), (k) and (n) or (13) to (17) and (33) to (37).

A further embodiment of the invention is a composition comprising one or more recombinant microorganisms of the invention as defined above. The composition may further comprise a medium that allows grow of the recombinant microorganism of the invention. The medium may additionally comprise a carbon source such as hexoses, pentoses or polyols for example sucrose, glucose, fructose, galactose, mannose, raffinose, xylose, arabinose, xylulose, glycerol, mannitol, arabitol, xylitol, starch, cellulose, lignocellulose or combinations thereof. Preferably the carbon source is glucose of sucrose, more preferably the carbon source is glucose.

In a preferred embodiment the composition comprises the microorganism of the invention and NBS medium, AM1 medium or PPM01 medium. More preferably the composition further comprises a carbon source, preferably a sugar. The ingredients of these media are known to a skilled person.

Preferably NBS medium comprises per liter
1-5g, preferably 3.5g KH_2PO_4 and
1-10g, preferably 5.0g K_2HPO_4 and
1-5g, preferably 3.5g $(NH_4)_2HPO_4$ and
0.1-1g, preferably 0.25g $MgSO_4 - 7\ H_2O$ and
5-25mg, preferably 15mg $CaCL_2$ - 2 H_2O and
0.1-1mg, preferably 0.5mg Thiamine and
0.1-5ml, preferably 1ml trace metal stock,
wherein the trace metal stock comprises 0.5-5g, preferably 1.6g $FeCL_3 - 6\ H_2O$; 0.05-0.5g, preferably 0.2g $CoCl_2 - 6\ H_2O$; 0.01-0.5g, preferably 0.1g $CuCl_2$ - 2 H_2O; 0.1-0.5g, preferably 0.2g $ZnCl_2$; 0.05-0.5g, preferably 0.2g $NaMoO_4 - 2\ H_2O$; 0.001-0.1g, preferably 0.05g H_3BO_3 per liter 0.01-1 M, preferably 0.1 M HCL.
The preferred carbon source in the NBS medium is glucose or sucrose, preferably 2%-18% glucose or 2%-16% sucrose.

Preferably AM 1 medium comprises per liter 0.1-10mM, preferably 1mM betain solution
1-10g, preferably 2.6g $(NH4)_2HPO_4$ and
0.1-5g, preferably 0.87g $NH_4H_2PO_4$ and
0.05-2.5 g, preferably 0.15g KCl and
0.05-5g, preferably 0.37g $MgSO_4$-$7H_2O$ and
0.1-5ml, preferably 1ml trace metal stock,
wherein the trace metal stock comprises per liter 0.01-1 M, preferably 0.12 M HCL, 1-5g, preferably 2.4g $FeCL_3$-$6H_2O$; 0.1-1g, preferably 0.3g $CoCl_2$-$6H_2O$; 0.1-1g, preferably 0.21g $CuCl_2$ - 2 H_2O; 0.1-1g, preferably 0.3g $ZnCl_2$; 0.1-1g, preferably 0.27g NaMoO4 – 2 H_2O; 0.01-0.5g, preferably 0.068g H_3BO_3 and 0.1-1g, preferably 0.5g $MnCl_2 - 4\ H_2O$,
and optionally 1-30g, preferably 15g $(NH_4)_2SO_4$.
The preferred carbon source in the NBS medium is glucose or sucrose, preferably 2%-18% glucose or 2%-16% sucrose.

Preferably PPM01 medium comprises per liter
0.05-5g, preferably 0.37g $MgSO_4 - 7\ H_2O$ and
0.1-10g, preferably 1g $(NH_4)_2SO_4$ and
0.05-5 g, preferably 0.46g betaine and
0.001-0.5g, preferably 0.05g Cyanocobalamin (B12) and
1-10g, preferably 3.74g KH_2PO_4 and
0.1-5ml, preferably 1ml trace metal stock,
wherein the trace metal stock comprises per liter 10-100 mM, preferably 60 mM sulfuric acid, 1-10g, preferably 3.48g $(NH_4)_2Fe(II)(SO_4)_2 - 7\ H_2O$; 0.1-1g, preferably 0.35g $CoSO_4 - 7\ H_2O$; 0.1-1g, preferably 0.31g $CuSO_4 - 5\ H_2O$; 0.1-5g, preferably 0.63g $ZnSO_4 - 7\ H_2O$; 0.1-1g, preferably 0.27g $MnSO_4 - H_2O$; 0.01-1g, preferably 0.07g $NaMoO_4 - 2\ H_2O$ and 0.1-5g, preferably 0.43g H_3BO_3.
The preferred carbon source in the PPM01 medium is glucose monohydrate, preferably 10-500g, more preferably 140g glucose monohydrate per liter medium.

A further embodiment of the invention is a method for producing a recombinant microorganism with enhanced alanine yield or productivity, which comprises the following steps:

(I) introducing, increasing or enhancing of one or more activity and/or expression of the asd gene or the gdhA gene or as defined above under (i) to (v) in a microorganism; and

(II) generating, identifying and isolating a recombinant microorganism with enhanced alanine yield or productivity compared to a corresponding microorganism without introduced, increased or enhanced activity and/or expression of the asd gene or the gdhA gene or as defined above under (i) to (v).

In a preferred embodiment of the method for producing a recombinant microorganism of the invention the method further comprises the step of reducing, repressing or deleting the activity and/or expression of at least one, at least two, at least three, at least four, at least five, at least six or all of the pflB gene, adhE gene, ldhA gene, pta gene, ackA gene, frdA gene or dadX gene for example as defined above under (A) to (Y) and (EE) to (II) and/or the step of introducing, increasing or enhancing activity and/or expression of an alaD gene, lpd gene, zipA gene and ygaW gene for example as defined above under (Z) to (DD), (8) to (17) and (33) to 37).

A more preferred method for producing a recombinant microorganism of the invention comprises the step of reducing, repressing or deleting the activity and/or expression of all of the pflB gene, adhE gene, ldhA gene, ackA gene and frdA gene and the step of introducing, increasing or enhancing activity and/or expression of an alaD gene, lpd gene, zipA gene and ygaW gene.

In one embodiment of the method for producing a recombinant microorganism of the invention the microorganism is selected from the group consisting of species of the genus

Corynebacterium, e.g. C. acetophilum, C. glutamicum, C. callunae, C. acetoacidophilum, C. acetoglutamicum, species of the genus Bacillus, e.g., B. thuringiensis, B. anthracis, B. megaterium, B. subtilis, B. lentils, B. circulans, B. pumilus, B. lautus, B.coagulans, B. brevis, B. firmus, B. alkaophius, B. licheniformis, B. clausii, B. stearothermophilus, B. halodurans, B. subtilis, B. pumilus, and B. amyloliquefaciens, species of the genus Erwinia, e.g., E. uredovora, E. carotovora, E. ananas, E. herbicola, E. punctate, E. terreus, species of the genus Escherichia, e.g., E. coli, species of the genus Pantoea, e.g., P. citrea, P. agglomerans, species of the genus Streptomyces, e.g., S. ambofaciens, S. achromogenes, S. avermitilis, S. coelicolor, S. aureofaciens, S. aureus, S. fungicidicus, S. griseus, S. lividans, species of the genus Zymomonas, e.g., Z. mobilis or Z. lipolytica species of the genus Rhodococcus, e.g. R. opacus, species of the genus Saccharomyces spec, such as Saccharomyces cerevisiae, species of the genus Hansenula spec, such as Hansenula polymorpha, species of the genus Schizosaccharomyces spec, such as Schizosaccharomyces pombe, species of the genus Kluyveromyces spec, such as Kluyveromyces lactis and Kluyveromyces marxianus, species of the genus Yarrowia spec, such as Yarrowia lipolytica, species of the genus Pichia spec, such as Pichia methanolica, Pichia stipites and Pichia pastoris, species of the genus Zygosaccharomyces spec, such as Zygosaccharomyces rouxii and Zygosaccharomyces bailii, species of the genus Candida spec, such as Candida boidinii, Candida utilis, Candida freyschussii, Candida glabrata and Candida sonorensis, species of the genus Schwanniomyces spec, such as Schwanniomyces occidentalis, species of the genus Arxula spec, such as Arxula adeninivorans, species of the genus Ogataea spec such as Ogataea minuta, species of the genus Klebsiella spec, such as Klebsiella pneumoniaand.

Preferably the microorganism is selected from the family of Enterobacteriaceae, preferably of the genus Escherichia, for example Escherichia coli (E. coli), preferably the strain E.coli W, which corresponds to DSMZ 1116, which corresponds to ATCC9637.

A further embodiment of the invention is a method of producing alanine, preferably L-alanine, comprising culturing one or more recombinant microorganism as defined above under conditions that allow for the production of alanine, preferably L-alanine.

In some embodiments, the recombinant microorganisms encompassed by the invention are grown under batch or continuous fermentations conditions. Classical batch fermentation is a closed system, wherein the compositions of the medium is set at the beginning of the fermentation and is not subject to artificial alterations during the fermentation. A variation of the batch system is a fed-batch fermentation. In this variation, the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is likely to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Batch and fed-batch fermentations are common and well known in the art. Continuous fermentation which also finds use in the present invention is a system where a defined fermentation medium is added continuously to a biore-

actor and an equal amount of conditioned medium (e.g., containing the desired end-products) is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in the growth phase where production of end products is enhanced. Continuous fermentation systems strive to maintain steady state growth conditions. Methods for modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology.

In some embodiments, fermentations are carried out in a temperature within the range of from about 10°C to about 60°C, from about 15°C to about 50°C, from about 20°C to about 45°C, from about 25°C to about 45°C, from about 30°C to about 45°C and from about 25°C to about 40°C. In a preferred embodiment the temperature is about 34°C, 35°C or 36°C. In a most preferred embodiment the temperature is about 37°C or 38°C.

In some other embodiments, the fermentation is carried out for a period of time within the range of from about 8 hours to 240 hours, from about 8 hours to about 168 hours, from about 10 hours to about 144 hours, from about 15 hours to about 120 hours, or from about 20 hours to about 72 hours. Preferably the fermentation is carried out from about 20 hours to about 40 hours.

In some other embodiments, the fermentation is carried out at a pH in the range of about 4 to about 9, in the range of about 4.5 to about 8.5, in the range of about 5 to about 8, or in the range of about 5.5 to about 7.5. Preferably the fermentation will be carried out at a pH of 7.

In one embodiment of the method of producing alanine, more preferably L-alanine, the microorganism is cultured in a medium comprising between 1% and 30% (w/v) of a sugar, between 5% and 25% (w/v) of a sugar, between 10% and 20% (w/v) of a sugar, between 12% and 18% (w/v) of a sugar. Preferably the microorganism is cultured in a medium comprising between 13% and 15% (w/v) of a sugar.

In another embodiment of the method for producing alanine, more preferably L-alanine the yield of alanine is at least 80% for example at least 81%, at least 82%, at least 83%, at least 84% or at least 85%. Preferably the yield is at least 86%, at least 87%, at least 88%, at least 89% or at least 90%. More preferably the yield is at least 90.5%, at least 91%, at least 91.5%, at least 92%, at least 92.5%, at least 93%, at least 93.5%, at least 94% or at least 94.5%. In an even more preferred embodiment the yield is at least 95% or at least 95.5%. In a most preferred embodiment, the yield is at least 96%. The percent yield is calculated as gram product produced from gram glucose in the medium. Hence, when the medium contained 100g glucose and the fermentation yielded 98 g alanine, the yield would be 98%.

In another embodiment of the method for producing alanine preferably L-alanine is produced, wherein the chiral purity of L-alanine is at least 90%, at least 91%, at least 92%, at least 93% or at least 94%. In a preferred embodiment the chiral purity of L-alanine is at least 95% or at least 95.5%. In a more preferred embodiment, the chiral purity of L-alanine is at least 96% or at least 96.5% or at least 97%. In an even more preferred embodiment the chiral purity of L-alanine is at least 97.5%, at least 98% or at least 98.5% for example at least 99%. Even more preferably the chiral purity of L-alanine is at least 99.5% or at least 99.6% for example at least 99.7%, at least 99.8%, or at least 99.9%. In a most preferred embodiment chiral pure L-alanine is produced.

Another embodiment of the invention is a method of culturing or growing any of the genetically modified microorganisms as defined above, the method comprising inoculating a culture medium with one or more genetically modified microorganism and culturing or growing said genetically modified microorganism in culture medium under conditions as defined above.

