

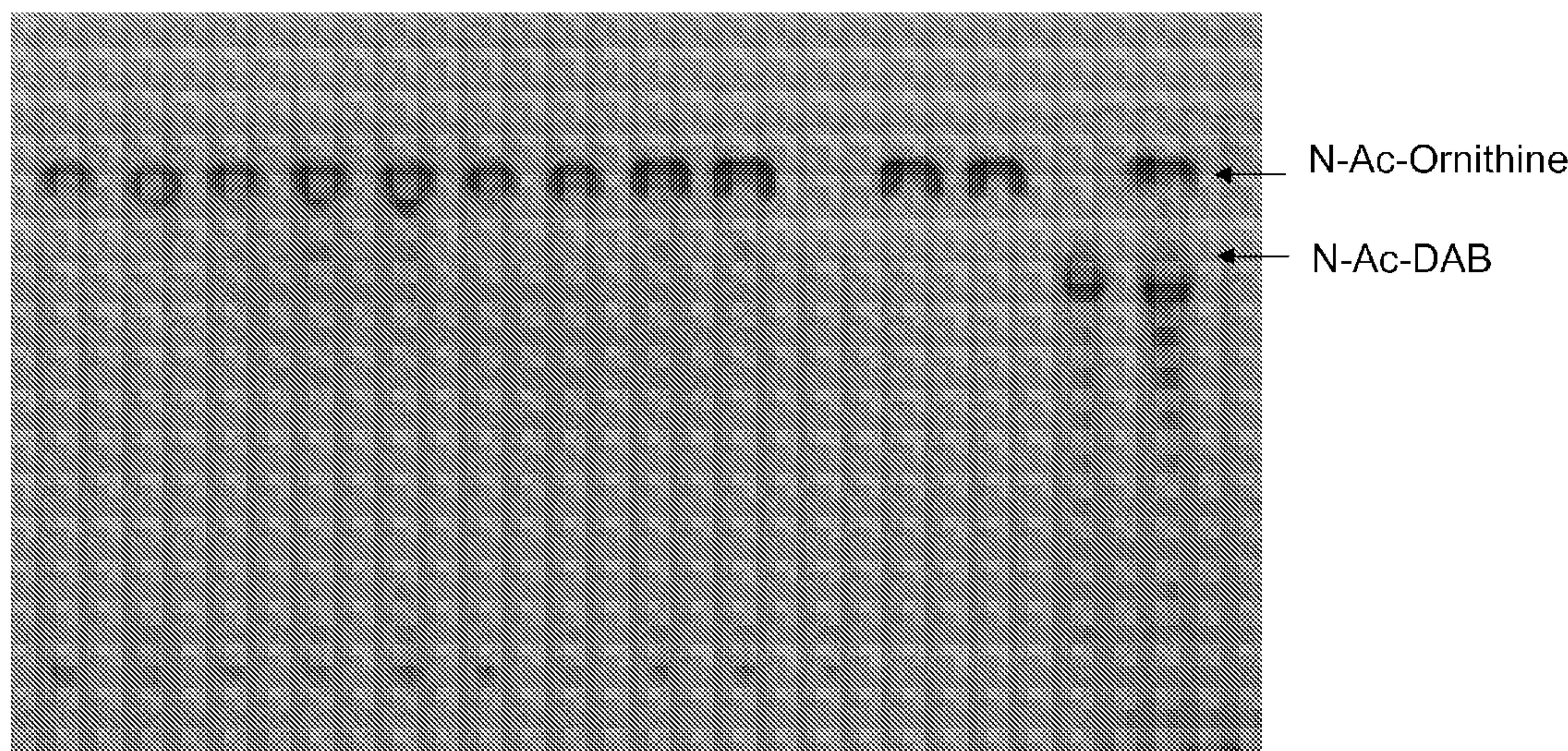


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(71) Demandeur/Applicant:
DSM IP ASSETS B.V., NL
(72) Inventeurs/Inventors:
WU, LIANG, NL;
RAEMAKERS-FRANKEN, PETRONELLA CATHARINA,
NL
(74) Agent: SMART & BIGGAR

