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(54) Title: PREPARATION OF ALPHA-AMINO-EPSILON-CAPROLACTAM VIA LYSINE CYCLISATION

(57) Abstract: The present invention relates to a method for preparing α -amino- ϵ -caprolactam, comprising converting lysine to α -amino- ϵ -caprolactam, wherein the conversion is catalysed by a biocatalyst. Further, the invention relates to a host cell comprising at least one recombinant vector comprising a nucleic acid sequence encoding a biocatalyst with lysine cyclase activity. Further a method is provided wherein α -amino- ϵ -caprolactam is used for preparing ϵ -caprolactam.



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Title: Preparation of alpha-amino-epsilon-caprolactam via lysine cyclisation

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The invention relates to a method for preparing α -amino- ϵ -caprolactam (hereinafter also referred to as ACL). The invention further relates to a method wherein ACL is used for preparing ϵ -caprolactam (hereafter referred to as 'caprolactam'). The invention further relates to a host cell which may be used in the preparation of ACL or caprolactam.

Caprolactam is a lactam which may be used for the production of polyamide, for instance nylon-6 or nylon-6,12 (a copolymer of caprolactam and laurolactam). Various manners of preparing caprolactam from bulk chemicals are known in the art and include the preparation of caprolactam from cyclohexanone, toluene, phenol, cyclohexanol, benzene or cyclohexane. These intermediate compounds are generally obtained from mineral oil. In view of a growing desire to prepare materials using more sustainable technology it would be desirable to provide a method wherein caprolactam is prepared from an intermediate compound that can be obtained from a biological source or at least from an intermediate compound that is converted into caprolactam using a biochemical method. Furthermore, it would be desirable to provide a method that requires less energy than conventional chemical processes making use of bulk chemicals from petrochemical origin.

It is known to prepare caprolactam from 6-aminocaproic acid (6-ACA), e.g. as described in US-A 6,194,572. As disclosed in WO 2005/068643, 6-ACA may be prepared biochemically by converting 6-aminohex-2-enoic acid (6-AHEA) in the presence of an enzyme having α,β -enoate reductase activity. The 6-AHEA may be prepared from lysine, e.g. biochemically or by pure chemical synthesis. Although, the preparation of 6-ACA via the reduction of 6-AHEA is feasible by the methods disclosed in WO 2005/068643, the inventors have found that – under the reduction reaction conditions – 6-AHEA may spontaneously and substantially irreversibly cyclise to form an undesired side-product, notably β -homoproline. This cyclisation may be a bottle neck in the production of 6-ACA, and lead to a considerable loss in yield.

It is an object of the invention to provide a novel method for preparing caprolactam that can serve as an alternative for known methods. It is in particular an

object to provide a novel method for preparing an intermediate compound that can be used to prepare caprolactam from.

It is a further object to provide a novel method that would overcome one or more of the drawbacks mentioned above.

5 It is a further object to provide a novel fermentative method for preparing caprolactam or an intermediate compound for preparing caprolactam.

One or more further objects which may be solved in accordance with the invention will follow from the description, below.

10 It has now been found possible to prepare caprolactam or an intermediate compound for preparing caprolactam, biocatalytically from a specific starting compound.

Accordingly, the invention relates to a method for preparing α -amino- ϵ -caprolactam (ACL), comprising converting lysine to α -amino- ϵ -caprolactam, wherein the conversion is catalysed by a biocatalyst.

15 The invention further relates to a method for preparing caprolactam from α -amino- ϵ -caprolactam.

The invention is based on the insight that it is possible to prepare caprolactam biocatalytically from lysine or from a product that can be obtained by the cyclisation of lysine.

20 The inventors consider it in particular unexpected that that a method according to the invention can also be carried out in an aqueous environment, such as in an intracellular environment. One would expect that the presence of a substantial quantity of water, as is typically present inside a (living) organism, would force the equilibrium of the reaction from lysine to ACL to the side of lysine. After all, in essence
25 the ring closure of lysine to is a reaction wherein a peptidic bond is formed. In general, in an aqueous environment, enzymatic hydrolysis is the kinetically favoured reaction over enzymatic peptidic bond formation.

The unexpected character of the invention is further illustrated by the fact that in a thorough search in scientific literature and in databases for natural micro-organisms did not result in finding any report of known organisms being capable of
30 converting lysine into ACL.

In accordance with the invention, no problems have been noticed with respect to an undesired cyclisation of an intermediate product, when forming ACL and optionally caprolactam, resulting in a loss of yield.

In an advantageous embodiment of the invention ACL or caprolactam is prepared fermentatively.

The term "or" as used herein means "and/or" unless specified otherwise.

5 The term "a" or "an" as used herein means "at least one" unless specified other wise.

 When referring to a noun (*e.g.* a compound, an additive *etc.*) in singular, the plural is meant to be included. Thus, when referring to a specific noun, *e.g.* "compound", this means "at least one" of that noun, *e.g.* "at least one compound",
10 unless specified otherwise.

 When referred to a compound of which stereoisomers exist, the compound may be any of such stereoisomers or a combination thereof. Thus, when referred to, *e.g.*, ACL or an amino acid of which enantiomers exist, the ACL or the amino acid may be the L-enantiomer, the D-enantiomer or a combination thereof. In
15 case a natural stereoisomer exists, the compound is preferably a natural stereoisomer.

 When referring herein to carboxylic acids or carboxylates, *e.g.* 6-ACA, another amino acid or a fatty acid, these terms are meant to include the protonated carboxylic acid, their corresponding carboxylate (their conjugated bases) as well as salts thereof. When referring herein to amino acids, *e.g.* 6-ACA, this
20 term is meant to include amino acids in their zwitterionic form (in which the amino group is in the protonated and the carboxylate group is in the deprotonated form), the amino acid in which the amino group is protonated and the carboxylic group is in its neutral form, and the amino acid in which the amino group is in its neutral form and the carboxylate group is in the deprotonated form, as well as salts thereof. Likewise, when
25 referring to an amine (*e.g.* lysine or another amino acid, or ACL), this is meant to include the protonated amine (typically cationic, *e.g.* $R-NH_3^+$) and the unprotonated amine (typically uncharged, *e.g.* $R-NH_2$).

 When an enzyme is mentioned with reference to an enzyme class (EC) between brackets, the enzyme class is a class wherein the enzyme is classified or
30 may be classified, on the basis of the Enzyme Nomenclature provided by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), which nomenclature may be found at <http://www.chem.qmul.ac.uk/iubmb/enzyme/>. Other suitable enzymes that have not (yet) been classified in a specified class but may be classified as such, are meant to be
35 included.

The term "homologue" is used herein in particular for polynucleotides or polypeptides having a sequence identity of at least 30 %, preferably at least 40 %, more preferably at least 60%, more preferably at least 65%, more preferably at least 70 %, more preferably at least 75%, more preferably at least 80%, in particular at least 85
5 %, more in particular at least 90 %, at least 91 %, at least 92 %, at least 93 %, at least 94 %, at least 95 %, at least 96 %, at least 97 %, at least 98 % or at least 99 %. The term homologue is also meant to include nucleic acid sequences (polynucleotide sequences) which differ from another nucleic acid sequence (polynucleotide sequence) due to the degeneracy of the genetic code and encode the same polypeptide
10 sequence.

Sequence identity or similarity is herein defined as a relationship between two or more polypeptide sequences or two or more nucleic acid sequences (polynucleotide sequences), as determined by comparing the sequences. Usually, sequence identities or similarities are compared over the whole length of the
15 sequences, but may however also be compared only for a part of the sequences aligning with each other. In the art, "identity" or "similarity" also means the degree of sequence relatedness between polypeptide sequences or nucleic acid sequences (polynucleotide sequences), as the case may be, as determined by the match between strings of such sequences. Preferred methods to determine identity or similarity are
20 designed to give the largest match between the sequences tested. In context of this invention a preferred computer program method to determine identity and similarity between two sequences includes BLASTP and BLASTN (Altschul, S. F. et al., J. Mol. Biol. **1990**, 215, 403-410, publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894). Preferred
25 parameters for polypeptide sequence comparison using BLASTP are gap open 10.0, gap extend 0.5, Blosom 62 matrix. Preferred parameters for nucleic acid sequence comparison using BLASTN are gap open 10.0, gap extend 0.5, DNA full matrix (DNA identity matrix).