The use of a recombinant microorganism as defined above or a composition as defined above for the fermentative production of alanine, preferably L-alanine is an additional embodiment of the invention.

The recombinant microorganism according to the present invention is characterized in that, compared to a respective reference microorganism for example a wild type or an alanine high production strain, the expression and/or the activity of the enzyme that is encoded by the *asd* gene or the *gdhA* gene is increased or enhanced.

Furthermore the recombinant microorganism according to the present invention is characterized in that, compared to a respective reference microorganism for example a wild type or an alanine high production strain the expression and/or the activity of both enzymes encoded by the *asd* gene and the *gdhA* gene are increased or enhanced.

In one embodiment the decrease of the expression and/or activity of a genes is achieved by a deactivation, mutation or knock-out of the gene. This could be done by deletion of part or total of the coding region and/or the promoter of the gene, by mutation of the gene such as insertion or deletion of a number of nucleotides for example one or two nucleotides leading to a frameshift in the coding region of the gene, introduction of stop codons in the coding region, inactivation of the promoter of the gene by for example deleting or mutating promoter boxes such as ribosomal entry sides, the TATA box and the like. The decrease may also be achieved by degrading the transcript of the gene for example by means of introduction of ribozymes, dsRNA, antisense RNA or antisense oligonucleotides. The decrease of the activity of a gene may be achieved by expressing antibodies or aptamers in the cell specifically binding the target enzyme. Other methods for the decrease of the expression and/or

activity of a gene are known to a skilled person.

The reduced expression and/or activity of the enzymes disclosed herein can be a reduction of the expression and/or enzymatic activity by at least 50%, compared to the expression and/or activity of said enzyme in a respective reference microorganism for example the wild type of the microorganism, or a reduction of the expression and/or enzymatic activity by at least 90%, or more preferably a reduction of expression and/or the enzymatic activity by at least 95%, or more preferably an expression and/or reduction of the enzymatic activity by at least 98%, or even more preferably a reduction of the expression and/or enzymatic activity by at least 99% or even more preferably a reduction of the expression and/or the enzymatic activity by at least 99.9%. In a most preferred embodiment the expression and/or activity of the enzymes is not detectable in the microorganism of the invention.

The enhanced or increased expression and/or activity of the enzymes disclosed herein can be an increase of the expression and/or enzymatic activity by at least 25%, compared to the expression and/or activity of said enzyme in a respective reference microorganism for example the wild type of the microorganism, or an increase of the expression and/or enzymatic activity by at least 50%, or more preferably an increase of expression and/or the enzymatic activity by at least 100%, or more preferably an increase of the expression and/or of the enzymatic activity by at least 3 fold, for example at least 5 fold, or even more preferably an increase of the expression and/or enzymatic activity by at least 10 fold or even more preferably an increase of the expression and/or the enzymatic activity by at least 20 fold.

The increase of the expression and/or activity of the asd gene or the gdhA gene leads to an improved yield and/ or productivity of alanine in the recombinant microorganism of the invention compared to a respective reference microorganism. Therefore the increase of the expression and/or activity of the asd gene or the gdhA gene may be determined by measuring alanine yield or productivity of the recombinant microorganism of the invention compared to a respective reference microorganism. Methods for fermentative production of metabolites, for example alanine are known to a skilled person and also described herein. Improved yield of e.g. alanine in fermentation by the microorganism of the invention compared to yield of alanine in fermentation by a respective reference microorganism is a measure for the increase of the expression and or activity of the asd gene or the gdhA gene.

Methods for determining the lactate dehydrogenase (ldhA) expression or activity are, for example, disclosed by Bunch et al. in “The ldhA gene encoding the fermentative lactate de hydrogenase of Escherichia Coli”, Microbiology (1997), Vol. 143, pages 187-155; or Bergmeyer, H.U., Bergmeyer J. and Grassl, M. (1983-1986) in “Methods of Enzymatic Analysis”, 3rd Edition, Volume III, pages 126-133, Verlag Chemie, Weinheim; or Enzymes in Industry: Production and Applications, Second Edition (2004), Wolfgang Aehle, page 23. Preferred is the last method.

Methods for determining the pyruvate formate lyase I (pflB) expression or activity are, for example, disclosed in Knappe J, Blaschkowski HP, Grobner P, Schmitt T (1974). "Pyruvate formate-lyase of Escherichia coli: the acetyl-enzyme intermediate." Eur J Biochem 1974;50(1);253-63. PMID: 4615902; in KNAPPE, Joachim, et al. "Pyruvate Formate-Lyase of Escherichia coli: the Acetyl-Enzyme Intermediate." European Journal of Biochemistry 50.1 (1974): 253-263; in Wong, Kenny K., et al. "Molecular properties of pyruvate formate-lyase activating enzyme." Biochemistry 32.51 (1993): 14102-14110 and in Nnyepi, Mbako R., Yi Peng, and Joan B. Broderick. "Inactivation of E. coli pyruvate formate-lyase: Role of AdhE and small molecules." Archives of biochemistry and biophysics 459.1 (2007): 1-9.

Methods for determining the bifunctional acetaldehyde-CoA dehydrogenase/iron-dependent alcohol dehydrogenase/pyruvate-formate lyase deactivase (adhE) expression or activity are, for example, disclosed in Membrillo-Hernández, Jorge, et al. "Evolution of the adhE Gene Product of Escherichia coli from a Functional Reductase to a Dehydrogenase GENETIC AND BIOCHEMICAL STUDIES OF THE MUTANT PROTEINS." Journal of Biological Chemistry 275.43 (2000): 33869-33875 and in Mbako R. Nnyepi, Yi Peng, Joan B. Broderick, Inactivation of E. coli pyruvate formate-lyase: Role of AdhE and small molecules, Archives of Biochemistry and Biophysics, Volume 459, Issue 1, 1 March 2007, Pages 1-9.

Methods for determining the phosphate acetyltransferase (pta) expression or activity are, for example, disclosed in Dittrich, Cheryl R., George N. Bennett, and Ka-Yiu San. "Characterization of the Acetate-Producing Pathways in Escherichia coli." Biotechnology progress 21.4 (2005): 1062-1067 and in Brown, T. D. K., M. C. Jones-Mortimer, and H. L. Kornberg. "The enzymic interconversion of acetate and acetyl-coenzyme A in Escherichia coli." Journal of general microbiology 102.2 (1977): 327-336.

Methods for determining the fumarate reductase (frdA) expression or activity are, for example, disclosed in Dickie, Peter, and Joel H. Weiner. "Purification and characterization of membrane-bound fumarate reductase from anaerobically grown Escherichia coli." Canadian journal of biochemistry 57.6 (1979): 813-821; in Cecchini, Gary, et al. "Reconstitution of quinone reduction and characterization of Escherichia coli fumarate reductase activity." Journal of Biological Chemistry 261.4 (1986): 1808-1814 or in Schröder, I., et al. "Identification of active site residues of Escherichia coli fumarate reductase by site-directed mutagenesis." Journal of Biological Chemistry 266.21 (1991): 13572-13579.

Methods for determining the alanine dehydrogenase (alaD) expression or activity are, for example, disclosed in Sakamoto, Y., Nagata, S., Esaki, N., Tanaka, H., Soda, K. "Gene cloning, purification and characterization of thermostable alanine dehydrogenase of Bacillus stearothermophilus" J Fermen. Bioeng. 69 (1990):154-158.

The term "*reduced expression of an enzyme*" includes, for example, the expression of the enzyme by said genetically manipulated (e.g., genetically engineered) microorganism at a

lower level than that expressed by a respective reference microorganism for example the wild type of said microorganism. Genetic manipulations for reducing the expression of an enzyme can include, but are not limited to, deleting the gene or parts thereof encoding for the enzyme, altering or modifying regulatory sequences or sites associated with expression of the gene encoding the enzyme (e.g., by removing strong promoters or repressible promoters), modifying proteins (e.g., regulatory proteins, suppressors, enhancers, transcriptional activators and the like) involved in transcription of the gene encoding the enzyme and/or the translation of the gene product, or any other conventional means of decreasing expression of a particular gene routine in the art (including, but not limited to, the use of antisense nucleic acid molecules or other methods to knock-out or block expression of the target protein). Further on, one may introduce destabilizing elements into the mRNA or introduce genetic modifications leading to deterioration of ribosomal binding sites (RBS) of the RNA. It is also possible to change the codon usage of the gene in a way, that the translation efficiency and speed is decreased.

A reduced activity of an enzyme can also be obtained by introducing one or more deleterious gene mutations which lead to a reduced activity of the enzyme. Furthermore, a reduction of the activity of an enzyme may also include an inactivation (or the reduced expression) of activating enzymes which are necessary in order to activate the enzyme the activity of which is to be reduced. By the latter approach the enzyme the activity of which is to be reduced is preferably kept in an inactivated state.

A deleterious mutation according to this application is any mutation within a gene comprising promoter and coding region that lead to a decreased or deleted protein activity of the protein encoded by the coding region of the gene. Such deleterious mutations comprise for example frameshifts, introduction of stop-codons in the coding region, mutation of promoter elements such as the TATA box that prevent transcription and the like.

Microorganisms having an increased or enhanced expression and/or activity of the enzyme encoded by the *asd* gene or the *gdhA* gene may occur naturally, i.e. due to spontaneous mutations. A microorganism can be modified to have significantly increased activity of the enzyme that is encoded by one or more of said genes by various techniques, such as chemical treatment or radiation. To this end, microorganisms will be treated by, e.g., a mutagenizing chemical agent, X-rays, or UV light. In a subsequent step, those microorganisms which have an increased expression and/or activity of the enzyme that is encoded by one or more of said genes will be selected. Recombinant microorganisms are also obtainable by homologous recombination techniques which aim to substitute one or more of said genes with a corresponding gene that encodes for an enzyme which, compared to the enzyme encoded by the wild type gene, has an increased expression and/or activity.

According to one embodiment of the recombinant microorganism according to the present invention, an increase of the expression and/or activity of the enzyme encoded by the *asd*

gene or the *gdhA* gene may be achieved by a modification of the *asd* gene or the *gdhA* gene, wherein this/these gene modification(s) is(are) preferably realized by multiplication of the copy-number of the asd gene or the *gdhA* gene in the genome of the microorganism, by introducing the gene on a self-replicating expression vector into the microorganism, by exchanging the promoter of the *asd* gene or the *gdhA* gene against a stronger promoter or by converting the endogenous promoter of the gene into a stronger promoter, e.g. by introducing point-mutations into the promoter sequence.

Further the activity of the *asd* gene or the *gdhA* gene may be enhanced by mutating the gene in order to achieve amino acid exchanges in the protein which improve activity of the gene. Such methods are known to a skilled person.

A mutation into the above-gene can be introduced, for example, by site-directed or random mutagenesis, followed by an introduction of the modified gene into the genome of the microorganism by recombination. Variants of the genes can be are generated by mutating the gene sequences by means of PCR. The “Quickchange Site-directed Mutagenesis Kit” (Stratagene) can be used to carry out a directed mutagenesis. A random mutagenesis over the entire coding sequence, or else only part thereof, can be performed with the aid of the “GeneMorph II Random Mutagenesis Kit” (Stratagene). The mutagenesis rate is set to the desired amount of mutations via the amount of the template DNA used. Multiple mutations are generated by the targeted combination of individual mutations or by the sequential performance of several mutagenesis cycles.

In the following, a suitable technique for recombination, in particular for introducing a mutation or for deleting sequences, is described.

This technique is also sometimes referred to as the “Campbell recombination” herein (Leenhouts *et al.*, *Appl Env Microbiol.* (1989), Vol. 55, pages 394-400). “Campbell in”, as used herein, refers to a transformant of an original host cell in which an entire circular double stranded DNA molecule (for example a plasmid) has integrated into a chromosome by a single homologous recombination event (a cross in event), and that effectively results in the insertion of a linearized version of said circular DNA molecule into a first DNA sequence of the chromosome that is homologous to a first DNA sequence of the said circular DNA molecule. “Campbelled in” refers to the linearized DNA sequence that has been integrated into the chromosome of a “Campbell in” transformant. A “Campbell in” contains a duplication of the first homologous DNA sequence, each copy of which includes and surrounds a copy of the homologous recombination crossover point.