(54) Titre : PROCEDE POUR LA PREPARATION DE 1,4-BUTANEDIAMINE PAR L'INTERMEDIAIRE DE
PRECURSEURS DE 1,4-BUTANEDIAMINE PROTEGES PAR N-ACYLE OU N-GUANIDYLE
(54) Title: PROCESS FOR THE PREPARATION OF 1,4-BUTANEDIAMINE VIA N-ACYL OR N-GUANIDYL PROTECTED
1,4-BUTANEDIAMINE PRECURSORS

FIGURE 1/1



(57) **Abrégé/Abstract:**

The present invention relates to a novel method for the preparation of 1,4-diaminobutane [DAB]. The method according to the present invention involves at least one biocatalytic step which comprises the biocatalytic production of at least one N-protected precursor of DAB. The present invention also relates to a method for the preparation of DAB involving at least one biocatalytic step, and comprising the steps of a) biocatalytically preparing an N-protected precursor of DAB yielding a - biocatalytic reaction mixture containing the N-protected precursor of DAB, b) recovering the N-protected precursor from the biocatalytic reaction mixture and c) converting the N-protected precursor into DAB. More in particular, the present invention relates to a method for the preparation of DAB, wherein the at least N-protected precursor of DAB is selected from the group consisting of N5-protected ornithine, N-protected DAB, and N-protected 4-aminobutyraldehyde.

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(71) Applicant (for all designated States except US): **DSM IP ASSETS B.V.** [NL/NL]; Het Overloon 1, NL-6411 TE Heerlen (NL).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **WU, Liang** [NL/NL]; Barenstraat 25, NL-2628 LC Delft (NL). **RAE-MAKERS-FRANKEN, Petronella, Catharina** [NL/NL]; Europalaan Zuid 124, NL-6021 KJ Budel (NL).

(74) Agent: **PLOEG VAN DER, Antonius**; P.O. Box 9, NL-6160 MA Geleen (NL).