In a method of the invention, a biocatalyst is used, *i.e.* at least one
30 reaction step in the method is catalysed by a biological material or moiety derived from a biological source, for instance an organism or a biomolecule derived there from. The biocatalyst may in particular comprise one or more enzymes. The biocatalyst may be used in any form. In an embodiment, one or more enzymes are used isolated from the natural environment (isolated from the organism it has been produced in), for instance
35 as a solution, an emulsion, a dispersion, (a suspension of) freeze-dried cells, as a

lysate, or immobilised on a support. In an embodiment, one or more enzymes form part of a living organism (such as living whole cells). The enzymes may perform a catalytic function inside the cell. It is also possible that the enzyme may be secreted into a medium, wherein the cells are present.

5 Living cells may be growing cells, resting or dormant cells (*e.g.* spores) or cells in a stationary phase. It is also possible to use an enzyme forming part of a permeabilised cell (*i.e.* made permeable to a substrate for the enzyme or a precursor for a substrate for the enzyme or enzymes).

A biocatalyst used in a method of the invention may in principle be
10 any organism, or be obtained or derived from any organism. The organism may be eukaryotic or prokaryotic. In particular the organism may be selected from animals (other than humans, at least in as far as the use of the organism *per se* is involved), plants, bacteria, archaea, yeasts and fungi. A suitable biocatalyst or part thereof may in principle also be of human origin. In particular an enzyme may be obtained or derived
15 from human cell material for use in method of the invention.

In an embodiment a biocatalyst, *e.g.* and enzyme, may originate from an animal, in particular from a part thereof – *e.g.* liver, pancreas, brain, kidney or other organ. The animal may in particular be selected from invertebrate marine animals, more in particular sponges (*Porifera*), in particular from *Demospongiae*, *Pachastrellidae*
20 or *Jaspidae*, *e.g.* *Jaspis sp.*, *Pachastrella sp.*, *Poecillastra sollasi*, *Choristidae* and mammals, more in particular mammals selected from the group of *Leporidae*, *Muridae*, *Suidae* and *Bovidae*.

Suitable bacteria may in particular be selected amongst the group of *Pseudomonas*, *Bacillus*, *Escherichia*, *Ochrobactrum*, *Citrobacter*; *Klebsiella*,
25 *Mycobacterium*, *Providencia*, *Achromobacter*, *Rhodococcus*, *Myxococcus*, *Enterobacter*; *Methylophilus*, *Streptomyces*, *Nocardia*, *Thermus* and *Alcaligenes*.

Suitable fungi may in particular be selected amongst the group of *Aspergillus*, *Tremella* and *Periconia*.

Suitable yeasts may in particular be selected amongst the group of
30 *Candida*, *Saccharomyces*, *Kluyveromyces*, *Cryptococcus* and *Trichosporon*.

It will be clear to the person skilled in the art that use can be made of a naturally occurring biocatalyst (wild type) or a mutant of a naturally occurring biocatalyst with suitable activity in a method according to the invention.

Properties of a naturally occurring biocatalyst may be improved by
35 biological techniques known to the skilled person in the art, such as *e.g.* molecular

evolution or rational design. Mutants of wild-type biocatalysts can for example be made by modifying the encoding DNA of an organism capable of acting as a biocatalyst or capable of producing a biocatalytic moiety (such as an enzyme) using mutagenesis techniques known to the person skilled in the art (random mutagenesis, site-directed
5 mutagenesis, directed evolution, gene recombination, *etc.*). In particular the DNA may be modified such that it encodes an enzyme that differs by at least one amino acid from the wild-type enzyme, so that it encodes an enzyme that comprises one or more amino acid substitutions, deletions and/or insertions compared to the wild-type, or such that the mutants combine sequences of two or more parent enzymes or by effecting the
10 expression of the thus modified DNA in a suitable (host) cell. The latter may be achieved by methods known to the skilled person in the art such as codon optimisation or codon pair optimisation, *e.g.* based on a method as described in WO 2008/000632. WO 2003/010183 discloses a particularly suitable process for the preparation of variant polynucleotides using a combination of mutagenesis of a starting population of
15 polynucleotides and recombination of the mutated polynucleotides.

A mutant biocatalyst may have improved properties, for instance with respect to one or more of the following aspects: selectivity towards the substrate, activity, stability, solvent tolerance, pH profile, temperature profile, substrate profile, susceptibility to inhibition, cofactor utilisation and substrate-affinity. Mutants with
20 improved properties can be identified by applying *e.g.* suitable high through-put screening or selection methods based on such methods known to the skilled person in the art.

When referred to a biocatalyst, in particular an enzyme, from a particular source, recombinant biocatalysts, in particular enzymes, originating from a
25 first organism, but actually produced in a (genetically modified) second organism, are specifically meant to be included as biocatalysts, in particular enzymes, from that first organism.

In accordance with a method of the invention, ACL is prepared by cyclising lysine, wherein the cyclisation is catalysed by a biocatalyst. In principle, D-
30 lysine, L-lysine or a mixture thereof can be used. By cyclising these, D-ACL, L-ACL or a mixture thereof is formed. In practice, L-lysine is preferred.

A biocatalyst used in this cyclisation reaction, preferably comprises an enzyme having lysine cyclase activity. For instance an enzyme having lysine cyclase activity may be used originating from an organism as identified above.

35 In particular, an enzyme capable of catalysing the cyclisation of lysine

to ACL, may be selected from the group of hydrolases (EC 3). The hydrolase preferably is selected from the group of hydrolases acting on ester bonds (esterases) (EC 3.1), and hydrolytic enzymes acting upon carbon-nitrogen bonds, other than peptide bonds (EC 3.5). An esterase may in particular be selected from the group of
5 carboxylic ester hydrolases (EC 3.1.1) and more in particular carboxyl esterases (EC 3.1.1.1), preferably from pig liver esterases. An enzyme of EC class 3.5 may in particular be selected from the group of hydrolases mainly acting on linear amides (EC 3.5.1).

A hydrolase mainly acting on linear amides, such as an amidase, may
10 in particular be such hydrolase from *Ochrobactrum*, *Rhodococcus*, *Enterobacter*, *Thermus*, *Klebsiella*, *Aspergillus*, *Methylophilus* or *Mycobacterium*. More in particular a hydrolase mainly acting on linear amides, such as an amidase, may be used from an organism selected from the group of *Ochrobactrum anthropi*, *Rhodococcus erythropolis*, *Enterobacter cloacae*, *Thermus sp.*, *Klebsiella terrigena*, *Klebsiella*
15 *oxytoca*, *Aspergillus nidulans*, *Methylophilus methylotrophus* and *Mycobacterium smegmatis*. An amidase originating from *Ochrobactrum anthropi* NCIMB 40321 or an amidase originating from *Rhodococcus erythropolis* NCIMB 11540 is particularly advantageous in a method wherein ACL is further used for the preparation of caprolactam. Such amidase may in particular comprise an amino acid sequence
20 according to Sequence ID 4, Sequence ID 6 or a homologue thereof.

Further, an amidase may be used as described in US 2005/0079595 or in EP-A 1 409 667 for the cyclisation reaction, the contents of which with respect to enzymes having lysine cyclase activity and genes coding for such enzymes are incorporated herein by reference.

25 Furthermore, an enzyme of EC class 3.5 may also in particular be selected from the group of hydrolases mainly acting on C-N bonds in cyclic amides (EC 3.5.2), which may also be referred to as a lactamase, and more in particular be selected from the group of lysine lactamases (EC 3.5.2.11).

In particular, a lactamase (*i.e.* a hydrolase acting in cyclic amides)
30 may be selected amongst L-lysine-1,6-lactam hydrolases (EC 3.5.2.11) and 6-aminohexanoate-cyclic dimer hydrolases (EC 3.5.2.12).