“Campbell out”, as used herein, refers to a cell descending from a “Campbell in” transformant, in which a second homologous recombination event (a cross out event) has occurred between a second DNA sequence that is contained on the linearized inserted DNA of the “Campbelled in” DNA, and a second DNA sequence of chromosomal origin, which is

homologous to the second DNA sequence of said linearized insert, the second recombination event resulting in the deletion (jettisoning) of a portion of the integrated DNA sequence, but, importantly, also resulting in a portion (this can be as little as a single base) of the integrated Campbelled in DNA remaining in the chromosome, such that compared to the original host cell, the "Campbell out" cell contains one or more intentional changes in the chromosome (for example, a single base substitution, multiple base substitutions, insertion of a heterologous gene or DNA sequence, insertion of an additional copy or copies of a homologous gene or a modified homologous gene, or insertion of a DNA sequence comprising more than one of these aforementioned examples listed above). A "Campbell out" cell is, preferably, obtained by a counter-selection against a gene that is contained in a portion (the portion that is desired to be jettisoned) of the "Campbelled in" DNA sequence, for example the *Bacillus subtilis sacB*-gene, which is lethal when expressed in a cell that is grown in the presence of about 5% to 10% sucrose. Either with or without a counter-selection, a desired "Campbell out" cell can be obtained or identified by screening for the desired cell, using any screenable phenotype, such as, but not limited to, colony morphology, colony color, presence or absence of antibiotic resistance, presence or absence of a given DNA sequence by polymerase chain reaction, presence or absence of an auxotrophy, presence or absence of an enzyme, colony nucleic acid hybridization, antibody screening, etc. The term "Campbell in" and "Campbell out" can also be used as verbs in various tenses to refer to the method or process described above.

It is understood that the homologous recombination events that leads to a "Campbell in" or "Campbell out" can occur over a range of DNA bases within the homologous DNA sequence, and since the homologous sequences will be identical to each other for at least part of this range, it is not usually possible to specify exactly where the crossover event occurred. In other words, it is not possible to specify precisely which sequence was originally from the inserted DNA, and which was originally from the chromosomal DNA. Moreover, the first homologous DNA sequence and the second homologous DNA sequence are usually separated by a region of partial non-homology, and it is this region of non-homology that remains deposited in a chromosome of the "Campbell out" cell.

Preferably, first and second homologous DNA sequence are at least about 200 base pairs in length, and can be up to several thousand base pairs in length. However, the procedure can be made to work with shorter or longer sequences. For example, a length for the first and second homologous sequences can range from about 500 to 2000 bases, and the obtaining of a "Campbell out" from a "Campbell in" is facilitated by arranging the first and second homologous sequences to be approximately the same length, preferably with a difference of less than 200 base pairs and most preferably with the shorter of the two being at least 70% of the length of the longer in base pairs.

In one embodiment the induction of the expression and/or activity of asd is achieved by an activation of the *asd* gene which encodes the aspartate-beta-semialdehyde dehydrogenase.

In one embodiment the induction of the expression and/or activity of gdhA is achieved by an activation of the *gdhA* gene which encodes the glutamate dehydrogenase.

The terms “alanine, pyruvate, succinate, aspartate, malate, lactate, valine and/or leucine”, as used in the context of the present invention, has to be understood in their broadest sense and also encompasses salts thereof, as for example alkali metal salts, like Na^+ and K^+-salts, or earth alkali salts, like Mg^{2+} and Ca^{2+}-salts, or ammonium salts or anhydrides of alanine, pyruvate, succinate, aspartate, malate, lactate, valine and/or leucine.

Preferably, alanine, pyruvate, succinate, aspartate, malate, lactate, valine and/or leucine, preferably succinate or alanine, more preferably alanine is produced under microaerobic conditions. Aerobic or anaerobic conditions may be also used.

Microaerobic means that the concentration of oxygen is less than that in air. According to one embodiment microaerobic means oxygen tension between 5 and 27 mm Hg, preferably between 10 and 20 Hg (Megan Falsetta et al. (2011), The composition and metabolic phenotype of Neisseria gonorrhoeae biofilms, Frontiers in Microbiology, Vol 2, page 1 to 11). Preferably the microaerobic conditions are established with 0.1 to 1 vvm air flow.

Anaerobic conditions may be established by means of conventional techniques, as for example by degassing the constituents of the reaction medium and maintaining anaerobic conditions by introducing carbon dioxide or nitrogen or mixtures thereof and optionally hydrogen at a flow rate of, for example, 0.1 to 1 or 0.2 to 0.5 vvm. Aerobic conditions may be established by means of conventional techniques, as for example by introducing air or oxygen at a flow rate of, for example, 0.1 to 1 or 0.2 to 0.5 vvm. If appropriate, a slight over pressure of 0.1 to 1.5 bar may be applied in the process.

According to one embodiment of the process according to the present invention the assimilable carbon source may be glucose, glycerin, glucose, maltose, maltodextrin, fructose, galactose, mannose, xylose, sucrose, arabinose, lactose, raffinose and combinations thereof.

In a preferred embodiment the assimilable carbon source is glucose, sucrose, xylose, arabinose, glycerol or combinations thereof. Preferred carbon sources are glucose, sucrose, glucose and sucrose, glucose and xylose and/or glucose, arabinose and xylose. According to one embodiment of the process according to the present invention the assimilable carbon source may be sucrose, glycerin and/or glucose.

The initial concentration of the assimilable carbon source, preferably the initial concentration is, preferably, adjusted to a value in a range of 5 to 250 g/l, preferably 50 to 200 g/l and more preferably 125 to 150 g/l, most preferably about 140g/l and may be maintained in said range during cultivation. The pH of the reaction medium may be controlled by addition of suitable bases as for example, gaseous ammonia, NH_4OH, NH_4HCO_3, $(NH_4)_2CO_3$, NaOH,

Na_2CO_3, $NaHCO_3$, KOH, K_2CO_3, $KHCO_3$, $Mg(OH)_2$, $MgCO_3$, $Mg(HCO_3)_2$, $Ca(OH)_2$, $CaCO_3$, $Ca(HCO_3)_2$, CaO, $CH_6N_2O_2$, C_2H_7N and/or mixtures thereof.

Another embodiment of the invention is a process for fermentative production of alanine, pyruvate, succinate, aspartate, malate, lactate, valine and/or leucine, preferably succinate or alanine, more preferably alanine, most preferably L-alanine comprising the steps of

I) growing the microorganism according to the invention as defined above in a fermenter and

II) recovering alanine, pyruvate, succinate, aspartate, malate, lactate, valine and/or leucine, preferably succinate or alanine, more preferably alanine, most preferably L-alanine from the fermentation broth obtained in I).

The fermentation step I) according to the present invention can, for example, be performed in stirred fermenters, bubble columns and loop reactors. A comprehensive overview of the possible method types including stirrer types and geometric designs can be found in Chmiel: "*Bioprozesstechnik: Einführung in die Bioverfahrenstechnik*", Volume 1. In the process according to the present invention, typical variants available are the following variants known to those skilled in the art or explained, for example, in Chmiel, Hammes and Bailey: "*Biochemical Engineering*", such as batch, fed-batch, repeated fed-batch or else continuous fermentation with and without recycling of the biomass. Depending on the production strain, sparging with air, oxygen, carbon dioxide, hydrogen, nitrogen or appropriate gas mixtures may be effected in order to achieve good yield (YP/S).

Particularly preferred conditions for producing alanine, pyruvate, succinate, aspartate, malate, lactate, valine and/or leucine, preferably succinate or alanine, more preferably alanine, most preferably L-alanine in process step I) are:

Assimilable carbon source:	glucose
Temperature:	30 to 45°C
pH:	6.0 to 7.0
	Microaerobic conditions

In process step II) the product is recovered from the fermentation broth obtained in process step I).

Usually, the recovery process comprises the step of separating the recombinant microorganisms from the fermentation broth as the so called "biomass". Processes for removing the biomass are known to those skilled in the art, and comprise filtration, sedimentation, flotation or combinations thereof. Consequently, the biomass can be removed, for example, with centrifuges, separators, decanters, filters or in a flotation apparatus. For maximum recovery of the product of value, washing of the biomass is often advisable, for example in the form of a diafiltration. The selection of the method is dependent upon the biomass content

in the fermentation broth and the properties of the biomass, and also the interaction of the biomass with the organic compound (e. the product of value). In one embodiment, the fermentation broth can be sterilized or pasteurized. In a further embodiment, the fermentation broth is concentrated. Depending on the requirement, this concentration can be done batch wise or continuously. The pressure and temperature range should be selected such that firstly no product damage occurs, and secondly minimal use of apparatus and energy is necessary. The skillful selection of pressure and temperature levels for a multistage evaporation in particular enables saving of energy.

The recovery process may further comprise additional purification steps in which the fermentation product is further purified. If, however, the fermentation product is converted into a secondary organic product by chemical reactions, a further purification of the fermentation product might, depending on the kind of reaction and the reaction conditions, not necessarily be required. For the purification of the fermentation product obtained in process step II) methods known to the person skilled in the art can be used, as for example crystallization, filtration, electrodialysis and chromatography. The resulting solution may be further purified by means of ion exchange chromatography in order to remove undesired residual ions.

DEFINITIONS

It is to be understood that this invention is not limited to the particular methodology or protocols. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. It must be noted that as used herein and in the appended claims, the singular forms "a," "and," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a vector" is a reference to one or more vectors and includes equivalents thereof known to those skilled in the art, and so forth. The term "about" is used herein to mean approximately, roughly, around, or in the region of. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20 percent, preferably 10 percent up or down (higher or lower). As used herein, the word "or" means any one member of a particular list and also includes any combination of members of that list. The words "comprise," "comprising," "include," "including," and "includes" when used in this specification and in the following claims are intended to specify the presence of one or more stated features, integers, components, or steps, but they do not preclude the presence or addition of one or more other features, integers, components, steps, or groups thereof. For clarity, certain terms used in the specification are defined and used as follows:

Coding region: As used herein the term "coding region" when used in reference to a structural gene refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. The coding region is bounded, in eukaryotes, on the 5'-side by the nucleotide triplet "ATG" which encodes the initiator methionine, prokaryotes also use the triplets “GTG” and “TTG” as startcodon. On the 3'-side it is bounded by one of the three triplets which specify stop codons (i.e., TAA, TAG, TGA). In addition a gene may include sequences located on both the 5'- and 3'-end of the sequences which are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5'-flanking region may contain regulatory sequences such as promoters and enhancers which control or influence the transcription of the gene. The 3'-flanking region may contain sequences which direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

Complementary: "Complementary" or "complementarity" refers to two nucleotide sequences which comprise antiparallel nucleotide sequences capable of pairing with one another (by the base-pairing rules) upon formation of hydrogen bonds between the complementary base residues in the antiparallel nucleotide sequences. For example, the sequence 5'-AGT-3' is complementary to the sequence 5'-ACT-3'. Complementarity can be "partial" or "total." "Partial" complementarity is where one or more nucleic acid bases are not matched according to the base pairing rules. "Total" or "complete" complementarity between nucleic acid molecules is where each and every nucleic acid base is matched with another base under the base pairing rules. The degree of complementarity between nucleic acid molecule strands has significant effects on the efficiency and strength of hybridization between nucleic acid molecule strands. A "complement" of a nucleic acid sequence as used herein refers to a nucleotide sequence whose nucleic acid molecules show total complementarity to the nucleic acid molecules of the nucleic acid sequence.

Endogenous: An "endogenous" nucleotide sequence refers to a nucleotide sequence, which is present in the genome of a wild type microorganism.

Enhanced expression: “enhance” or “increase” the expression of a nucleic acid molecule in a microorganism are used equivalently herein and mean that the level of expression of a nucleic acid molecule in a microorganism is higher compared to a reference microorganism, for example a wild type. The terms "enhanced” or “increased" as used herein mean herein higher, preferably significantly higher expression of the nucleic acid molecule to be expressed. As used herein, an “enhancement” or “increase” of the level of an agent such as a protein, mRNA or RNA means that the level is increased relative to a substantially identical microorganism grown under substantially identical conditions. As used herein, “enhancement” or “increase” of the level of an agent, such as for example a preRNA, mRNA, rRNA, tRNA, expressed by the target gene and/or of the protein product encoded by it, means that the level is increased 50% or more, for example 100% or more, preferably 200% or more,

more preferably 5 fold or more, even more preferably 10 fold or more, most preferably 20 fold or more for example 50 fold relative to a suitable reference microorganism. The enhancement or increase can be determined by methods with which the skilled worker is familiar. Thus, the enhancement or increase of the nucleic acid or protein quantity can be determined for example by an immunological detection of the protein. Moreover, techniques such as protein assay, fluorescence, Northern hybridization, nuclease protection assay, reverse transcription (quantitative RT-PCR), ELISA (enzyme-linked immunosorbent assay), Western blotting, radioimmunoassay (RIA) or other immunoassays and fluorescence-activated cell analysis (FACS) can be employed to measure a specific protein or RNA in a microorganism. Depending on the type of the induced protein product, its activity or the effect on the phenotype of the microorganism may also be determined. Methods for determining the protein quantity are known to the skilled worker. Examples, which may be mentioned, are: the micro-Biuret method (Goa J (1953) Scand J Clin Lab Invest 5:218-222), the Folin-Ciocalteau method (Lowry OH et al. (1951) J Biol Chem 193:265-275) or measuring the absorption of CBB G-250 (Bradford MM (1976) Analyt Biochem 72:248-254).