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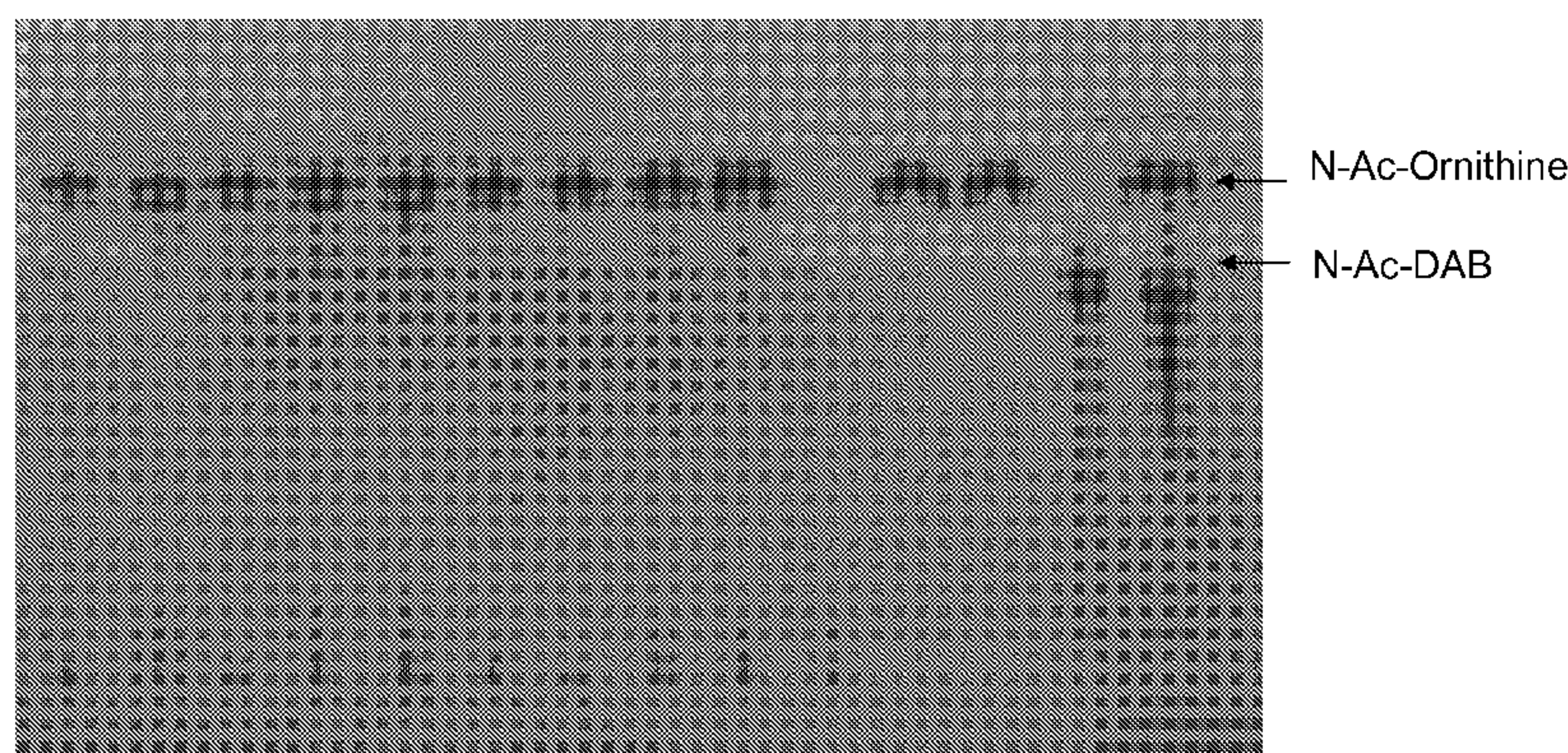
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(54) Title: PROCESS FOR THE PREPARATION OF 1,4-BUTANEDIAMINE VIA N-ACYL OR N-GUANIDYL PROTECTED 1,4-BUTANEDIAMINE PRECURSORS

FIGURE 1/1



(57) Abstract: The present invention relates to a novel method for the preparation of 1,4-diaminobutane [DAB]. The method according to the present invention involves at least one biocatalytic step which comprises the biocatalytic production of at least one N-protected precursor of DAB. The present invention also relates to a method for the preparation of DAB involving at least one biocatalytic step, and comprising the steps of a) biocatalytically preparing an N-protected precursor of DAB yielding a - biocatalytic reaction mixture containing the N-protected precursor of DAB, b) recovering the N-protected precursor from the biocatalytic reaction mixture and c) converting the N-protected precursor into DAB. More in particular, the present invention relates to a method for the preparation of DAB, wherein the at least N-protected precursor of DAB is selected from the group consisting of N5-protected ornithine, N-protected DAB, and N-protected 4-aminobutyraldehyde.

WO 2011/009859 A1

PROCESS FOR THE PREPARATION OF 1,4-BUTANEDIAMINE VIA N-ACYL OR N-GUANIDYL
PROTECTED 1,4-BUTANEDIAMINE PRECURSORS

The present invention relates to a method for the preparation of 1,4-diaminobutane [DAB] involving at least one biocatalytic step.

5 The compound DAB is an important raw material for the production of some of the major engineering plastics: polyamide-4,6, either in the form of a homopolymer, or copolymerized, for example, with about 5 wt.% of polyamide-6 monomer (caprolactam). The homopolymer polyamide-4,6 (nylon-4,6) was described as early as 1938 (US-A-2,130,948, Carothers). It is the polycondensation product of the
10 monomers DAB and adipic acid. Presently, especially compounds of polyamide-4,6 are being produced and sold by DSM in the Netherlands under the trade name STANYL®.