In an embodiment the lactamase, in particular an L-lysine lactamase, is selected amongst the group of lactamases from *Aspergillus*, *Cryptococcus*, *Candida*, *Citrobacter*, *Trichosporon*, *Tremella* and *Providencia*. More in particular, said
35 lactamase may be selected amongst the group of lactamases originating from

Aspergillus ustus, *Aspergillus niger*, *Cryptococcus laurentii*, *Candida humicola*,
Citrobacter freundii, *Trichosporon cutaneum*, *Tremella fuciformis*, *Tremella aurentia*,
Tremella foliacea, *Tremella subanomalia* and *Providencia alcalifaciens*.

In an embodiment the lactamase, in particular a 6-aminohexanoate-
5 cyclic dimer hydrolase (EC 3.5.2.12) is a lactamase from *Alcaligenes*, such as from
Alcaligenes lactamlytics or from *Achromobacter*, such as from *Achromobacter xerosis*
or *Achromobacter guttatus*.

A lipase may in particular be selected from lipases originating from a
mammal, such as porcine lipase, bovine lipase or the like. In particular, a lipase used in
10 a method of the invention may be a pancreatic lipase. Lipases are commercially
available, e.g. porcine pancreas lipase may be obtained from Röhms (catalogue number
7023C) or from Sigma (catalogue number L-3126). It is known to the person skilled in
the art that commercial pig liver esterase (PLE) preparations, e.g. available from Sigma
as a suspension (catalog number E2884) or in powder form (catalog number E3019),
15 usually are a mixture of enzymes, amongst others, isoenzymes, of pig liver esterase. It
is contemplated that one or more of these isoenzymes in the PLE preparation are
responsible for the bioconversion of lysine to ACL. A person skilled in the art knows
how to isolate, clone and/or express a pig liver esterase isoenzyme into a suitable host,
if desired.

20 In an embodiment, one may use a non-ribosomal peptide synthase
(NRPS) for cyclisation of lysine. It is known for secondary metabolite producers to
synthesise peptides via non-ribosomal peptide synthases (NRPSs). NRPSs are in
detail described in, e.g., "Assembly-Line Enzymology for Polyketide and Nonribosomal
Peptide Antibiotics: Logic, Machinery, and Mechanisms" Michael A. Fischbach and
25 Christopher T. Walsh, Chem. Rev. **2006**, 106, 3468-3496, and in WO/00/58478. In
some instances biocatalysts analogous to some parts of NRPSs are also used for
production of modified amino acids (e.g. amino coumarin in e.g. novobiocin and β -
hydroxyhistidine as precursor for the imidazolone moiety in nikkomycin X) as building
blocks for secondary metabolites. In bacteria and lower fungi biosynthetic genes
30 required for production of secondary metabolites are typically clustered in one locus on
the genome. In particular, in an embodiment wherein an NRPS is used, the NRPS may
be a modular non-ribosomal peptide synthase comprising a lysine specific adenylation
domain, a peptidyl carrier domain and a thioesterase/ cyclisation domain.

In a specific embodiment a biocatalyst for cyclisation of lysine to ACL
35 can be found in a gene cluster encoding the biosynthesis of bengamides,

nocardiamycins, capuramycins, circinatins or any other ACL or ACL-derivative containing secondary metabolite. Such a gene cluster may be present in any microorganism producing such a compound or a microbial endosymbiont thereof. Such a gene cluster can readily be identified by methods generally known in the art such as

5 genome scanning, whole genome sequencing, PCR using degenerated primers, or Southern hybridisation using information from known biosynthetic pathways. A specific biocatalyst may consist of a truncated NRPS module consisting of an adenylation domain specific for the activation of lysine, a peptidyl carrier domain, and a specific cyclisation domain. This cyclisation domain is expected to be homologous to known

10 thioesterases catalysing the macrocyclisation of cyclic non-ribosomal peptides such as e.g. tyrocidin. It is expected that a cyclisation domain specific for cyclisation of lysine contains specific signature motifs allowing its differentiation from other cyclising thioesterases or thioesterase domains. The domain required for cyclisation of lysine may be encoded by one open reading frame resulting in a modular biocatalyst or in

15 separate open reading frames resulting in separate proteins, which together form the biocatalyst. In the present invention use of such a biocatalyst may be advantageous, since the reaction is coupled to the hydrolysis of ATP and thus (at least substantially) irreversible.

ACL prepared in a method of the invention may be used for the

20 preparation of caprolactam. This may be accomplished chemically, e.g. by deamination using hydroxyl amine-*o*-sulphonic acid and potassium hydroxide or sodium hydroxide in water, an alcohol or a mixture thereof. A suitable preparation method and a subsequent purification step is e.g. described in WO 07/99029.

In an embodiment, ACL – prepared in a method of the invention – is

25 converted into (Z)-6,7-dihydro-1*H*-azepin-2(5*H*)-one (6,7-DAO). This may be accomplished chemically, or catalysed by a biocatalyst. 6,7-DAO may be used as an intermediate compound for the preparation of caprolactam.

6,7-DAO may in particular be prepared from ACL in a method comprising biocatalytically removing the α -amino group from α -amino- ϵ -caprolactam by

30 biocatalytic elimination of ammonia from ACL catalysed by a biocatalyst having ammonia lyase activity, thereby forming 6,7-DAO or removal of the α -amino group from ACL by another biocatalyst able of catalysing such elimination or another biocatalyst able of catalysing such removal of the ammonia group.

Further, the chemical preparation of 6,7-DAO may be based on, e.g., Reimschuessel, H.K. *et al.* J. Org. Chem. (1969), 34, 969, of which publication the contents are incorporated herein by reference, in particular with respect to reaction conditions. Based on this methodology, the skilled person will be able to prepare 6,7-DAO from ACL by diazotising ACL with NaNO₂ in the presence of HCl or HBr (or the like) by which the formed diazonium ACL derivative is transformed in situ to α -chloro- or α -bromocaprolactam, respectively. The latter compounds (or a similar compound if a different acid is used) can be converted into 6,7-DAO in an elimination reaction, using 2,6-lutidine as described in said reference.

Further, removal of the α -amino group of ACL to yield 6,7-DAO may e.g. be accomplished by ammonia elimination, or by subsequent transamination, keto-group reduction and dehydration. The removal reaction may be catalysed by one or more biocatalysts.

In particular the removal of the α -amino group may be catalysed by a biocatalyst comprising a lyase (EC 4). Preferably, a C-N lyase (EC 4.3) is used, more preferably an ammonia lyase (EC 4.3.1) is used.

A biocatalyst catalysing the conversion of ACL to 6,7-DAO may for instance originate from an organism, as mentioned above.

It is also possible to select a suitable biocatalyst for the conversion of ACL to 6,7-DAO using a selection method, as described, next.

For instance, one may select for a biocatalyst using a library comprising a collection of potential biocatalysts for removal of the α -amino group from ACL. In a selection method for finding a suitable biocatalyst, the candidate biocatalysts are contacted with a culture medium wherein as a sole nitrogen source ACL and/or at least one functional analogue of ACL is present. Only those micro-organisms will be able to grow, which can use the ACL-analogue as a nitrogen source.

Thereafter, one or more samples are selected that show growth in such culture medium (the so called 'growing cultures'). Thereafter, one or more of these growing cultures are tested for having activity towards converting ACL to 6,7-DAO. Optionally, in particular in case only one or more ACL-analogues have been used as the sole nitrogen source, the growing cultures are first checked for showing activity towards converting the ACL-analogue or analogues, after which one or more cultures showing such activity are tested for their activity towards converting ACL to 6,7-DAO.

Thus, in a specific aspect of the invention, the invention relates to a method of finding a biocatalyst capable of catalysing the removal of the α -amino group from ACL, comprising

- providing a library comprising a plurality of candidate biocatalysts in one or more cell
- 5 cultures, which cultures comprise a culture medium containing α -amino- ϵ -caprolactam and/or one or more analogues thereof as sole nitrogen source;
- selecting one or more candidate biocatalysts which grow in said culture medium; and
- screening for a biocatalyst which grows in said culture having catalytic activity in removing the α -amino group from ACL.

10 The term "selecting" as used herein is defined as a method in which one or more biocatalysts are tested for growth using certain specific conditions, which growth is an indication for the presence of the desired biocatalytic activity.

The term "screening" as used herein is defined as a method in which one or more biocatalysts are tested for one (or more) desired biocatalytic

15 conversion(s).