Expression: "Expression" refers to the biosynthesis of a gene product, preferably to the transcription and/or translation of a nucleotide sequence, for example an endogenous gene or a heterologous gene, in a cell. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and - optionally - the subsequent translation of mRNA into one or more polypeptides. In other cases, expression may refer only to the transcription of the DNA harboring an RNA molecule.

Foreign: The term "foreign" refers to any nucleic acid molecule (e.g., gene sequence) which is introduced into a cell by experimental manipulations and may include sequences found in that cell as long as the introduced sequence contains some modification (e.g., a point mutation, the presence of a selectable marker gene, etc.) and is therefore different relative to the naturally-occurring sequence.

Functional linkage: The term "functional linkage" or "functionally linked" is equivalent to the term “operable linkage” or “operably linked” and is to be understood as meaning, for example, the sequential arrangement of a regulatory element (e.g. a promoter) with a nucleic acid sequence to be expressed and, if appropriate, further regulatory elements (such as e.g., a terminator) in such a way that each of the regulatory elements can fulfill its intended function to allow, modify, facilitate or otherwise influence expression of said nucleic acid sequence. As a synonym the wording “operable linkage” or “operably linked” may be used. The expression may result depending on the arrangement of the nucleic acid sequences in relation to sense or antisense RNA. To this end, direct linkage in the chemical sense is not necessarily required. Genetic control sequences such as, for example, enhancer sequences, can also exert their function on the target sequence from positions which are further away, or indeed from other DNA molecules. Preferred arrangements are those in which

the nucleic acid sequence to be expressed recombinantly is positioned behind the sequence acting as promoter, so that the two sequences are linked covalently to each other. In a preferred embodiment, the nucleic acid sequence to be transcribed is located behind the promoter in such a way that the transcription start is identical with the desired beginning of the chimeric RNA of the invention. Functional linkage, and an expression construct, can be generated by means of customary recombination and cloning techniques as described (e.g., in Maniatis T, Fritsch EF and Sambrook J (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor (NY); Silhavy et al. (1984) Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor (NY); Ausubel et al. (1987) Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley Interscience; Gelvin et al. (Eds) (1990) Plant Molecular Biology Manual; Kluwer Academic Publisher, Dordrecht, The Netherlands). However, further sequences, which, for example, act as a linker with specific cleavage sites for restriction enzymes, or as a signal peptide, may also be positioned between the two sequences. The insertion of sequences may also lead to the expression of fusion proteins. Preferably, the expression construct, consisting of a linkage of a regulatory region for example a promoter and nucleic acid sequence to be expressed, can exist in a vector-integrated form or can be inserted into the genome, for example by transformation.

Gene: The term "gene" refers to a region operably linked to appropriate regulatory sequences capable of regulating the expression of the gene product (e.g., a polypeptide or a functional RNA) in some manner. A gene includes untranslated regulatory regions of DNA (e.g., promoters, enhancers, repressors, etc.) preceding (up-stream) and following (down-stream) the coding region (open reading frame, ORF). The term "structural gene" as used herein is intended to mean a DNA sequence that is transcribed into mRNA which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Genome and genomic DNA: The terms “genome” or “genomic DNA” is referring to the heritable genetic information of a host organism. Said genomic DNA comprises the DNA of the nucleoid but also the DNA of the self replicating plasmid.

Heterologous: The term "heterologous” with respect to a nucleic acid molecule or DNA refers to a nucleic acid molecule which is operably linked to, or is manipulated to become operably linked to, a second nucleic acid molecule to which it is not operably linked in nature, or to which it is operably linked at a different location in nature. A heterologous expression construct comprising a nucleic acid molecule and one or more regulatory nucleic acid molecule (such as a promoter or a transcription termination signal) linked thereto for example is a constructs originating by experimental manipulations in which either a) said nucleic acid molecule, or b) said regulatory nucleic acid molecule or c) both (i.e. (a) and (b)) is not located in its natural (native) genetic environment or has been modified by experimental manipulations, an example of a modification being a substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. Natural genetic environment refers to the

natural genomic locus in the organism of origin, or to the presence in a genomic library. In the case of a genomic library, the natural genetic environment of the sequence of the nucleic acid molecule is preferably retained, at least in part. The environment flanks the nucleic acid sequence at least at one side and has a sequence of at least 50 bp, preferably at least 500 bp, especially preferably at least 1,000 bp, very especially preferably at least 5,000 bp, in length. A naturally occurring expression construct - for example the naturally occurring combination of a promoter with the corresponding gene - becomes a transgenic expression construct when it is modified by non-natural, synthetic “artificial” methods such as, for example, mutagenization. Such methods have been described (US 5,565,350; WO 00/15815). For example a protein encoding nucleic acid molecule operably linked to a promoter, which is not the native promoter of this molecule, is considered to be heterologous with respect to the promoter. Preferably, heterologous DNA is not endogenous to or not naturally associated with the cell into which it is introduced, but has been obtained from another cell or has been synthesized. Heterologous DNA also includes an endogenous DNA sequence, which contains some modification, non-naturally occurring, multiple copies of an endogenous DNA sequence, or a DNA sequence which is not naturally associated with another DNA sequence physically linked thereto. Generally, although not necessarily, heterologous DNA encodes RNA or proteins that are not normally produced by the cell into which it is expressed.

Hybridization: The term "hybridization" as used herein includes "any process by which a strand of nucleic acid molecule joins with a complementary strand through base pairing." (J. Coombs (1994) Dictionary of Biotechnology, Stockton Press, New York). Hybridization and the strength of hybridization (i.e., the strength of the association between the nucleic acid molecules) is impacted by such factors as the degree of complementarity between the nucleic acid molecules, stringency of the conditions involved, the Tm of the formed hybrid, and the G:C ratio within the nucleic acid molecules. As used herein, the term "Tm" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the Tm of nucleic acid molecules is well known in the art. As indicated by standard references, a simple estimate of the Tm value may be calculated by the equation: Tm=81.5+0.41(% G+C), when a nucleic acid molecule is in aqueous solution at 1 M NaCl [see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985)]. Other references include more sophisticated computations, which take structural as well as sequence characteristics into account for the calculation of Tm. Stringent conditions, are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Suitable hybridization conditions are for example hybridizing under conditions equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 2 X SSC, 0.1% SDS at 50°C (low stringency) to a nucleic acid molecule comprising at least 50, preferably at least 100, more preferably at least 150, even more preferably at least 200, most preferably at least 250 consecutive nucleotides of the complement of

a sequence. Other suitable hybridizing conditions are hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 1 X SSC, 0.1% SDS at 50°C (medium stringency) or 65°C (high stringency) to a nucleic acid molecule comprising at least 50, preferably at least 100, more preferably at least 150, even more preferably at least 200, most preferably at least 250 consecutive nucleotides of a complement of a sequence. Other suitable hybridization conditions are hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 65°C (very high stringency) to a nucleic acid molecule comprising at least 50, preferably at least 100, more preferably at least 150, even more preferably at least 200, most preferably at least 250 consecutive nucleotides of a complement of a sequence.

"Identity": "Identity" when used in respect to the comparison of two or more nucleic acid or amino acid molecules means that the sequences of said molecules share a certain degree of sequence similarity, the sequences being partially identical.

To determine the percentage identity (homology is herein used interchangeably if referring to nucleic acid sequences) of two amino acid sequences or of two nucleic acid molecules, the sequences are written one underneath the other for an optimal comparison (for example gaps may be inserted into the sequence of a protein or of a nucleic acid in order to generate an optimal alignment with the other protein or the other nucleic acid).

The amino acid residues or nucleic acid molecules at the corresponding amino acid positions or nucleotide positions are then compared. If a position in one sequence is occupied by the same amino acid residue or the same nucleic acid molecule as the corresponding position in the other sequence, the molecules are identical at this position. The percentage identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e. % identity = number of identical positions/total number of positions x 100). The terms "homology" and "identity" are thus to be considered as synonyms when referring to nucleic acid sequences. When referring to amino acid sequences the term identity refers to identical amino acids at a specific position in a sequence, the term homology refers to homologous amino acids at a specific position in a sequence. Homologous amino acids are amino acids having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art.

A nucleic acid molecule encoding a protein homologous to a protein of the invention can be created by introducing one or more nucleotide substitutions, additions or deletions into a nucleotide sequence such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into one of the sequences of the invention by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid sub-

stitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a protein of the invention is preferably replaced with another amino acid residue from the same side chain family.

Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for the respective activity described herein to identify mutants that retain their activity. Following mutagenesis of one of the sequences of the invention, the encoded protein can be expressed recombinantly and the activity of the protein can be determined using, for example, assays described herein.

For the determination of the percentage identity of two or more amino acids or of two or more nucleotide sequences several computer software programs have been developed. The identity of two or more sequences can be calculated with for example the software fasta, which presently has been used in the version fasta 3 (W. R. Pearson and D. J. Lipman, PNAS 85, 2444(1988); W. R. Pearson, Methods in Enzymology 183, 63 (1990); W. R. Pearson and D. J. Lipman, PNAS 85, 2444 (1988); W. R. Pearson, Enzymology 183, 63 (1990)). Another useful program for the calculation of identities of different sequences is the standard blast program, which is included in the Biomax pedant software (Biomax, Munich, Federal Republic of Germany). This leads unfortunately sometimes to suboptimal results since blast does not always include complete sequences of the subject and the query. Nevertheless as this program is very efficient it can be used for the comparison of a huge number of sequences. The following settings are typically used for such a comparisons of sequences:

-p Program Name [String]; -d Database [String]; default = nr; -i Query File [File In]; default = stdin; -e Expectation value (E) [Real]; default = 10.0; -m alignment view options: 0 = pairwise; 1 = query-anchored showing identities; 2 = query-anchored no identities; 3 = flat query-anchored, show identities; 4 = flat query-anchored, no identities; 5 = query-anchored no identities and blunt ends; 6 = flat query-anchored, no identities and blunt ends; 7 = XML Blast output; 8 = tabular; 9 tabular with comment lines [Integer]; default = 0; -o BLAST report Output File [File Out] Optional; default = stdout; -F Filter query sequence (DUST with blastn, SEG with others) [String]; default = T; -G Cost to open a gap (zero invokes default behavior) [Integer]; default = 0; -E Cost to extend a gap (zero invokes default behavior) [Integer]; default = 0; -X X dropoff value for gapped alignment (in bits) (zero invokes default

behavior); blastn 30, megablast 20, tblastx 0, all others 15 [Integer]; default = 0; -I Show GI's in deflines [T/F]; default = F; -q Penalty for a nucleotide mismatch (blastn only) [Integer]; default = -3; -r Reward for a nucleotide match (blastn only) [Integer]; default = 1; -v Number of database sequences to show one-line descriptions for (V) [Integer]; default = 500; -b Number of database sequence to show alignments for (B) [Integer]; default = 250; -f Threshold for extending hits, default if zero; blastp 11, blastn 0, blastx 12, tblastn 13; tblastx 13, megablast 0 [Integer]; default = 0; -g Perfom gapped alignment (not available with tblastx) [T/F]; default = T; -Q Query Genetic code to use [Integer]; default = 1; -D DB Genetic code (for tblast[nx] only) [Integer]; default = 1; -a Number of processors to use [Integer]; default = 1; -O SeqAlign file [File Out] Optional; -J Believe the query defline [T/F]; default = F; -M Matrix [String]; default = BLOSUM62; -W Word size, default if zero (blastn 11, megablast 28, all others 3) [Integer]; default = 0; -z Effective length of the database (use zero for the real size) [Real]; default = 0; -K Number of best hits from a region to keep (off by default, if used a value of 100 is recommended) [Integer]; default = 0; -P 0 for multiple hit, 1 for single hit [Integer]; default = 0; -Y Effective length of the search space (use zero for the real size) [Real]; default = 0; -S Query strands to search against database (for blast[nx], and tblastx); 3 is both, 1 is top, 2 is bottom [Integer]; default = 3; -T Produce HTML output [T/F]; default = F; -I Restrict search of database to list of GI's [String] Optional; -U Use lower case filtering of FASTA sequence [T/F] Optional; default = F; -y X dropoff value for ungapped extensions in bits (0.0 invokes default behavior); blastn 20, megablast 10, all others 7 [Real]; default = 0.0; -Z X dropoff value for final gapped alignment in bits (0.0 invokes default behavior); blastn/megablast 50, tblastx 0, all others 25 [Integer]; default = 0; -R PSI-TBLASTN checkpoint file [File In] Optional; -n MegaBlast search [T/F]; default = F; -L Location on query sequence [String] Optional; -A Multiple Hits window size, default if zero (blastn/megablast 0, all others 40 [Integer]; default = 0; -w Frame shift penalty (OOF algorithm for blastx) [Integer]; default = 0; -t Length of the largest intron allowed in tblastn for linking HSPs (0 disables linking) [Integer]; default = 0.