 For the synthesis of DAB a number of chemical routes are known. These chemical routes suffer from the disadvantage that starting materials have to be obtained from sources that are considered to be non-renewable. There exists, however,
15 a substantial need for providing new and feasible routes for the synthesis of DAB starting from renewable carbon sources and using biochemical processes (also referred to as "biotransformation").

 A method for the preparation of DAB involving at least one fermentative step has been described in PCT applications published as
20 WO2006/005603 and WO2006/00504. Both documents describe the fermentative production of DAB in a micro-organism having an increased level of an ornithine decarboxylase activity.

 The present method relates to an alternative method for the preparation of DAB. The method according to the present invention involves at least
25 one biocatalytic step which comprises the biocatalytic production of at least one N-protected precursor of DAB and subsequent *in vitro* conversion of the N-protected precursor into DAB.

 It has been found that the recovery of DAB after biocatalytic production meets with considerable difficulties. In WO2007/079944 the recovery of an
30 organic amine, such as DAB has been described. In a particular embodiment described therein, a cell-free broth containing a sulfate or phosphate salt of the amine (hence, e.g. DAB-disulfate) is concentrated, and a base, like ammonia is added. Depending on the conditions a two-layer system is formed. From the layer containing mainly the organic compounds, the desired amine can be recovered.

Detailed description of the invention

According to one embodiment the method for the preparation of DAB involves at least one biocatalytic step, and comprises the steps of (a) biocatalytically preparing an N-protected precursor of DAB yielding a biocatalytic reaction mixture
5 containing the N-protected precursor of DAB, (b) recovering the N-protected precursor from the biocatalytic reaction mixture, and (c) converting the N-protected precursor into DAB.

According to a particular embodiment the present invention for the production of DAB involves at least one biocatalytic step, which comprises the
10 biocatalytic production of at least one N-protected precursor of DAB selected from the group consisting of N⁵-protected ornithine, N-protected DAB, and N-protected 4-aminobutyraldehyde and subsequent *in vitro* conversion of the N-protected precursor into DAB.

With “*in vitro* conversion” is meant here conversion of an N-protected
15 precursor of DAB into DAB in a medium outside a cell. The *in vitro* conversion can be a conversion by at least one biocatalyst or can be a chemical conversion involving at least one chemical step, or can be a combination of at least one biocatalytic and at least one chemical step.

With an “N-protected precursor of DAB” is meant here a compound
20 containing a protected amino group and which can be converted into DAB by way of at least one chemical or biocatalytic reaction or a combination of chemical and biocatalytic reactions.

With “N⁵-protected ornithine” is meant here the ornithine molecule which has a protecting group at its N⁵ atom; with “N-protected DAB” is meant here the
25 DAB molecule which has a protecting group at one of its amino groups; and with “N-protected 4-aminobutyraldehyde” is meant here the 4-aminobutyraldehyde molecule which has a protecting group at the amino group.

The protecting groups referred to above may be selected from the group consisting of acyl species having 1-6 carbon atoms or may be a guanidyl group.
30 Such a protecting group should be selected to allow for at least one of biocatalytic production, ease of recovery of the N-protected precursor from the biocatalytic reaction mixture (e.g. fermentation broth) and subsequent biocatalytic and/or chemical reactions to ultimately produce DAB.

N-protected DAB precursors can be prepared by acylation of for
35 example 4-aminobutyraldehyde or ornithine. For examples, by acylation with acetic

acid anhydride in formic acid to introduce a formyl protecting group or by reaction of C2-C6 carboxylic acid anhydride or acyl chloride to introduce a N-acetyl, N-propionyl, N-butyryl, N-valeryl or N-caproyl protecting group, respectively.