The library may in particular be a metagenomic library, comprising genomic fragments of micro-organisms, which fragments may have been identified or which may be unidentified, and which fragments have been cloned into a suitable micro-organism for expression such as *Escherichia*, *Pseudomonas*, *Bacillus*,

20 *Streptomyces*, or *Saccharomyces*. The fragments may in principle originate from any organism and one or more organisms. The organism(s) may be culturable or un-culturable under the existing conditions, may have a specific habitat, requiring specific environmental factors (e.g. temperature, pH, light, oxygen, nutrients) or symbiotic partners. In particular the organisms may be endosymbionts of a multicellular organism

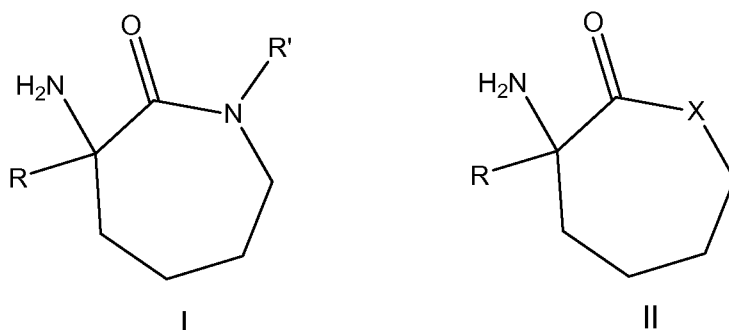
25 such as a sponge, insect, mammal, or plant.

In an embodiment, the library comprises a variety of environmental samples containing candidate biocatalysts, in particular a variety of water samples (e.g. waste water samples), compost samples and/or soil samples. Such samples comprise a variety of wild-type micro-organisms.

30 The term "functional analogue of ACL" is used herein to indicate that the analogue comprises a functional group that may be recognised by the biocatalyst. In particular a functional analogue may have the L- or D- configuration or a mixture thereof in any ratio, consists of a seven-membered α -amino lactam or α -amino

(thio)lactone with an additional carbon substituent at the α -position and optionally at the lactam nitrogen.

Preferably, an ACL-analogue is chosen which i) elicits the desired ACL ammonia lyase activity or alike activity leading to removal of the α -amino group from ACL and ii) has a low tendency towards eliciting side-reactions. In particular, the sole nitrogen source may consist of one or more compounds represented by formula I or II:



10

Herein, R and R' independently represent a hydrogen atom, or an organic moiety - which optionally comprises of one or more heteroatoms. Heteroatoms in the organic moieties R and R' may in particular be selected from N, S, O, F, Cl, Br, and I atoms. The organic moieties R and R' may in particular be independently selected from substituted and unsubstituted C1-C6 alkyl groups. X represents an O atom or an S atom.

The use of one or more functional analogues as the sole nitrogen source is preferred because the inventors have contemplated that the chance of finding a false positive would be higher when using ACL.

Another suitable selection method for finding a biocatalyst capable of catalysing the conversion of ACL to 6,7-DAO is based on lysine auxotrophy complementation. Herein a suitable host cell, which is lysine auxotroph, and which contains ACL-hydrolase activity is used for expression screening of genomic or metagenomic libraries. Such a host cell may be naturally occurring or can be engineered e.g. by inactivating the *lysA* gene in *E. coli* and expressing a suitable ACL-hydrolase. Such a host cell is then used for constructing a library as described above resulting in various host cells containing different DNA fragments. Various cells comprise different cloned genes.

The host cells are contacted with a culture medium comprising 6,7-DAO as sole lysine precursor. Then, one or more host cells are selected which grow in this medium. Thereafter, one or more growing host cells are usually tested for having catalytic activity for the conversion of 6,7-DAO to ACL. Thereafter one or more growing
5 host cells (usually selected from those having catalytic activity for the conversion of 6,7-DAO to ACL) are tested for having catalytic activity for the conversion of ACL to 6,7-DAO. A host cell having such activity can be used as a biocatalyst, or be used to obtain a biocatalyst therefrom.

Accordingly, the invention further relates to a method of detecting a
10 biocatalyst capable of catalysing the removal of the α -amino group of α -amino- ϵ -caprolactam, comprising

- providing lysine auxotrophic host cells, the host cells comprising a gene encoding an enzyme capable of catalysing the conversion of α -amino- ϵ -caprolactam into L-lysine, the host cells comprising a candidate gene encoding for an enzyme having lysine
15 cyclase activity;
- contacting the host cells with a library comprising various vectors containing a candidate gene encoding for an enzyme capable of catalysing the conversion of 6,7-DAO to ACL, whereby at least a part of the host cells are provided with said vector;
- contacting the host cells, provided with said vector, with (Z)-6,7-dihydro-1H-azepin-
20 2(5H)-one and an ammonia source;
- selecting one or more cultures which grow in said culture medium; and
- screening for one or more of said cultures which grow for having catalytic activity with respect to biocatalytic removal of the α -amino group of α -amino- ϵ -caprolactam, as the culture providing the biocatalyst capable of catalysing the elimination of the α -amino
25 group of α -amino- ϵ -caprolactam.

Another suitable screening method contemplated by the inventors is based on using a molecular receptor and reporter system in a suitable host organism. Several such systems have been described in the art (Beggah, S.; Vogne, C.; Zenaro, E.; van der Meer, J. R. *Microbial Biotechnology* **2008**, 1(1), 68-78; Sint Fiet, S.; van
30 Beilen, J. B.; Witholt, B. *Proceedings of the National Academy of Sciences* **2006**, 103(6), 1693-1698.). In such a system a suitable transcriptional regulator, herein also referred to as receptor, is able to bind a compound of interest such as 6,7-DAO or an analogue. Such a receptor may be a naturally occurring receptor having the desired properties in regards to e.g. specificity and binding affinity towards the compound of

interest. In most cases these properties have to be optimized for the specific compound of interest and receptor interaction by protein engineering methods generally known in the art. Upon binding the receptor elicits transcription from a suitable promoter, which is linked to a suitable reporter gene, herein also referred to as reporter. Suitable reporters may in principle be a gene which elicits a detectible, and preferably quantifiable, phenotype on the host strain such as production of a pigment, a fluorescent protein, an enzyme complementing an auxotrophy, or an antibiotic resistance marker.

Such a receptor/ reporter system may be established in a host and subsequently be used for screening (e.g. if using a fluorescent protein such as a green fluorescent protein as reporter) or selection (e.g. if using an antibiotic resistance gene as reporter) of a suitable biocatalyst for conversion of ACL to 6,7-DAO. The host cells are contacted with a culture medium comprising ACL or an analogue thereof. Then, one or more host cell cultures are selected or screened for which elicit a phenotype corresponding to the expression of the reporter. Thereafter, one or more such host cell cultures are usually tested for having catalytic activity for the conversion of ACL to 6,7-DAO. A host cell having such activity can be used as a biocatalyst, or be used to obtain a biocatalyst therefrom.

Accordingly, the invention also relates to a method of finding a biocatalyst capable of catalysing the removal of the α -amino group of α -amino- ϵ -caprolactam, comprising

- identifying or engineering a receptor to specifically bind 6,7-DAO;
- linking said receptor to a suitable reporter such as a β -galactosidase, green fluorescent protein, or an antibiotic resistance gene;
- optionally optimising the binding of 6,7-DAO to the receptor via one or more rounds of protein engineering to obtain desired specificity (i.e. no or low signal from the natural ligand and/ or ACL or analogues) and desired affinity towards 6,7-DAO or analogues thereof;
- expressing such a receptor/ reporter in a host suitable for metagenomic screening;
- contacting the host cells with a library comprising various vectors containing a candidate gene encoding for a biocatalyst (such as an enzyme) capable of catalysing the conversion of 6,7-DAO to ACL, whereby at least a part of the host cells comprise said vector;
- contacting the host cells, comprising said vector, with ACL or an analogue thereof;

- selecting or screening for one or more cultures which show the desired phenotype based on expression of the chosen reporter; and
 - screening for one or more of said cultures for having catalytic activity with respect to biocatalytic removal of the α -amino group of α -amino- ϵ -caprolactam, as the culture
- 5 providing the biocatalyst capable of catalysing the elimination of the α -amino group of α -amino- ϵ -caprolactam.