Results of high quality are reached by using the algorithm of Needleman and Wunsch or Smith and Waterman. Therefore programs based on said algorithms are preferred. Advantageously the comparisons of sequences can be done with the program PileUp (J. Mol. Evolution., 25, 351 (1987), Higgins et al., CABIOS 5, 151 (1989)) or preferably with the programs “Gap” and “Needle”, which are both based on the algorithms of Needleman and Wunsch (J. Mol. Biol. 48; 443 (1970)), and “BestFit”, which is based on the algorithm of Smith and Waterman (Adv. Appl. Math. 2; 482 (1981)). “Gap” and “BestFit” are part of the GCG software-package (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711 (1991); Altschul et al., (Nucleic Acids Res. 25, 3389 (1997)), “Needle” is part of the The European Molecular Biology Open Software Suite (EMBOSS) (Trends in Genetics 16 (6), 276 (2000)). Therefore preferably the calculations to determine the percentages of sequence identity are done with the programs “Gap” or “Needle” over the whole range of the sequences. The following standard adjustments for the comparison of nucleic

acid sequences were used for “Needle”: matrix: EDNAFULL, Gap_penalty: 10.0, Extend_penalty: 0.5. The following standard adjustments for the comparison of nucleic acid sequences were used for “Gap”: gap weight: 50, length weight: 3, average match: 10.000, average mismatch: 0.000.

For example a sequence, which is said to have 80% identity with sequence SEQ ID NO: 1 at the nucleic acid level is understood as meaning a sequence which, upon comparison with the sequence represented by SEQ ID NO: 1 by the above program “Needle” with the above parameter set, has a 80% identity. Preferably the identity is calculated on the complete length of the query sequence, for example SEQ ID NO: 1.

Isolated: The term "isolated" as used herein means that a material has been removed by the hand of man and exists apart from its original, native environment and is therefore not a product of nature. An isolated material or molecule (such as a DNA molecule or enzyme) may exist in a purified form or may exist in a non-native environment such as, for example, in a transgenic host cell. For example, a naturally occurring nucleic acid molecule or polypeptide present in a living cell is not isolated, but the same nucleic acid molecule or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such nucleic acid molecules can be part of a vector and/or such nucleic acid molecules or polypeptides could be part of a composition, and would be isolated in that such a vector or composition is not part of its original environment. Preferably, the term "isolated" when used in relation to a nucleic acid molecule, as in "an isolated nucleic acid sequence" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in its natural source. Isolated nucleic acid molecule is nucleic acid molecule present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acid molecules are nucleic acid molecules such as DNA and RNA, which are found in the state they exist in nature. For example, a given DNA sequence (e.g., a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs, which encode a multitude of proteins. However, an isolated nucleic acid sequence comprising for example SEQ ID NO: 1 includes, by way of example, such nucleic acid sequences in cells which ordinarily contain SEQ ID NO: 1 where the nucleic acid sequence is in a genomic or plasmid location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid sequence may be present in single-stranded or double-stranded form. When an isolated nucleic acid sequence is to be utilized to express a protein, the nucleic acid sequence will contain at a minimum at least a portion of the sense or coding strand (i.e., the nucleic acid sequence may be single-stranded). Alternatively, it may contain both the sense and anti-sense strands (i.e., the nucleic acid sequence may be double-stranded).

Non-coding: The term "non-coding" refers to sequences of nucleic acid molecules that do

not encode part or all of an expressed protein. Non-coding sequences include but are not limited enhancers, promoter regions, 3' untranslated regions, and 5' untranslated regions.

Nucleic acids and nucleotides: The terms "nucleic acids" and "Nucleotides" refer to naturally occurring or synthetic or artificial nucleic acid or nucleotides. The terms “nucleic acids” and "nucleotides” comprise deoxyribonucleotides or ribonucleotides or any nucleotide analogue and polymers or hybrids thereof in either single- or double-stranded, sense or antisense form. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. The term "nucleic acid" is used inter-changeably herein with "gene", "cDNA, "mRNA", "oligonucleotide," and "nucleic acid molecule". Nucleotide analogues include nucleotides having modifications in the chemical structure of the base, sugar and/or phosphate, including, but not limited to, 5-position pyrimidine modifications, 8-position purine modifications, modifications at cytosine exocyclic amines, substitution of 5-bromo-uracil, and the like; and 2'-position sugar modifications, including but not limited to, sugar-modified ribonucleotides in which the 2'-OH is replaced by a group selected from H, OR, R, halo, SH, SR, NH2, NHR, NR2, or CN. Short hairpin RNAs (shRNAs) also can comprise non-natural elements such as non-natural bases, e.g., ionosin and xanthine, non-natural sugars, e.g., 2'-methoxy ribose, or non-natural phosphodiester linkages, e.g., methylphosphonates, phosphorothioates and peptides.

Nucleic acid sequence: The phrase "nucleic acid sequence" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5'- to the 3'-end. It includes chromosomal DNA, self-replicating plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role. "Nucleic acid sequence" also refers to a consecutive list of abbreviations, letters, characters or words, which represent nucleotides. In one embodiment, a nucleic acid can be a "probe" which is a relatively short nucleic acid, usually less than 100 nucleotides in length. Often a nucleic acid probe is from about 50 nucleotides in length to about 10 nucleotides in length. A "target region" of a nucleic acid is a portion of a nucleic acid that is identified to be of interest. A "coding region" of a nucleic acid is the portion of the nucleic acid, which is transcribed and translated in a sequence-specific manner to produce into a particular polypeptide or protein when placed under the control of appropriate regulatory sequences. The coding region is said to encode such a polypeptide or protein.

Oligonucleotide: The term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof, as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases. An oligonucleotide preferably includes

two or more nucleomonomers covalently coupled to each other by linkages (e.g., phosphodiesters) or substitute linkages.

Overhang: An "overhang" is a relatively short single-stranded nucleotide sequence on the 5'- or 3'-hydroxyl end of a double-stranded oligonucleotide molecule (also referred to as an "extension," "protruding end," or "sticky end").

Polypeptide: The terms "polypeptide", "peptide", "oligopeptide", "polypeptide", "gene product", "expression product" and "protein" are used interchangeably herein to refer to a polymer or oligomer of consecutive amino acid residues.

Promoter: The terms "promoter", or "promoter sequence" are equivalents and as used herein, refer to a DNA sequence which when operably linked to a nucleotide sequence of interest is capable of controlling the transcription of the nucleotide sequence of interest into RNA. A promoter is located 5' (i.e., upstream), proximal to the transcriptional start site of a nucleotide sequence of interest whose transcription into mRNA it controls, and provides a site for specific binding by RNA polymerase and other transcription factors for initiation of transcription. The promoter does not comprise coding regions or 5´ untranslated regions. The promoter may for example be heterologous or homologous to the respective cell. A nucleic acid molecule sequence is "heterologous to" an organism or a second nucleic acid molecule sequence if it originates from a foreign species, or, if from the same species, is modified from its original form. For example, a promoter operably linked to a heterologous coding sequence refers to a coding sequence from a species different from that from which the promoter was derived, or, if from the same species, a coding sequence which is not naturally associated with the promoter (e.g. a genetically engineered coding sequence or an allele from a different ecotype or variety). Suitable promoters can be derived from genes of the host cells where expression should occur or from pathogens for this host.

Purified: As used herein, the term "purified" refers to molecules, either nucleic or amino acid sequences that are removed from their natural environment, isolated or separated. "Substantially purified" molecules are at least 60% free, preferably at least 75% free, and more preferably at least 90% free from other components with which they are naturally associated. A purified nucleic acid sequence may be an isolated nucleic acid sequence.

Recombinant: The term "recombinant" with respect to nucleic acid molecules refers to nucleic acid molecules produced by recombinant DNA techniques. The term also comprises nucleic acid molecules which as such does not exist in nature but are modified, changed, mutated or otherwise manipulated by man. Preferably, a "recombinant nucleic acid molecule" is a non-naturally occurring nucleic acid molecule that differs in sequence from a naturally occurring nucleic acid molecule by at least one nucleic acid. A “recombinant nucleic acid molecule” may also comprise a “recombinant construct” which comprises, preferably operably linked, a sequence of nucleic acid molecules not naturally occurring in that order.

Preferred methods for producing said recombinant nucleic acid molecule may comprise cloning techniques, directed or non-directed mutagenesis, synthesis or recombination techniques.

Significant increase: An increase for example in enzymatic activity, gene expression, productivity or yield of a certain product, that is larger than the margin of error inherent in the measurement technique, preferably an increase by about 10% or 25% preferably by 50% or 75%, more preferably 2-fold or-5 fold or greater of the activity, expression, productivity or yield of the control enzyme or expression in the control cell, productivity or yield of the control cell, even more preferably an increase by about 10-fold or greater.

Significant decrease: A decrease for example in enzymatic activity, gene expression, productivity or yield of a certain product, that is larger than the margin of error inherent in the measurement technique, preferably a decrease by at least about 5% or 10%, preferably by at least about 20% or 25%, more preferably by at least about 50% or 75%, even more preferably by at least about 80% or 85%, most preferably by at least about 90%, 95%, 97%, 98% or 99%.

Substantially complementary: In its broadest sense, the term "substantially complementary", when used herein with respect to a nucleotide sequence in relation to a reference or target nucleotide sequence, means a nucleotide sequence having a percentage of identity between the substantially complementary nucleotide sequence and the exact complementary sequence of said reference or target nucleotide sequence of at least 60%, more desirably at least 70%, more desirably at least 80% or 85%, preferably at least 90%, more preferably at least 93%, still more preferably at least 95% or 96%, yet still more preferably at least 97% or 98%, yet still more preferably at least 99% or most preferably 100% (the latter being equivalent to the term “identical” in this context). Preferably identity is assessed over a length of at least 19 nucleotides, preferably at least 50 nucleotides, more preferably the entire length of the nucleic acid sequence to said reference sequence (if not specified otherwise below). Sequence comparisons are carried out using default GAP analysis with the University of Wisconsin GCG, SEQWEB application of GAP, based on the algorithm of Needleman and Wunsch (Needleman and Wunsch (1970) J Mol. Biol. 48: 443-453; as defined above). A nucleotide sequence "substantially complementary " to a reference nucleotide sequence hybridizes to the reference nucleotide sequence under low stringency conditions, preferably medium stringency conditions, most preferably high stringency conditions (as defined above).

Transgene: The term "transgene" as used herein refers to any nucleic acid sequence, which is introduced into the genome of a cell by experimental manipulations. A transgene may be an "endogenous DNA sequence," or a "heterologous DNA sequence" (i.e., "foreign DNA"). The term "endogenous DNA sequence" refers to a nucleotide sequence, which is naturally found in the cell into which it is introduced so long as it does not contain some

modification (e.g., a point mutation, the presence of a selectable marker gene, etc.) relative to the naturally-occurring sequence.

Transgenic: The term transgenic when referring to an organism means transformed, preferably stably transformed, with a recombinant DNA molecule that preferably comprises a suitable promoter operatively linked to a DNA sequence of interest.

Vector: As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid molecule to which it has been linked. One type of vector is a genomic integrated vector, or "integrated vector", which can become integrated into the genomic DNA of the host cell. Another type of vector is an episomal vector, i.e., a plasmid or a nucleic acid molecule capable of extra-chromosomal replication. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In the present specification, "plasmid" and "vector" are used interchangeably unless otherwise clear from the context.