5 N-guanidyl protected precursors are for example the proteinogenic arginine or N-guanidyl-aminobutyraldehyde or N-guanidyl-DAB. A fermentative route is described, for example, in EP1260588, which describes the biochemical production of agmatine from arginine under influence of an arginine decarboxylase. Agmatine is N-guanidyl-protected DAB. Agmatine (N-guanidyl-protected DAB) can be smoothly deprotected to DAB by acidic hydrolysis, for instance by refluxing agmatine in an
10 aqueous concentrated mineral acid solution such as concentrated hydrochloric or sulfuric acid. This gives the diacid salt of DAB and the by-products carbon dioxide and ammonia (the latter in the form of its ammonium salt of the mineral acid which is used). To obtain the DAB in its free amine form, the formed diacid salt should be isolated, redissolved and neutralized with a base.

15 According to a further particular embodiment the invention relates to a method for the preparation of DAB wherein at least one N-protected precursor of DAB is produced, which N-protected precursor is selected from the group consisting of N⁵-acetyl ornithine, N-acetyl DAB, and N-acetyl 4-aminobutyraldehyde.

According to one particular embodiment the method for the
20 preparation of DAB involving at least one biocatalytic step comprises the steps of (a) biocatalytically preparing N⁵-acetyl ornithine yielding a biocatalytic reaction mixture containing N⁵-acetyl ornithine, (b) recovering N⁵-acetyl ornithine from the biocatalytic reaction mixture, and (c) converting N⁵-acetyl ornithine into DAB.

According to one particular embodiment the method for the
25 preparation of DAB involving at least one biocatalytic step comprises the steps of (a) biocatalytically preparing N-acetyl DAB yielding a biocatalytic reaction mixture containing N-acetyl DAB, (b) recovering N-acetyl DAB from the biocatalytic reaction mixture, and (c) converting N-acetyl DAB into DAB.

According to one particular embodiment the method for the
30 preparation of DAB involving at least one biocatalytic step comprises the steps of (a) biocatalytically preparing N-acetyl 4-aminobutyraldehyde yielding a biocatalytic reaction mixture containing N-acetyl 4-aminobutyraldehyde, (b) recovering N-acetyl 4-aminobutyraldehyde from the biocatalytic reaction mixture, and (c) converting N-acetyl 4-aminobutyraldehyde into DAB.

35 When referred herein explicitly or implicitly to an amine or an

N-protected amine , *e.g.* N-protected DAB, these terms are meant to include the neutral amine group, the corresponding charged protonated amine as well as salts thereof.

5 Definitions

The term “or” as used herein is defined as “and/or” unless specified otherwise.

The term “a” or “an” as used herein is defined as “at least one” unless specified otherwise.

10 When referring to a noun (*e.g.* a compound, an additive, *etc.*) in the singular, the plural is meant to be included.

When referring 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.*, an amino acid of which enantiomers exist, the amino acid may be the
15 L-enantiomer or the D-enantiomer or a combination thereof. In case a natural stereoisomer exists, the compound is preferably a natural stereoisomer.

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 may be classified on the basis of the Enzyme Nomenclature provided by the
20 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 included.

25 The term “homologous” or “homolog” or “ortholog” refers to related sequences that have a functional relationship and is generally determined based on degree of sequence identity. These terms may describe the relationship between a gene found in one species, subspecies, variety, cultivar or strain and the corresponding or equivalent gene in another species, subspecies, variety, cultivar or strain. They may
30 also describe the relationship between a gene found in nature and an artificially constructed gene, or between two artificially constructed genes. A functional relationship may be indicated in any one of a number of ways, including, but not limited to, (a) degree of sequence identity; (b) same or similar biological function. Preferably, both (a) and (b) are indicated. The term homolog is also meant to include nucleic acid
35 sequences (polynucleotide sequences) which differ from another nucleic acid

sequence due to the degeneracy of the genetic code and encode the same polypeptide sequence.

Wherever the term “homolog” is used herein in relation to a polypeptide, this is intended to indicate a polypeptide having the same or a similar biological function and a sequence identity of a certain degree with a reference polypeptide. In particular it is intended to indicate 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 %, 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 %.