The gene encoding a biocatalyst, such as an enzyme, capable of catalysing the conversion of α -amino- ϵ -caprolactam into lysine may suitably be incorporated in the host cells using a vector, by conventional means.

- 10 The candidate gene encoding a biocatalyst having lysine cyclase activity may suitably be incorporated in the host cells using a vector, which may be the same or different as the vector encoding a biocatalyst capable of catalysing the conversion of α -amino- ϵ -caprolactam into lysine.

- 15 Alternatively, 6,7-DAO may be prepared chemically from ACL obtained in a method of the invention.

In a method according to the invention, ϵ -caprolactam may be prepared by reducing the unsaturated carbon-carbon double bond of (Z)-6,7-dihydro-1H-azepin-2(5H)-one prepared in a method of the invention, yielding caprolactam.

- 20 Such reduction can be carried out in the presence of a biocatalyst, capable of catalysing the reduction. Preferably such biocatalyst has reductase activity, in particular 6,7-DAO enone reductase activity, *i.e.* the catalyst is able to catalyse the reduction of the carbon-carbon double bond in 6,7-DAO, thereby forming caprolactam.

- In particular, the biocatalyst may comprise an enzyme selected from the group of oxidoreductases (EC1), more in particular the oxidoreductase may be an
- 25 oxidoreductase acting on the CH-CH group of donors (EC1.3) or an oxidoreductase that acts on NADH or NADPH (EC 1.6).

More specifically an oxidoreductase from EC 1.3.1 may be used, such as a 2-enone reductase (EC 1.3.1.33).

- A specific example of class an EC 1.6 enzyme is old yellow enzyme 1
- 30 (OYE1) is EC 1.6.99.1. The biocatalyst for reducing 6,7-DAO may be used in combination with a cofactor, suitable cofactors are known in the art, depending on the biocatalyst (enzyme) that is used.

A biocatalyst capable of catalysing said reduction may originate from an organism such as mentioned above. In particular, said biocatalyst may originate

from yeasts, plants, bacteria, fungi, archaea or mammals. More in particular a suitable biocatalyst capable of catalyzing said reduction may originate from a micro-organism selected from *Candida macedoniensis*, *Kluyveromyces lactis*, *Pseudomonas fluorescens*, *Pseudomonas syringae* pv. *glycinea*, *Escherichia coli*, *Saccharomyces cerevisiae* and *Bacillus subtilis*.

Reaction conditions for any biocatalytic step in the context of the present invention may be chosen depending upon known conditions for the biocatalyst, in particular the enzyme, the information disclosed herein and optionally some routine experimentation.

In principle, the pH of the reaction medium used may be chosen within wide limits, as long as the biocatalyst is active under the pH conditions. Alkaline, neutral or acidic conditions may be used, depending on the biocatalyst and other factors. In case the method includes the use of a micro-organism, e.g. for expressing an enzyme catalysing a method of the invention, the pH is selected such that the micro-organism is capable of performing its intended function or functions. The pH may in particular be chosen within the range of four pH units below neutral pH and two pH units above neutral pH, i.e. between pH 3 and pH 9 in case of an essentially aqueous system at 25 °C. A system is considered aqueous if water is the only solvent or the predominant solvent (> 50 wt. %, in particular > 90 wt. %, based on total liquids), wherein e.g. a minor amount of alcohol or another solvent (< 50 wt. %, in particular < 10 wt. %, based on total liquids) may be dissolved (e.g. as a carbon source) in such a concentration that micro-organisms which may be present remain active. In particular in case a yeast and/or a fungus is used, acidic conditions may be preferred, in particular the pH may be in the range of pH 3 to pH 8, based on an essentially aqueous system at 25 °C. If desired, the pH may be adjusted using an acid and/or a base or buffered with a suitable combination of an acid and a base.

In principle, the incubation conditions can be chosen within wide limits as long as the biocatalyst shows sufficient activity and/ or growth. Conditions may be selected from the group of aerobic, oxygen limited and anaerobic conditions.

Anaerobic conditions are herein defined as conditions without any oxygen or in which substantially no oxygen is consumed by the biocatalyst, in particular a micro-organism, and usually corresponds to an oxygen consumption of less than 5 mmol/l.h, in particular to an oxygen consumption of less than 2.5 mmol/l.h, or less than 1 mmol/l.h.

Aerobic conditions are conditions in which a sufficient level of oxygen

for unrestricted growth is dissolved in the medium, able to support a rate of oxygen consumption of at least 10 mmol/l.h, more preferably more than 20 mmol/l.h, even more preferably more than 50 mmol/l.h, and most preferably more than 100 mmol/l.h.

Oxygen-limited conditions are defined as conditions in which the
5 oxygen consumption is limited by the oxygen transfer from the gas to the liquid. The lower limit for oxygen-limited conditions is determined by the upper limit for anaerobic conditions, *i.e.* usually at least 1 mmol/l.h, and in particular at least 2.5 mmol/l.h, or most specifically at least 5 mmol/l.h. The upper limit for oxygen-limited conditions is determined by the lower limit for aerobic conditions, *i.e.* less than 100 mmol/l.h, less
10 than 50 mmol/l.h, less than 20 mmol/l.h, or less than to 10 mmol/l.h.

Whether conditions are aerobic, anaerobic or oxygen limited is dependent on the conditions under which the method is carried out, in particular by the amount and composition of ingoing gas flow, the actual mixing/mass transfer properties of the equipment used, the type of micro-organism used and the micro-organism
15 density.

In principle, the temperature used is not critical, as long as the biocatalyst, in particular the enzyme, shows substantial activity. Generally, the temperature may be at least 0 °C, in particular at least 15 °C, more in particular at least 20 °C. A desired maximum temperature depends upon the biocatalyst. In general such
20 maximum temperature is known in the art, *e.g.* indicated in a product data sheet in case of a commercially available biocatalyst, or can be determined routinely based on common general knowledge and the information disclosed herein. The temperature is usually 90 °C or less, preferably 70 °C or less, in particular 50 °C or less, more in particular or 40 °C or less.

25 In particular if a biocatalytic reaction is performed outside a host organism, a reaction medium comprising an organic solvent may be used in a high concentration (*e.g.* more than 50 wt. %, or more than 90 wt. %, based on total liquids), in case an enzyme is used that retains sufficient activity in such a medium.

In an advantageous method caprolactam is prepared making use of a
30 whole cell biotransformation of the substrate for caprolactam or an intermediate for forming caprolactam (ACL, 6,7-DAO), comprising the use of a micro-organism wherein a lysine cyclase, and an ammonia lyase and/or biocatalyst with activity for removal the α -amino group from ACL, and a 6,7-DAO enone reductase and/or other biocatalyst capable of reducing 6,7-DAO to caprolactam are produced, and a carbon source for
35 the micro-organism.

The carbon source may in particular contain at least one compound selected from the group of monohydric alcohols, polyhydric alcohols, carboxylic acids, carbon dioxide, fatty acids, glycerides, including mixtures comprising any of said compounds. Suitable monohydric alcohols include methanol and ethanol. Suitable
5 polyols include glycerol and carbohydrates. Suitable fatty acids or glycerides may in particular be provided in the form of an edible oil, preferably of plant origin.

In particular a carbohydrate may be used, because usually carbohydrates can be obtained in large amounts from a biologically renewable source, such as an agricultural product, preferably an agricultural waste-material. Preferably a
10 carbohydrate is used selected from the group of glucose, fructose, sucrose, lactose, saccharose, starch, cellulose and hemi-cellulose. Particularly preferred are glucose, oligosaccharides comprising glucose and polysaccharides comprising glucose.

It is contemplated that the lysine concentration may be in the nanomolar range (1-1000 nmol/l), the micromolar range (1-1000 μ mol/l) or the mmol/l
15 range (1-1000 mmol), or in a concentration exceeding 1 mol/l.

In particular, in case preparation and conversion of lysine take place intracellularly in the same cell or extracellularly in one a pot-type process, a concentration of 1 nmol/l or more, 100 nmol/l or more, 1 μ mol/l or more, 10 μ mol/l or
20 more, or 100 μ mol/l or more may already provide lysine in a sufficient concentration for acceptable or advantageous conversion rates. In case the preparation of lysine takes place intracellularly in the same cell as the conversion thereof, said concentrations in particular may be the intracellular concentration of lysine. Extracellular concentrations of lysine may be considerably lower in such embodiment; even 0 (*i.e.* below detection limit).