Wild type: The term "wild type", "natural" or "natural origin" means with respect to an organism that said organism is not changed, mutated, or otherwise manipulated by man. With respect to a polypeptide or nucleic acid sequence, that the polypeptide or nucleic acid sequence is naturally occurring or available in at least one naturally occurring organism which is not changed, mutated, or otherwise manipulated by man.
A wild type of a microorganism refers to a microorganism whose genome is present in a state as before the introduction of a genetic modification of a certain gene. The genetic modification may be e.g. a deletion of a gene or a part thereof or a point mutation or the introduction of a gene.

The terms "production" or "productivity" are art-recognized and include the concentration of the fermentation product (for example, alanine) formed within a given time and a given fermentation volume (e.g., kg product per hour per liter). The term "efficiency of production" includes the time required for a particular level of production to be achieved (for example, how long it takes for the cell to attain a particular rate of output of a fine chemical).

The term "yield" or "product/carbon yield" is art-recognized and includes the efficiency of the conversion of the carbon source into the product (i.e., fine chemical). This is generally written as, for example, kg product per kg carbon source. By increasing the yield or production of the compound, the quantity of recovered molecules or of useful recovered molecules of that compound in a given amount of culture over a given amount of time is increased.

The term "*recombinant microorganism*" includes microorganisms which have been genetically modified such that they exhibit an altered or different genotype and/or phenotype (e. g., when the genetic modification affects coding nucleic acid sequences of the microorganism) as compared to the wild type microorganism from which it was derived. A recombinant

microorganism comprises at least one recombinant DNA molecule.

The term "*recombinant*" with respect to DNA refers to DNA molecules produced by man using recombinant DNA techniques. The term comprises DNA molecules which as such do not exist in nature or do not exist in the organism from which the DNA is derived, but are modified, changed, mutated or otherwise manipulated by man. Preferably, a "recombinant DNA molecule" is a non-naturally occurring nucleic acid molecule that differs in sequence from a naturally occurring nucleic acid molecule by at least one nucleic acid. A “recombinant DNA molecule” may also comprise a “recombinant construct” which comprises, preferably operably linked, a sequence of nucleic acid molecules not naturally occurring in that order. Preferred methods for producing said recombinant DNA molecule may comprise cloning techniques, directed or non-directed mutagenesis, gene synthesis or recombination techniques.

The term “directed evolution” is used synonymously with the term “metabolic evolution” herein and involves applying a selection pressure that favors the growth of mutants with the traits of interest. The selection pressure can be based on different culture conditions, ATP and growth coupled selection and redox related selection. The selection pressure can be carried out with batch fermentation with serial transferring inoculation or continuous culture with the same pressure.

An example of such a recombinant DNA is a plasmid into which a heterologous DNA-sequence has been inserted or a gene or promoter which has been mutated compared to gene or promoter from which the recombinant DNA derived. The mutation may be introduced by means of directed mutagenesis technologies known in the art or by random mutagenesis technologies such as chemical, UV light or x-ray mutagenesis or directed evolution technologies.
The term “expression” or “gene expression” means the transcription of a specific gene(s) or specific genetic vector construct. The term “expression” or “gene expression” in particular means the transcription of gene(s) or genetic vector construct into mRNA. The process includes transcription of DNA and may include processing of the resulting RNA-product. The term “expression” or “gene expression” may also include the translation of the mRNA and therewith the synthesis of the encoded protein, i.e. protein expression.

Figure 1
Alanine formation in a batch fermentation of E. coli QZ20 with empty plasmid control and E. coli QZ20/pACYC-asd in 500 mL AM 1 medium with 120 g/L glucose. The fermentation was controlled at 37 C, 400 rpm, at pH 6.8 with 5 N NH4OH without aeration.

Figure 2
Alanine formation in a batch fermentation of E. coli QZ20 with empty plasmid control and E. coli QZ20/pACYC-gdhA in 500 mL AM 1 medium with 120 g/L glucose. The fermentation

was controlled at 37 C, 400 rpm, at pH 6.8 with 5 N NH4OH without aeration.

EXAMPLES

Chemicals and common methods

Unless indicated otherwise, cloning procedures carried out for the purposes of the present invention including restriction digest, agarose gel electrophoresis, purification of nucleic acids, ligation of nucleic acids, transformation, selection and cultivation of bacterial cells are performed as described (Sambrook et al., 1989). Sequence analyses of recombinant DNA are performed with a laser fluorescence DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the Sanger technology (Sanger et al., 1977). Unless described otherwise, chemicals and reagents are obtained from Sigma Aldrich (Sigma Aldrich, St. Louis, USA), from Promega (Madison, WI, USA), Duchefa (Haarlem, The Netherlands) or Invitrogen (Carlsbad, CA, USA). Restriction endonucleases are from New England Biolabs (Ipswich, MA, USA) or Roche Diagnostics GmbH (Penzberg, Germany). Oligonucleotides are synthesized by Eurofins MWG Operon (Ebersberg, Germany).

Example 1:

E. coli W (LU17032) was engineered for L-alanine production by inactivation of the ackA, adhE, frdABCD and pflB ORFs and replacement of the ldhA ORF by a codon-optimized variant of the alaD gene (alaD-gstear).

The ackA, adhE, frdABCD and pflB ORFs were inactivated by insertion of an FRT-flanked kanamycin resistance cassette, followed by removal of the antibiotic resistance cassette by FLP recombination.

The ldhA gene was replaced by alaD-gstear and a downstream FRT-flanked zeocin resistance cassette, which was finally removed by FLP recombination.

The procedure has been described previously for example in WO2015/044818 which is hereby incorporated by reference.

Disruption of ackA has been described previously for example in Causey et al (2004), PNAS, 101 (8) pages 2235 – 2240.

Further, in said strain comprising an inactivated ackA, adhE, frdABCD, pflB and ldhA and an introduced alaD activity, the activity of the genes lpd, zipA and ygaW have been increased as described previously in WO2015/044818 and the activity of the alaD has been further enhanced by mutating the promoter controlling said gene as described previously in WO2015/044818.

Bacterial culture

E. coli W (LU17032) was cultured in Luria-Bertani (LB) liquid medium or on Luria-Bertani solid medium. Occasionally, clones were passaged over M9 minimal agar containing 10 mM Sucrose to confirm W strain identity. Antibiotics were added to the liquid and solid media as appropriate, to final concentrations of 15 µg/ml (kanamycin, chloramphenicol), 25 µg/ml (zeocin) or 3 µg/ml (tetracyclin).

Red/ET recombination

Red/ET recombination was performed using standard protocols of Gene Bridges GmbH (www.genebridges.com). Briefly, Red/ET-proficient E. coli W was aerobically grown at 30°C to an OD600nm of ~0.3. Expression of red genes was induced by adding 50 µl of 10% (w/v) L-arabinose, followed by a temperature increase to 37°C. Arabinose was omitted from uninduced control cultures. After 35 min of incubation at 37°C the cells were washed twice with ice cold 10% (v/v) glycerol and electroporated with 500 ng of PCR product at 1.35 kV, 10µF, 600Ω. The cells were then resuspended in 1 ml ice-cold LB medium and aerobically grown at 37°C for approximately 1.5 h. Cultures were then plated on LB agar containing 15 µg/ml kanamycin (knockouts) or 25 µg/ml zeocin (knockin).

FLP recombination

Flanking FRT sites allowed removal of antibiotic resistance markers by FLP recombination following modification of the E. coli chromosome. FLP recombination leaves a single FRT site (34 bp) as well as short flanking sequences (approx. 20 bp each) which are used as primer binding sites in the amplification of the cassettes.

To perform FLP recombination, plasmid 708-FLPe (Tab. 1) encoding FLP recombinase was introduced into the Red/ET recombinants by electroporation. KanR CmR or ZeoR CmR transformants were used to inoculate 0.2 ml LB cultures, which were incubated at 30°C for 3 h. FLP activity was then induced by a temperature shift to 37°C, followed by a three-hour incubation at 37°C. Single colonies obtained from these cultures were subsequently screened for a CmS and KanS or ZeoS phenotype.

DNA preparation and analysis

E. coli genomic DNA (gDNA) was isolated from overnight cultures with the Gentra Puregene Yeast/Bact. Kit B (Qiagen, Hilden, Germany). PCR products harbouring knockout or knockin cassettes were amplified from template plasmids with PRECISOR high-fidelity DNA polymerase (BioCat, Heidelberg) and analytical PCR reactions were performed with the PCR Extender System (5PRIME GmbH, Hamburg, Germany), according to the manufacturer's recommendations. PCR amplicons were purified using the GeneJET PCR Purification Kit or the GeneJET Gel Extraction Kit (Fermentas, St. Leon-Rot, Germany) and sequencing was performed by GATC BioTech (Konstanz, Germany) or BioSpring (Frankfurt am Main, Germany).

Table 1. Plasmids and primers

	Relevant characteristics / oligo sequences (5′→ 3′)	Source
plasmids		
pRed/ET	*red* expression plasmid, pSC101-based, Tc^R	Gene Bridges
708-FLPe	FLP recombinase expression plasmid, pSC101-based, Cm^R	Gene Bridges
primers (BioSpring)	SEQ ID NO	
pACYC-asd-F	35	
pACYC-asd-R	36	
pACYC-gdhA-F	40	
pACYC-gdhA-R	41	

Example 2 HPLC detection and quantification of alanine

The following HPLC method for the alanine detection in the cell culture media was used:
Column: Aminex HPX-87C column (Bio-Rad), 300×7.8 mm, i.d. particle size 9 µm
Mobile phase: Ca(NO3)2 at 0.1mol/L 90%, Acetonitrile 10%
Flow rate : 0.6 mL/min
Column temperature: 60°C
Detection: Refractive index detector

Under above method, major estimated components in the cell culture sample matrix can be well separated from alanine, without interfering alanine's quantitation.

The amount of the alanine in the sample was determined by external standard calibration method. Standard samples containing alanine from 0.5 to 10.0 g/L were injected and the peak areas were used for calibration. Linear regression coefficient of the calibration curve was 0.9995.

Samples are injected once at 20 µL. Peak areas are used to calculate the amount presenting in the sample by Waters LC Millenium software.

Example 3 HPLC detection and quantification of of glucose, succinate, lactate, formate, acetate and ethanol

HPLC method used
Column: Aminex HPX-87H column (Bio-Rad), 300×7.8 mm, i.d. particle size 9 µm
Mobile phase: H2SO4 4 mM

Flow rate : 0.4 mL/min
Column temperature: 45°C
Detection: Refractive index detector

The amount of the analytes was determined by external standard calibration method. Standard samples containing glucose from 0.1 to 38.0 g/L, succinate, lactate, formate, acetate and ethanol from 0.05 to 10.0 g/L were injected and the peak areas were used for calibration. Linear regression coefficients for all six calibration curves were better than 0.999.

Samples are injected once at 20 µL. Peak areas are used to calculate the amount presenting in the sample by Waters LC Millenium software.

Example 4 Effect of the increased expression of the asd gene on alanine productivity

An additional copy of the asd gene (SEQ ID NO: 25) was introduced into the pACYC184 plasmid under the control of an IPTG-inducible Ptrc promoter. The vector, designated as pACYC-asd (SEQ ID NO: 34), was constructed via commercial InFusion cloning technology (Clontech, Mountain View, CA, USA). The pACYC184 vector (NEB) was linearized with HindIII and SalI restriction endonucleases (NEB). The generated vector backbone was purified by agarose gel extraction. The asd gene was PCR amplified from wild-type E. coli W genomic DNA with primers asd-pACYC_F (SEQ ID NO: 35) and asd-pACYC_R (SEQ ID NO: 36). The primers contained additional 15 bp homologous overhangs to the vector backbone and a double-stranded DNA fragment with the Ptrc promoter that was synthesized by IDT. The amplified asd gene, the upstream Ptrc promoter and the pACYC184 vector backbone were cloned together according to the InFusion cloning manual. The resulting pACYC-asd vector was transformed into E. coli via electroporation and selected for on LB chloramphenicol plates. Positive constructs were confirmed by DNA sequencing.