“Sequence identity” or “sequence similarity” is herein defined as a relationship between two or more polypeptide sequences or two or more nucleic acid sequences, as determined by comparing the sequences. Usually, sequence identities or similarities are compared over the whole length of the 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, as the case may be, as determined by the match between such sequences. Preferred methods to determine identity or similarity are 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 parameters for polypeptide sequence comparison using BLASTP are gap open 10.0, gap extend 0.5, Blosum 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).

With “biotransformation” or “biocatalytic reaction” is meant here a biochemical reaction wherein an enzyme is used as a catalyst. Wherever in accordance with the invention herein, it is indicated that a biocatalyst is used, at least one reaction step in the method is catalyzed by a biological material or moiety derived from a biological source, for instance an organism or a biomolecule derived there from. In particular, the biotransformation may be a fermentation step. The biocatalyst may in particular comprise one or more enzymes. The biocatalyst may be used in any form. In

a particular embodiment, one or more enzymes are used isolated from the natural environment (isolated from the organism it has been produced in), for instance as a solution, an emulsion, a dispersion, (a suspension of) freeze-dried cells, as a lysate, or immobilized 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.

With "biocatalytic reaction mixture" is meant here the environment in which the biocatalytic reaction takes place. This may be a cellular environment (for intracellular or extracellular biocatalytic reactions) or a cell-free environment.

With "fermentative step" is meant here a process step wherein the formation or conversion of a particular chemical entity takes place in a unicellular host, more in particular in a micro-organism in a cell culture. "Fermentatively preparing" means here producing a particular chemical entity in a micro-organism comprising a biocatalyst in a cell culture with a fermentable carbon source, wherein the carbon source contains any of said compounds which are to be converted into the particular chemical entity to be prepared or wherein the cells prepare the compound to be converted into the particular chemical entity to be prepared from the carbon source. The micro-organism may be a natural producer of the particular chemical entity or it may have obtained the capability to produce the particular chemical entity by transformation with a gene encoding at least one suitable enzyme using recombinant DNA techniques. The natural producer of the particular chemical entity may also be transformed with a gene encoding at least one suitable enzyme using recombinant DNA technology in order to increase the production of the desired particular chemical entity and/or to diminish the production of components which could interfere with the productivity of the desired particular chemical entity or which would interfere with the further steps in the process according to the present invention.

Preferred micro-organisms for fermentatively preparing the N-protected precursor of DAB may be of eukaryotic or prokaryotic origin. In particular it may be selected from animal (including human) cells, plant cells, bacteria, archaea, yeasts and fungi. More in particular the micro-organism may be selected from the group consisting of bacteria, such as *Bacillus* (in particular *B. subtilis*), *Brevibacterium* (in particular *B. ketoglutamicum*), corynebacteria (in particular *C. glutamicum*), *Escherichia* (in particular *E. coli*), *Klebsiella* (in particular *K. pneumoniae*), lactobacilli (in particular *L. lactis*), propionibacterium, pseudomonas (in particular *P. putida*),

Rodococcus (in particular *R. erythropolis*, *Streptomyces* (in particular *S. coelicor* and *S. clavuligerus*), yeasts such as *Kluyveromyces* (in particular *K. lactis*), *Penicillium* (in particular *P. chrysogenum*), *Saccharomyces* (in particular *S. cerevisiae*), *Aspergillus* (in particular *A. niger*), *Pichia* (in particular *P. pastoris*), *Hansenula*, *Schizosaccharomyces* (in particular *S. pombe*), *Yarrowia* (in particular *Y. lipolytica*), fungi, such as *Talaromyces*.

In the most preferred embodiment, the fermentative production of the N-protected precursor is performed in a micro-organism wherein the N-protected precursor is being formed *in vivo*. Preferably, the formation of the N-protected precursor according to the present invention is a biotransformation into the N-protected precursor from any suitable carbon source.