In case lysine is converted inside an organism, but the preparation of lysine has taken place outside that organism, or in case the preparation of lysine has taken place in a different reaction system and for the lysine conversion to ACL use is made of an enzyme isolated from an organism, the concentration of 6,7-DAO usually is
25 at least 1 μ mol/l, in particular at least 100 μ mol/l, more in particular at least 1 mmol/l or at least 10 mmol/l (extracellular concentration in the medium wherein the organism is present if an organism is used; or concentration in the reaction medium wherein lysine is converted in case an enzyme is used isolated from an organism).
30

The upper limit for the lysine concentration is not particularly critical. The lysine concentration may be exceeding 1 mol/l, 1 mol/l or less, in particular 0.5 mol/l or less or 0.1 mol/l or less.

A cell, in particular a recombinant cell, comprising one or more
5 enzymes for catalysing a reaction step in a method of the invention can be constructed using molecular biological techniques, which are known in the art *per se*. For instance, if one or more biocatalysts are to be produced in a recombinant cell (which may be a heterologous system), such techniques can be used to provide a vector which comprises one or more genes encoding one or more of said biocatalysts. One or more
10 vectors may be used which each comprise one or more genes. One or more vectors may be used, each vector comprising one or more of such genes. Such vector can comprise one or more regulatory elements, e.g. one or more promoters, which may be operably linked to a gene encoding a biocatalyst.

As used herein, the term "operably linked" refers to a linkage of
15 polynucleotide elements (or coding sequences or nucleic acid sequence) in a functional relationship. A nucleic acid sequence is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence.

20 As used herein, the term "promoter" refers to a nucleic acid fragment that functions to control the transcription of one or more genes, located upstream with respect to the direction of transcription of the transcription initiation site of the gene, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but
25 not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one of skilled in the art to act directly or indirectly to regulate the amount of transcription from the promoter. A "constitutive" promoter is a promoter that is active under most environmental and developmental conditions. An "inducible" promoter is a promoter that is active under
30 environmental or developmental regulation. The term "homologous" when used to indicate the relation between a given (recombinant) nucleic acid or polypeptide molecule and a given host organism or host cell, is understood to mean that in nature the nucleic acid or polypeptide molecule is produced by a host cell or organisms of the same species, preferably of the same variety or strain.

35 The promoter that could be used to achieve the expression of the

nucleic acid sequences coding for a biocatalyst for use in a method of the invention, in particular a lysine cyclase and optionally at least one biocalalyst selected from the group of ammonia lyases and 6,7-DAO enone reductases, such as described herein above may be native to the nucleotide sequence coding for the biocatalyst to be
5 expressed, or may be heterologous to the nucleotide sequence (coding sequence) to which it is operably linked. Preferably, the promoter is homologous, *i.e.* endogenous to the host cell.

If a heterologous promoter (to the nucleic acid sequence encoding the biocatalyst of interest) is used, the heterologous promoter is preferably capable of
10 producing a higher steady state level of the transcript comprising the coding sequence (or is capable of producing more transcript molecules, *i.e.* mRNA molecules, per unit of time) than is the promoter that is native to the coding sequence. Suitable promoters in this context include both constitutive and inducible natural promoters as well as engineered promoters, which are known to the person skilled in the art.

15 A "strong constitutive promoter" is a promotor which causes mRNAs to be initiated at high frequency compared to a native host cell. Examples of such strong constitutive promoters in Gram-positive micro-organisms include SP01-26, SP01-15, *veg*, *pyc* (pyruvate carboxylase promoter), and *amyE*.

Examples of inducible promoters in Gram-positive micro-organisms
20 include, the IPTG inducible Pspac promoter, the xylose inducible PxylA promoter.

Examples of constitutive and inducible promoters in Gram-negative microorganisms include, but are not limited to, *tac*, *tet*, *trp-tet*, *lpp*, *lac*, *lpp-lac*, *lacIq*, *T7*, *T5*, *T3*, *gal*, *trc*, *ara* (P_{BAD}), SP6, λ - P_R , and λ - P_L .

Promoters for (filamentous) fungal cells are known in the art and can
25 be, for example, the glucose-6-phosphate dehydrogenase *gpdA* promoters, protease promoters such as *pepA*, *pepB*, *pepC*, the glucoamylase *glaA* promoters, amylase *amyA*, *amyB* promoters, the catalase *catR* or *catA* promoters, glucose oxidase *goxC* promoter, beta-galactosidase *lacA* promoter, alpha-glucosidase *aglA* promoter, translation elongation factor *tefA* promoter, xylanase promoters such as *xlnA*, *xlnB*,
30 *xlnC*, *xlnD*, cellulase promoters such as *eglA*, *eglB*, *cbhA*, promoters of transcriptional regulators such as *areA*, *creA*, *xlnR*, *pacC*, *prtT*, etc or any other, and can be found among others at the NCBI website (<http://www.ncbi.nlm.nih.gov/entrez/>).

The term "heterologous" when used with respect to a nucleic acid (DNA or RNA) or protein refers to a nucleic acid or protein that does not occur naturally
35 as part of the organism, cell, genome or DNA or RNA sequence in which it is present,

or that is found in a cell or location or locations in the genome or DNA or RNA sequence that differ from that in which it is found in nature. Heterologous nucleic acids or proteins are not endogenous to the cell into which it is introduced, but has been obtained from another cell or synthetically or recombinantly produced. Generally, 5 though not necessarily, such nucleic acids encode proteins that are not normally produced by the cell in which the DNA is transcribed or expressed. Similarly exogenous RNA encodes for proteins not normally expressed in the cell in which the exogenous RNA is present. Heterologous nucleic acids and proteins may also be referred to as foreign nucleic acids or proteins. Any nucleic acid or protein that one of 10 skill in the art would recognize as heterologous or foreign to the cell in which it is expressed is herein encompassed by the term heterologous nucleic acid or protein.

A method according to the invention may be carried out in a host organism, which may be novel. Accordingly, the invention also relates to a novel host cell comprising one or more biocatalysts capable of catalysing the conversion of lysine 15 to ACL. The invention also relates to a novel vector comprising one or more genes encoding one or more biocatalysts (in particular enzymes) capable of catalysing the conversion of lysine to ACL and to a novel host cell comprising one or more vectors comprising one or more genes encoding one or more biocatalysts (in particular enzymes) capable of catalysing the conversion of lysine to ACL.

20 One or more suitable genes for a host cell or vector according to the invention may in particular be selected amongst genes encoding a biocatalyst (such as an enzyme) as mentioned herein above. Such gene may in particular comprise a nucleic acid sequence encoding a biocatalyst represented by Sequence ID 4, Sequence ID 6, or a homologue of any of these sequences. Examples of suitable 25 nucleic acid sequences are given in Sequence ID 3 and Sequence ID 5. The nucleic acid sequence may be from a wild-type organism. It is also possible to use a non-wild type sequence wherein one or more codons have been optimised, for improved expression in a host organism of interest.

A method according to the invention may partially or fully be carried 30 out in a host organism. Accordingly, the invention also relates to a novel vector comprising one or more genes encoding one or more biocatalysts (in particular enzymes) capable of catalysing one or more reaction steps and to a novel host cell comprising one or more vectors comprising one or more genes encoding one or more biocatalysts (in particular enzymes) capable of catalysing one or more reaction steps.

35 In an embodiment, a host cell according to the invention comprises at

least one recombinant vector comprising a nucleic acid sequence encoding a biocatalyst (in particular an enzyme) with lysine cyclase activity. Optionally the cell comprises a nucleic acid sequence encoding a biocatalyst (in particular an enzyme) with ammonia lyase activity. In a specific embodiment a recombinant vector comprising
5 a nucleic acid sequence encoding a biocatalyst (in particular an enzyme) with ammonia lyase activity is present, which sequence can be in the same or a different vector as the sequence encoding the biocatalyst having lysine cyclase activity. Optionally, the cell comprises a nucleic acid sequence encoding a biocatalyst (in particular an enzyme) with 6,7-DAO enone reductase activity. In a specific embodiment a recombinant vector
10 comprising a nucleic acid sequence encoding a biocatalyst (in particular an enzyme) with 6,7-DAO enone reductase activity is present, which sequence can be in the same or a different vector as the sequence encoding the biocatalyst having lysine cyclase activity.