The effect of asd overexpression on alanine productivity was tested by comparative cultivation of E. coli with an empty control pACYC vector and E. coli harbouring the asd overexpression plasmid pACYC-asd (SEQ ID NO: 34). Precultures were grown in shake flasks with LB medium, 20% filling volume at 37 C and 200 rpm overnight. The fermentation was performed in the DASGIP 1.5 L parallel bioreactor system (Eppendorf) in 500 mL AM 1 medium (2.6 g/L (NH4)2HPO4, 0.87 g/L NH4H2PO4, 0.15 g/L KiII, 0.37 g/L MgSO4 · 7 H2O, 15 g/L (NH4)2SO4, 1 mM betaine, 1ml/L trace metal stock solution). The trace metal stock comprised 1.6g/L FeCL3 · 6 H2O; 0.2g/L CoCl2 · 6 H2O; 0.1g/L CuCl2 · 2 H2O; 0.2g/L ZnCl2; 0.2g/L NaMoO4 · 2 H2O; 0.05g/L H3BO3, 0.1 M HCL. 120 g/L Glucose were used as carbon source in the fermentation medium. 25 ug/mL chloramphenicol were added to stably maintain the plasmid. Expression of the asd gene from the Ptrc promoter was induced with 250 uM isopropyl β-D-1-thiogalactopyranoside (IPTG) during the early logarithmic growth phase. Each strain was run in duplicates at 37 C and 400 rpm stirrer speed. 5N NH4OH was used to control the pH to 6.8 and provide the culture with ammonium as an alanine precursor throughout the fermentation. No air was sparged during the fermentation

and the vessel was not pressurized so that after the initial consumption of dissolved oxygen in the medium by the cells the fermentation was run under microaerobic conditions. Samples were taken throughout the fermentation and analyzed by HPLC for alanine and glucose concentrations.

After 72 h of fermentation time *E. coli* QZ20 in which the asd gene (SEQ ID NO: 25) was overexpressed from the pACYC-asd plasmid (SEQ ID NO: 34) reached a significantly higher L-alanine titer of 66.58 ± 0.50 g/L compared to the strain harbouring the empty control plasmid with 46.00 ± 1.85 g/L (Figure 1).

Example 5 Effect of the increased expression of the gdhA gene on alanine productivity

An additional copy of the gdhA gene (SEQ ID NO: 37) was introduced into the pACYC184 plasmid under the control of an IPTG-inducible Ptrc promoter. The vector, designated as pACYC-gdhA (SEQ ID NO: 39), was constructed via commercial InFusion cloning technology (Clontech, Mountain View, CA, USA). The pACYC184 vector (NEB) was linearized with HindIII and SalI restriction endonucleases (NEB). The generated vector backbone was purified by agarose gel extraction. The gdhA gene was PCR amplified from wild-type E. coli W genomic DNA with primers gdhA-pACYC_F (SEQ ID NO: 40) and gdhA-pACYC_R (SEQ ID NO: 41). The primers contained additional 15 bp homologous overhangs to the vector backbone and a double-stranded DNA fragment with the Ptrc promoter that was synthesized by IDT. The amplified gdhA gene, the upstream Ptrc promoter and the pACYC184 vector backbone were cloned together according to the InFusion cloning manual. The resulting pACYC-gdhA vector was transformed into E. coli via electroporation and selected for on LB chloramphenicol plates. Positive constructs were confirmed by DNA sequencing.

The effect of gdhA overexpression on alanine productivity was tested by comparative cultivation of E. coli with an empty control pACYC vector and E. coli harbouring the gdhA overexpression plasmid pACYC-gdhA (SEQ ID NO: 39). Precultures were grown in shake flasks with LB medium, 20% filling volume at 37 C and 200 rpm overnight. The fermentation was performed in the DASGIP 1.5 L parallel bioreactor system (Eppendorf) in 500 mL AM 1 medium (2.6 g/L (NH4)2HPO4, 0.87 g/L NH4H2PO4, 0.15 g/L Kill, 0.37 g/L MgSO4 · 7 H2O, 15 g/L (NH4)2SO4, 1 mM betaine, 1ml/L trace metal stock solution). The trace metal stock comprised 1.6g/L FeCL3 · 6 H2O; 0.2g/L CoCl2 · 6 H2O; 0.1g/L CuCl2 · 2 H2O; 0.2g/L ZnCl2; 0.2g/L NaMoO4 · 2 H2O; 0.05g/L H3BO3, 0.1 M HCL. 120 g/L Glucose were used as carbon source in the fermentation medium. 25 ug/mL chloramphenicol were added to stably maintain the plasmid. Expression of the gdhA gene from the Ptrc promoter was induced with 250 uM isopropyl β-D-1-thiogalactopyranoside (IPTG) during the early logarithmic growth phase. Each strain was run in duplicates at 37 C and 400 rpm stirrer speed. 5N NH4OH was used to control the pH to 6.8 and provide the culture with ammonium as an alanine precursor throughout the fermentation. No air was sparged during the fermentation and the vessel was not pressurized so that after the initial consumption of dissolved oxygen

in the medium by the cells the fermentation was run under microaerobic conditions. Samples were taken throughout the fermentation and analyzed by HPLC for alanine and glucose concentrations.

After 72 h of fermentation time *E. coli* QZ20 in which the gdhA gene (SEQ ID NO: 37) was overexpressed from the pACYC-gdhA plasmid (SEQ ID NO: 39) reached a significantly higher L-alanine titer of 62.18 ± 0.37 g/L compared to the strain harbouring the empty control plasmid with 50.96 ± 3.41 g/L (Figure 2).

We claim:

1. A recombinant microorganism comprising a reduced, repressed or deleted activity and/or expression of an asd gene or a gdhA gene.

2. The recombinant microorganism of claim 1 further comprising an introduced, increased or enhanced activity and/or expression of an alaD gene.

3. The recombinant microorganism of claim 1 or 2 further comprising a reduced, repressed or deleted activity and/or expression of a pflB gene.

4. The recombinant microorganism of any of claim 1 to 3 further comprising a reduced, repressed or deleted activity and/or expression of an adhE gene.

5. The recombinant microorganism of any of claim 1 to 4 further comprising a reduced, repressed or deleted activity and/or expression of an ldhA gene.

6. The recombinant microorganism of any of claim 1 to 5 further comprising a reduced, repressed or deleted activity and/or expression of a pta gene and/ or an ackA gene.

7. The recombinant microorganism of any of claim 1 to 6 further comprising a reduced, repressed or deleted activity and/or expression of an frdA.

8. The recombinant microorganism of any of claim 1 to 7 further comprising a reduced, repressed or deleted activity and/or expression of a dadX.

9. The recombinant microorganism of any of claim 1 to 8 further comprising an introduced, increased or enhanced activity and/or expression of an ygaW gene.

10. The recombinant microorganism of any of claim 1 to 9 further comprising an introduced, increased or enhanced activity and/or expression of a zipA gene.

11. The recombinant microorganism of any of claim 1 to 10 further comprising an introduced, increased or enhanced activity and/or expression of an lpd gene.

12. The recombinant microorganism of any of claim 1 to 11 further comprising a reduced, repressed or deleted activity and/or expression of a brnQ gene.

13. The recombinant microorganism of any of claim 1 to 12 further comprising a mutated lpxD gene.

14. The recombinant microorganism of any of claim 1 to 13 further comprising an

introduced, increased or enhanced activity and/or expression of a gcvA gene.

15. The recombinant microorganism of any of claim 1 to 14 further comprising a reduced, repressed or deleted activity and/or expression of a gcvB gene.

16. The recombinant microorganism of any of claim 1 to 15, wherein the asd gene or the gdhA gene is selected from the group of
    (i) a nucleic acid molecule comprising a sequence of SEQ ID NO: 25 or SEQ ID NO: 37, or
    (ii) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 25 or SEQ ID NO: 37, or
    (iii) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 25 or SEQ ID NO: 37 under stringent conditions, or
    (iv) a nucleic acid molecule encoding the polypeptide of SEQ ID NO: 26 or SEQ ID NO: 38, or
    (v) a nucleic acid molecule encoding a polypeptide having at least 60% homology to the polypeptide of SEQ ID NO: 26 or SEQ ID NO: 38.

17. The recombinant microorganism of any of claim 2 to 16, wherein the alaD gene is selected from the group consisting of
    (AA) a nucleic acid molecule comprising a sequence of SEQ ID NO: 1, or
    (BB) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 1, or
    (CC) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 1 under stringent conditions, or
    (DD) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 2, or
    (EE) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 2.

18. The recombinant microorganism of any of claim 3 to 17, wherein the pflB gene is selected from the group consisting of
    (A) a nucleic acid molecule comprising a sequence of SEQ ID NO: 5, or
    (B) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 5, or
    (C) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 5 under stringent conditions, or
    (D) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 6, or
    (E) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 6.

19. The recombinant microorganism of any of claim 4 to 18, wherein the adhE gene is selected from the group consisting of

(F) a nucleic acid molecule comprising a sequence of SEQ ID NO: 7, or

(G) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 7, or

(H) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 7 under stringent conditions, or

(I) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 8, or

(J) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 8.

20. The recombinant microorganism of any of claim 5 to 19, wherein the ldhA gene is selected from the group consisting of

(K) a nucleic acid molecule comprising a sequence of SEQ ID NO: 9, or

(L) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 9, or

(M) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 9 under stringent conditions, or

(N) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 10, or

(O) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 10.

21. The recombinant microorganism of any of claim 6 to 20, wherein the pta gene is selected from the group consisting of

(P1) a nucleic acid molecule comprising a sequence of SEQ ID NO: 11, or

(Q1) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 11, or

(R1) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 11 under stringent conditions, or

(S1) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 12, or

(T1) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 12 and wherein the ackA gene is selected from the group consisting of

(P2) a nucleic acid molecule comprising a sequence of SEQ ID NO: 32, or

(Q2) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 32, or

(R2) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 32 under stringent conditions, or

(S2) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 33, or

(T2) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 33.

22. The recombinant microorganism of any of claim 7 to 21, wherein the frdA gene is selected from the group consisting of

(U) a nucleic acid molecule comprising a sequence of SEQ ID NO: 13, or

(V) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 13, or

(W) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 13 under stringent conditions, or

(X) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 14, or

(Y) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 14.

23. The recombinant microorganism of any of claim 8 to 22, wherein the dadX gene is selected from the group consisting of

(Z) a nucleic acid molecule comprising a sequence of SEQ ID NO: 15, or

(FF) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 15, or

(GG) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 15 under stringent conditions, or

(HH) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 16, or

(II) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 16.

24. The recombinant microorganism of any of claim 9 to 23, wherein the ygaW gene is selected from the group consisting of

(JJ) a nucleic acid molecule comprising a sequence of SEQ ID NO: 17, or

(KK) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 17, or

(LL) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 17 under stringent conditions, or

(MM) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 18, or

(NN) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 18.

25. The recombinant microorganism of any of claim 10 to 24, wherein the zipA gene is selected from the group consisting of

(OO) a nucleic acid molecule comprising a sequence of SEQ ID NO: 19, or

(PP) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 19, or

(QQ) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 19 under stringent conditions, or

(RR) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 20, or

(SS) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 20.

26. The recombinant microorganism of any of claim 11 to 25, wherein the lpd gene is selected from the group consisting of
(TT) a nucleic acid molecule comprising a sequence of SEQ ID NO: 21, or
(UU) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 21, or
(VV) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 21 under stringent conditions, or
(WW) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 22, or
(XX) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 22.

27. The recombinant microorganism of any of claim 12 to 26, wherein the brnQ gene is selected from the group consisting of
(YY) a nucleic acid molecule comprising a sequence of SEQ ID NO: 23, or
(ZZ) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 23, or
(AAA) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 23 under stringent conditions, or
(BBB) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 24, or
(CCC) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 24.

28. The recombinant microorganism of any of claim 13 to 27, wherein the lpxD gene is selected from the group consisting of
(DDD) a nucleic acid molecule comprising a sequence of SEQ ID NO: 27, or
(EEE) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 28.

29. The recombinant microorganism of any of claim 14 to 28, wherein the gcvA gene is selected from the group consisting of
(FFF) a nucleic acid molecule comprising a sequence of SEQ ID NO: 29, or
(GGG) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule of SEQ ID NO: 29, or
(HHH) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 29 under stringent conditions, or
(III) a nucleic acid molecule encoding a polypeptide of SEQ ID NO: 30, or
(JJJ) a nucleic acid molecule encoding a polypeptide having at least 60% homology to a polypeptide of SEQ ID NO: 30.

30. The recombinant microorganism of any of claim 15 to 29, wherein the gcvB gene is selected from the group consisting of
(KKK) a nucleic acid molecule comprising a sequence of SEQ ID NO: 31, or
(LLL) a nucleic acid molecule having at least 80% identity to a nucleic acid molecule

of SEQ ID NO: 31, or

(MMM) a nucleic acid molecule hybridizing to the complement of a nucleic acid molecule having SEQ ID NO: 31 under stringent conditions.