In an advantageous embodiment, a cell of the invention comprises a
15 nucleic acid sequence encoding a biocatalyst with lysine cyclase activity, a nucleic acid sequence encoding a biocatalyst with ammonia lyase activity, and a nucleic acid sequence encoding a biocatalyst with 6,7-DAO enone reductase activity. Such cell is particularly suitable for a method wherein caprolactam is prepared from lysine, wherein purely chemical (*i.e.* not biocatalysed) reaction steps are avoided are at least
20 considerably reduced. Thus, the cell may be used as a biocatalyst for all reaction steps to prepare caprolactam from lysine, which steps may take place intracellularly in at least some embodiments. Such as cell may be a natural micro-organism or a recombinant organism. In the recombinant organism at least one, at least two or at least three recombinant nucleic acid sequences are present for encoding any of said
25 biocatalysts (usually enzymes).

The host cell may for instance be selected from the group of bacteria, yeasts and fungi. In particular from the genera selected from the group of *Aspergillus*, *Penicillium*, *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Candida*, *Hansenula*, *Bacillus*, *Corynebacterium*, *Pseudomonas*, *Gluconobacter* and *Escherichia*, in which one or
30 more encoding nucleic acid sequences as mentioned above have been cloned and expressed.

In particular, the host cell may be selected from the group of *Escherichia coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Aspergillus niger*, *Penicillium chrysogenum*, *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Candida albicans*, *Kluyveromyces lactis*, *Pichia stipitis* and *Pichia pastoris* host cells. In a
35

preferred embodiment, the host cell is capable of producing lysine (as a precursor).

The host cell may be in principle a naturally occurring organism or may be an engineered organism. Such an organism can be engineered using a mutation screening or metabolic engineering strategies known in the art. For instance
5 such a host cell may be selected of the genus *Corynebacterium*, in particular *C. glutamicum*, enteric bacteria, in particular *Escherichia coli*, *Bacillus*, in particular *B. subtilis* and *B. methanolicus* and *Saccharomyces*, in particular *S. cerevisiae*. Especially preferred are *C. glutamicum* or *B. methanolicus* strains which have been developed for the industrial production of lysine.

10 In a specific embodiment, the host cell naturally comprises (or is capable of producing) one or more of the enzymes suitable for catalysing a reaction step in a method of the invention.

The invention will now be illustrated by the following example.

15 **EXAMPLE**

General:

Molecular and genetic techniques

Standard genetic and molecular biology techniques are generally
20 known in the art and have been previously described (Maniatis et al. **1982** "Molecular cloning: a laboratory manual". Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; Miller **1972** "Experiments in molecular genetics", Cold Spring Harbor Laboratory, Cold Spring Harbor; Sambrook and Russell **2001** "Molecular cloning: a laboratory manual" (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory
25 Press; F. Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York **1987**).

Identification of plasmids and inserts

Plasmids carrying the different genes were identified by genetic,
30 biochemical, and/or phenotypic means generally known in the art, such as resistance of transformants to antibiotics, PCR diagnostic analysis of transformant or purification of plasmid DNA, restriction analysis of the purified plasmid DNA or DNA sequence analysis.

Example 1: Biocatalytic synthesis of ACL from lysine

1.1 HPLC-MS analysis for the determination of lysine and ACL

5 The calibration was performed by an external calibration line of both Lys and ACL. Lys elutes at a retention time (Rt) of 2.4 min (ESI(-)-MS, m/z 145) and ACL elutes at 4.4 min. (ESI(+)-MS, m/z 129).

 The LC-UV-MS experiments were performed on an Agilent 1100, equipped with a quaternary pump, degasser, autosampler, column oven, diode-array
10 detector (DAD) with 10-mm cell and a time-of-flight MS (Agilent, Waldbronn, Germany).

 The LC-UV-MS conditions were:

 Column: 50 x 4.6 mm Nucleosil C18, 5 µm (Machery & Nagel) precolumn
 coupled to a 250 x 4.6 mm id. Prevail C18, 5 µm (Alltech)
 Eluent: 0.1(% v/v) formic acid in ultrapure water
15 Flow: 1 ml/min., before entering the MS the flow is split 1:3
 Gradient: No gradient
 Injection volume: 5 µl
 UV detection: no UV used for detection
 MS detection: ESI-MS, using the negative mode at Rt 0-4 minutes and the positive
20 mode at 4-10 minutes. The electrospray ionization (ESI) used the
 following conditions; m/z 50-3600, 175 V fragmentor, 350 °C drying
 gas temperature, 10 L N₂/min drying gas, 50 psig nebuliser pressure
 and 2.5 kV capillary voltage.

25 1.2 Construction of biocatalyst

Isolation of chromosomal DNA from *R. erythropolis* NCIMB11540

 Chromosomal DNA from *Rhodococcus erythropolis* NCIMB 11540
was isolated following the general protocol of the QIAGEN Genomic DNA Handbook
30 (QIAGEN, Hilden, Germany) for the isolation of chromosomal DNA from gram positive
bacteria. The raw preparation was purified by using a QIAGEN Genomic-tip 500/G
column (QIAGEN, Hilden, Germany) and the manufacturer's procedure.

PCR amplification of the *R. erythropolis* lysine cyclase gene:

The sequences of the primers used for amplification of the *R. erythropolis* NCIMB 11540 lysine cyclase PCR-reaction contained restriction sites (underlined) for *Nde*I (forward-primer) and *Sph*I (reverse primer) to allow the subsequent cloning into plasmid pMS470Δ8 (Balzer *et al.*, Nucleic Acids Research, 1992, 20 (8): 1851-1858).

R. erythropolis -forward [SEQ ID No. 1]:

10 5' - CTCATATGGC GACAATCCGA CCTGACG - 3'

R. erythropolis -reverse [SEQ ID No. 2]:

5' - CTGCATGCTT GTTGTCTGAC AGTGCGTC - 3'

15 Synergy[®]-polymerase (GeneCraft, Cologne, Germany) was used according to the supplier's manual to allow TA-cloning of PCR-products. The PCR temperature profile was as follows: 1) 15 min 95°C; 2) 1 min 94°C, 0.5 min 60°C, 4 min 72°C (30 x); 3) 10 min 72°C. The product of the PCR-reaction formed a clear band of the expected size on the analytical agarose gels.

20

Cloning of the PCR-product into pCR[®]II-vector (Invitrogen)

15 μl of the PCR product were purified by preparative agarose gel electrophoresis using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany). 2 μl DNA-solution served as insert for the Invitrogen TA-Topo cloning procedure into the pCR[®]II plasmid with subsequent transformation of *E. coli* Top10F'. Positive clones were selected by white/blue screening on LB/ampicilline/IPTG/X-Gal plates. White colonies were picked and struck out for plasmid isolation. Restriction analysis with *Eco*RI showed clone pCR-33/3/1 to carry an insert of the desired size. DNA sequencing with M13f(-20) and M13rev-primers confirmed that the right fragment for the target lysine cyclase gene [SEQ ID. No. 3] coding for a lysine cyclase from *Rhodococcus erythropolis* NCIMB 11540 [SEQ ID No. 4] had been cloned.

30

Cloning of the pCR-33/3/1-insert into pMS470Δ8

Plasmid pMS470Δ8 (Balzer *et al.*, *Nucleic Acids Research*, **1992**, 20 (8): 1851-1858) was isolated from *E. coli* by standard procedures. Double restriction with *Nde*I and *Sph*I resulted in two fragments, from which the 4 kb part was eluted from an agarose gel. pCR-33/3/1 was digested with *Nde*I and *Sph*I. A 1.6 kb fragment was isolated and purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany). Ligation of the linearized pMS470 fragment and the *Sph*I/*Nde*I gene fragment was performed with T4-DNA-ligase (Invitrogen) at 16°C over night. Transformation of *E. coli* DH10B and restriction analysis of the plasmids of the ampicillin resistant clones with *Eco*RI resulted in a clone carrying the pMS470-33/3/1/11 plasmid.