31. The recombinant microorganism of any one of claim 1 to 30, wherein the microorganism is selected from a genus of the group consisting of Corynebacterium, Bacillus, Erwinia, Escherichia, Pantoea, Streptomyces, Zymomonas, Rhodococcus Saccharomyces, Candida or Pichia.

32. A composition comprising one or more recombinant microorganisms according to any one of claims 1 to 31.

33. The composition of claim 32 further comprising a medium and a carbon source.

34. A method for producing a recombinant microorganism with enhanced alanine yield and/or productivity, which comprises the following steps:

(I) reducing, repressing or deleting the activity and/or expression of a gene as defined in claim 1 or 16 in a microorganism; and

(II) generating a recombinant microorganism with enhanced alanine yield and/or productivity compared to a reference microorganism without reduced, repressed or deleted activity and/or expression of a gene as defined in claim 1 or 16.

35. The method of claim 34, wherein the recombinant microorganism used in said method further comprises a changed activity and/or expression of at least one gene as defined in claims 2 to 15 and 17 to 30.

36. The method of any one of claim 34 or 35, wherein the microorganism is selected from a genus of the group consisting of Corynebacterium, Bacillus, Erwinia, Escherichia, Pantoea, Streptomyces, Zymomonas, Rhodococcus, Saccharomyces, Candida or Pichia.

37. A method of producing alanine comprising culturing one or more recombinant microorganism according to any one of claim 1 to 30 under conditions that allow for the production of alanine.

38. The method according to claim 37, wherein the microorganism is cultured in a medium comprising between 0.5% and 30% (w/v) of a sugar.

39. The method according to claim 37 or 38, wherein the yield of alanine is at least 80%.

40. The method according to any one of claim 37 to 39, wherein the chiral purity of L-alanine is at least 95%.

41. The method according to any one of claim 37 to 40, wherein chiral pure L-alanine is produced.

42. A method of culturing or growing a genetically modified microorganism comprising inoculating a culture medium with one or more genetically modified microorganism according to any one of claim 1 to 30 and culturing or growing said genetically modified microorganism in culture medium.

43. A use of a recombinant microorganism according to any one of claim 1 to 30 or a composition according to any one of claim 32 or 33 for the fermentative production of alanine.

44. A process for fermentative production of alanine comprising the steps of
I) growing the microorganism according to any one of claims 1 to 30 in a fermenter and
II) recovering alanine from the fermentation broth obtained in I).

Figure 1

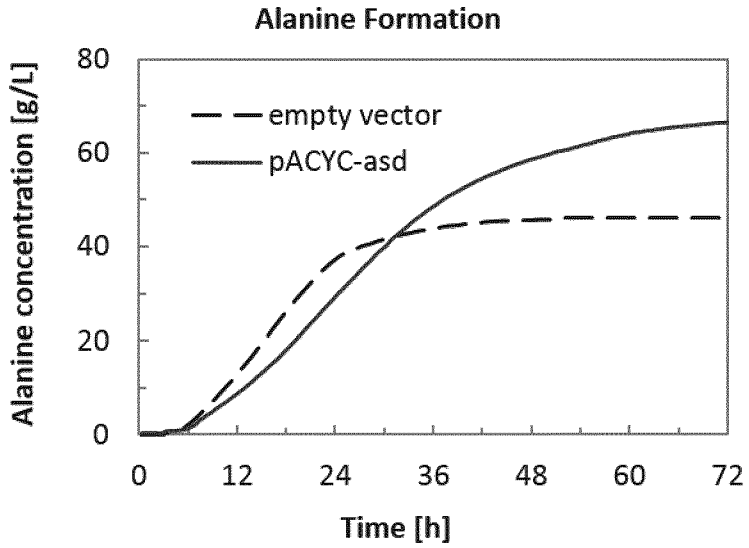
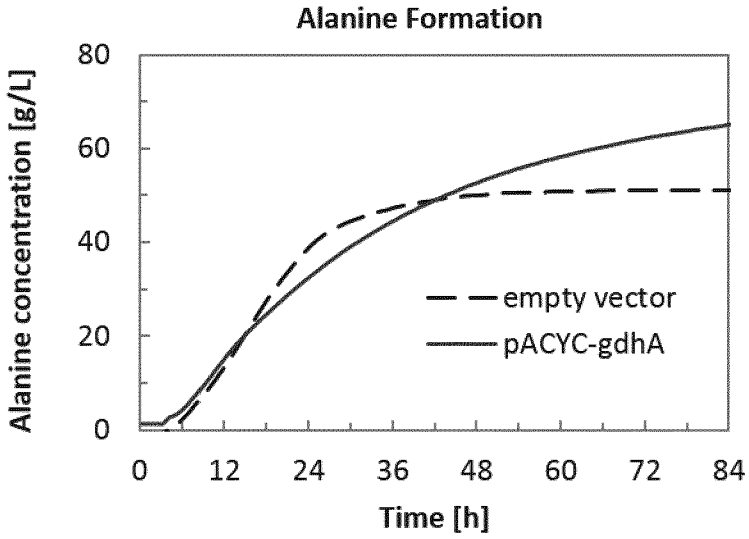


Figure 2



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/063172

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P13/06 C12N9/02 C12N9/06
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)
C12P C12N

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, Sequence Search, FSTA, 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 2008/119009 A2 (UNIV FLORIDA [US]; ZHANG XUELI ZHANG [US]; JANTAMA KAEMWICH [US]; MOOR) 2 October 2008 (2008-10-02) cited in the application the whole document example 1 table 1	1-15, 31-44
X	WO 89/03427 A1 (UNIV WASHINGTON [US]) 20 April 1989 (1989-04-20) the whole document	1,31-33, 42

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

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 September 2016	09/11/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	van de Kamp, Mart

2

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/141226 A2 (UNIV ARIZONA [US]; UNIV WASHINGTON [US]; CURTISS ROY III [US]) 20 November 2008 (2008-11-20) the whole document paragraphs [0033], [0039], [0045], [0053] example 1 claims 1,4,5,10,11,16,17	1,31-33, 42
A	RACZYNSKA-PAWELEC A. ET AL: "Characterization of Campylobacter jejuni asd gene cloned in Escherichia coli", ACTA MICROBIOLOGICA POLONICA, vol. 44, no. 3-4, 1995, pages 227-241, XP002194221, ISSN: 0137-1320 the whole document	1,31-33, 42
A	WO 2009/022754 A1 (AJINOMOTO KK [JP]; ALTMAN IRINA BORISOVNA [RU]; PTITSYN LEONID ROMANOV) 19 February 2009 (2009-02-19) the whole document page 7, line 20 - page 8, line 30 claims 5,7	1,32,34, 37,42-44
A	WO 2014/009435 A1 (ADISSEO FRANCE SAS [FR]) 16 January 2014 (2014-01-16) the whole document page 11, line 29 - page 12, line 27 page 13, line 32 - page 14, line 2 example 5 claims 14,18 figure 1	1,32,34, 37,42-44
A	CAHYANTO M.N. ET AL: "Construction of Lactobacillus plantarum strain with enhanced L-lysine yield", JOURNAL OF APPLIED MICROBIOLOGY, vol. 102, no. 3, 24 October 2006 (2006-10-24), pages 674-679, XP002732022, ISSN: 1364-5072, DOI: 10.1111/J.1365-2672.2006.03174.X the whole document	1,32,34, 37,42-44

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NAI-CHEN WANG ET AL: "Molecular cloning of the aspartate 4-decarboxylase gene from Pseudomonas sp. ATCC 19121 and characterization of the bifunctional recombinant enzyme", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 73, no. 2, 8 June 2006 (2006-06-08), pages 339-348, XP019458709, ISSN: 1432-0614, DOI: 10.1007/S00253-006-0475-6 the whole document	1,32,34, 37,42-44
A	C-C CHEN ET AL: "Cloning, expression and characterization of L-aspartate beta-decarboxylase gene from Alcaligenes faecalis CCRC 11585", JOURNAL OF INDUSTRIAL MICROBIOLOGY AND BIOTECHNOLOGY, vol. 25, no. 3, 1 September 2000 (2000-09-01), pages 132-140, XP055300386, GB ISSN: 1367-5435, DOI: 10.1038/sj.jim.7000043 the whole document	1,32,34, 37,42-44
A	US 5 019 509 A (ROZZELL J DAVID [US]) 28 May 1991 (1991-05-28) the whole document	1,32,34, 37,42-44

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/063172

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 16-30
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-44(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 16-30

No search is carried out for the subject-matter of claims 16-30 because of lack of correspondence between the SEQ ID NOs recited in said claims and the sequence listing which has been filed for the present application.

This is explained in some detail for claim 16 as follows:

- SEQ ID NO:25 in the sequence listing is a 894-nt sequence annotated as E. coli argP,
- SEQ ID NO:37 in the sequence listing is a 30-nt sequence annotated as Artificial Primer K244A REV,
- SEQ ID NO:26 in the sequence listing is a 297-aa sequence annotated as E. coli argP,
- SEQ ID NO:38 in the sequence listing is a 36-nt sequence annotated as Artificial Primer I237N FWD.

According to claim 16, SEQ ID NOs 25 and 26 would correspond to asd nucleic acid and polypeptide sequences, and SEQ ID NOs 37 and 38 would correspond to gdhA nucleic acid and polypeptide sequences.

Mutatis mutandis, similar observations can be made for the SEQ ID NOs recited in claims 17-30.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-44(partially)

A recombinant microorganism comprising a reduced, repressed or deleted activity and/or expression of an asd gene. A composition comprising said recombinant microorganism. A method for producing a recombinant microorganism with enhanced alanine yield and/or productivity, which comprises reducing, repressing or deleting the activity and/or expression of an asd gene in a microorganism. A method of producing alanine comprising culturing said recombinant microorganism under conditions that allow for the production of alanine. A method of culturing or growing a genetically modified microorganism comprising inoculating a culture medium with said genetically modified microorganism and culturing or growing said genetically modified microorganism in culture medium. A use of said recombinant microorganism or said composition for the fermentative production of alanine. A process for fermentative production of alanine comprising the steps of I) growing said microorganism in a fermenter and II) recovering alanine from the fermentation broth obtained in I).

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2. claims: 1-44(partially)

A recombinant microorganism comprising a reduced, repressed or deleted activity and/or expression of a gdhA gene. A composition comprising said recombinant microorganism. A method for producing a recombinant microorganism with enhanced alanine yield and/or productivity, which comprises reducing, repressing or deleting the activity and/or expression of a gdhA gene in a microorganism. A method of producing alanine comprising culturing said recombinant microorganism under conditions that allow for the production of alanine. A method of culturing or growing a genetically modified microorganism comprising inoculating a culture medium with said genetically modified microorganism and culturing or growing said genetically modified microorganism in culture medium. A use of said recombinant microorganism or said composition for the fermentative production of alanine. A process for fermentative production of alanine comprising the steps of I) growing said microorganism in a fermenter and II) recovering alanine from the fermentation broth obtained in I).

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/063172

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008119009 A2	02-10-2008	NONE	
WO 8903427 A1	20-04-1989	AT 121785 T	15-05-1995
		AU 637969 B2	17-06-1993
		CA 1339412 C	02-09-1997
		DE 3853683 D1	01-06-1995
		DE 3853683 T2	31-08-1995
		EP 0381706 A1	16-08-1990
		JP H04501351 A	12-03-1992
		JP H11235191 A	31-08-1999
		US 5672345 A	30-09-1997
		US 5840483 A	24-11-1998
		WO 8903427 A1	20-04-1989
WO 2008141226 A2	20-11-2008	EP 2152883 A2	17-02-2010
		US 2010317084 A1	16-12-2010
		WO 2008141226 A2	20-11-2008
WO 2009022754 A1	19-02-2009	AT 554179 T	15-05-2012
		EP 2185683 A1	19-05-2010
		WO 2009022754 A1	19-02-2009
WO 2014009435 A1	16-01-2014	AR 091726 A1	25-02-2015
		CN 104471069 A	25-03-2015
		EP 2872640 A1	20-05-2015
		JP 2015524253 A	24-08-2015
		KR 20150035726 A	07-04-2015
		RU 2014153354 A	27-08-2016
		US 2015147793 A1	28-05-2015
		WO 2014009435 A1	16-01-2014
US 5019509 A	28-05-1991	NONE	