Cultivation of *E. coli* DH10B pMS470-33/3/1/11-1

Fermentation for the production of the *R. erythropolis* NCIMB 11540 lysine cyclase was carried out on ten litre scale in an ISF-200 laboratory fermentor (Infors, Bottmingen, Switzerland). For the inoculation of the fermentor an over night (24 h) starter culture in 0.5 l Terrific Broth (TB; 12 g/l tryptone, 24 g/l yeast extract, 4 g/l glycerol, 2.31 g/l KH₂PO₄, 12.54 g/l K₂HPO₄, pH 7.0 containing 100 µg/ml carbenicillin) was used, which itself had been inoculated with 0.1 ml of the respective glycerol stock culture of *E. coli* DH10B pMS470-33/3/1/11-1.

The expression of *R. erythropolis* NCIMB 11540 lysine cyclase was induced by addition of 0.5 mM IPTG (final concentration) at a cell density of OD₆₂₀ = 0.8. After 20.5 hours of cultivation (OD₆₂₀ = 6.4) the cells were harvested by centrifugation (12 minutes at 12,227x g at 4°C).

Preparation of cell free extract of *E. coli* DH10B pMS470-33/3/1/11-1

The wet cells of *E. coli* DH10B pMS470-33/3/1/11-1 (117 g) were washed with 20 mM HEPES buffer (pH 7.0) and resuspended in 350 ml 0.1 M potassium phosphate buffer (pH 7.0). Cells were disrupted in a nanojet homogeniser (Haskel, Wesel, Germany) at 1300 bar and subsequently centrifuged (32,000x g for 60 min at 4°C) to obtain the cell-free extracts (supernatant). The cell free extract was frozen in 10 ml portions and stored at -20°C until further use.

Fermentation of *E. coli* expressing the nucleic acid sequence as presented in [SEQ ID No. 5]

Escherichia coli cells expressing the nucleic acid sequence as presented in [SEQ ID. 5] encoding lysine cyclase as presented in [SEQ ID No. 6] were
 5 fermented as described in US 7,241,602, whereby feed profile used to introduce feed 1 was used as described in Table 1 of US 7,241,602.

Preparation of enzyme solution LAM0011 from cells of *E. coli*

Enzyme solution LAM0011 containing lysine cyclase as presented in
 10 [SEQ ID No. 6] from cells of *E. coli* expressing the nucleic acid sequence as presented in [SEQ ID No. 5] was prepared as described in US 7,241,602.

Biocatalytic synthesis of ACL

A substrate solution of 70 mM L-lysine.HCl and 1 mM ZnSO₄ in 100
 15 mM sodium phosphate buffer (pH 7.0, containing 1 mM ZnSO₄) was prepared. To start the reaction, 1 ml of the cell free extract of *E. coli* DH10B pMS470-33/3/1/11-1 or 1 ml of enzyme solution LAM0011 were added to 9 ml substrate solution. Reaction mixtures were incubated on a shaker at 37°C for 96 h. Furthermore, a chemical blank mixture (without cell free extract) and a biological blank (consisting of 1 ml cell free extract of *E.*
 20 *coli* DH10B pMS470-33/3/1/11-1 or 1 ml enzyme solution LAM0011 added to 9 ml 50 mM sodium phosphate buffer, pH 7.0 without L-lysine.HCl) were incubated under the same conditions. Samples were taken after 96 hours of incubation and analysed by HPLC-MS. The results are summarised in the following Table.

25 Table 1: ACL formation from L-lysine in the presence of enzyme solution LAM0011 and cell free extract of *E. coli* DH10B pMS470-33/3/1/11-1

| Biocatalyst | ACL concentration [mg/kg] |
|--|---------------------------|
| Enzyme solution LAM0011 | 4.2 |
| Cell free extract of <i>E. coli</i> DH10B pMS470-33/3/1/11-1 | 0.2 |

It is shown that the conversion of L-lysine to ACL is catalysed by each of the biocatalysts mentioned in Table 1. No ACL was detected in the chemical and
 30 biological blank samples.

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CLAIMS

1. Method for preparing α -amino- ϵ -caprolactam, comprising converting lysine to α -
5 amino- ϵ -caprolactam, wherein the conversion is catalysed by a biocatalyst.
2. Method according to claim 1, wherein the biocatalyst has lysine cyclase activity.
3. Method according to claim 1 or 2, wherein the biocatalyst comprises an enzyme
selected from the group of hydrolases (EC 3), in particular an enzyme selected
from the group of hydrolases acting on ester bonds (EC 3.1), and hydrolytic
10 enzymes acting upon carbon-nitrogen bonds, other than peptide bonds (EC
3.5).
4. Method according to claim 3 wherein the hydrolase is selected from the group
of carboxylic-ester hydrolases (EC 3.1.1), hydrolases acting on linear amides
(EC 3.5.1) and hydrolases acting on cyclic amides (EC 3.5.2).
- 15 5. Method according to claim 4 wherein the hydrolase is selected from the group
of pig liver esterase (EC 3.1.1.1), hydrolases acting on linear amides (EC
3.5.1), L-lysine-1,6-lactam hydrolases (EC 3.5.2.11), and 6-aminohexanoic-
cyclic dimer hydrolases (3.5.2.12).
- 20 6. Method according to claims 3, 4, or 5, wherein the enzyme is selected from the
group of enzymes capable of catalysing the cyclisation of lysine to α -amino- ϵ -
caprolactam from an organism or part of an organism selected from the group
of mammals, *Jaspis*, *Poechillastra*, *Periconia*, *Pachastrella*, *Myxococcus*,
Nocardia, *Streptomyces*, *Ochrobactrum*, *Rhodococcus*, *Enterobacter*, *Thermus*,
Aspergillus, *Methylophilus*, *Mycobacterium*, *Citrobacter*, *Alcaligenes*,
25 *Achromobacter*, deep sea isolate PC12/1000-B4, *Providencia*, *Tremella*,
Cryptococcus, *Candida* and *Trichosporon*.
7. Method according to claim 6, wherein said organism is selected from the group
of organism selected from the group of *Ochrobactrum*, *Rhodococcus*,
Aspergillus, *Citrobacter*, *Providencia*, *Tremella*, *Cryptococcus*, *Candida* and
30 *Trichosporon*.
8. Method according to any of the preceding claims, wherein the biocatalyst
comprises an amino acid sequence represented by SEQ ID No. 4, SEQ ID No.
6 or a homologue of any of these sequences.
9. Method according to claim 8, wherein said amino acid sequence has a

sequence identity with any of said Sequence ID's of at least 80 %, in particular of at least 90 %, more in particular of at least 95 %.

10. Method according to any of the preceding claims, wherein the method is carried out in an aqueous environment.
- 5 11. Method according to any of the preceding claims comprising the use of a peptide synthase for converting lysine to α -amino- ϵ -caprolactam.
12. Method for preparing (Z)-6,7-dihydro-1*H*-azepin-2(5*H*)-one comprising removing the α -amino group from α -amino- ϵ -caprolactam after preparing the α -amino- ϵ -caprolactam in a method according to any of the preceding claims.
- 10 13. Method for preparing ϵ -caprolactam comprising reducing the carbon-carbon double bond of (Z)-6,7-dihydro-1*H*-azepin-2(5*H*)-one after preparing the (Z)-6,7-dihydro-1*H*-azepin-2(5*H*)-one in a method according to claim 12.
14. A host cell comprising at least one recombinant vector comprising a nucleic acid sequence encoding a biocatalyst with lysine cyclase activity.
- 15 15. A host cell according to claim 14, comprising a nucleic acid sequence encoding a biocatalyst with ammonia lyase activity.
16. Host cell according to claim 14 or 15, wherein the host cell is selected from the group of genera consisting of *Aspergillus*, *Penicillium*, *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Candida*, *Hansenula*, *Bacillus*, *Corynebacterium* and
20 *Escherichia*.
17. Host cell according to any of the claims 14-16, wherein the host cell comprises a nucleic acid sequence encoding a biocatalyst comprising an amino acid sequence represented by SEQ ID No. 4, SEQ ID No. 6 or a homologue of any of these sequences.