The carbon source for the fermentation process 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 polyols include glycerol and carbohydrates. Suitable fatty acids or glycerides may in particular be provided in the form of 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 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.

Also, as a carbon source may be used amino acids or derivatives thereof, glutamate or derivatives thereof and/or ornithine or derivatives thereof.

As nitrogen source may be used inorganic nitrogen-containing compounds, such as ammonia, ammonia salts, ureum, nitrate and nitrite, or organic nitrogen-containing compounds, such as amino acids or derivatives thereof, more in particular glutamate or derivatives thereof and/or ornithine or derivatives thereof.

When reference is made here to a biocatalyst it may refer to an organism expressing at least one enzyme relevant for the biocatalytic function, or it may refer to at least one enzyme obtained or derived from an organism. The organism may be eukaryotic or prokaryotic. In particular the organism may be selected from animals (including humans), plants, bacteria, archaea, yeasts and fungi.

In one embodiment the biocatalyst originates from an animal, in particular from a part thereof – e.g. liver, pancreas, brain, kidney, heart or other organ. The animal may in particular be selected from the group of mammals, more in particular selected from the group of primates (like *Homo sapiens*), *Leporidae*,
 5 *Muridae*, *Suidae* and *Bovidae*.

Suitable plants as origin of the biocatalyst are, in particular, plants selected from the group of *Asplenium*; *Cucurbitaceae*, in particular *Cucurbita*, e.g. *Cucurbita moschata* (squash), or *Cucumis*; *Mercurialis*, e.g. *Mercurialis perennis*; *Hydnocarpus*; and *Ceratonia*.

10 Suitable bacteria as origin of the biocatalyst may in particular be selected amongst the group of *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Clostridium*, *Corynebacterium*, *Deinococcus*, *Enterobacter*, *Enterococcus*, *Erwinia*, *Escherichia*, *Geobacillus*, *Klebsiella*, *Lactobacillus*, *Lactococcus*, *Legionella*, *Mycobacterium*, *Neisseria*, *Nitrosomonas*, *Novosphingobium*,
 15 *Paracoccus*, *Proteus*, *Pseudomonas*, *Ralstonia*, *Rhodobacter*, *Rhodopseudomonas*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Thermus*, *Vibrio* and *Zymomonas*.

Suitable archaea as origin of the biocatalyst may in particular be selected amongst the group of *Aeropyrum*, *Archaeoglobus*, *Halobacterium*,
 20 *Methanobacterium*, *Methanobrevibacter*, *Methanocaldococcus*, *Methanococcus*, *Methanopyrus*, *Methanosarcina*, *Methanosphaera*, *Pyrobaculum* and *Thermoplasma*.

Suitable fungi as origin of the biocatalyst may in particular be selected amongst the group of *Aspergillus*, *Neurospora*, *Penicillium*, *Rhizopus* and *Trichoderma*.

A suitable yeast as origin of the biocatalyst may in particular be
 25 selected amongst the group of *Candida*, *Cytophagia*, *Hansenula*, *Humicola*, *Kluyveromyces*, *Mucor*, *Rhizoctonia*, *Saccharomyces* and *Yarrowia*.

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
 30 naturally occurring biocatalyst may be improved by 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
 35 in the art (random mutagenesis, site-directed 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
5 sequences of two or more parent enzymes or by effecting the 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 optimization or codon pair optimization, *e.g.* based on a method as described in WO 2008/000632.

A mutant biocatalyst may have improved properties, for instance with
10 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 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
15 the art.

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

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.

25 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 catalyzing a method of the invention, the pH is selected such that the micro-organism is
30 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
35 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
5 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.

The incubation conditions can be chosen within wide limits as long as the biocatalyst shows sufficient activity and/ or growth. This includes aerobic, micro-aerobic, oxygen limited and anaerobic conditions.

10 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.

15 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
20 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 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 than 50 mmol/l.h,
25 less than 20 mmol/l.h, or less than 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
30 density.

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 maximum
35 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.

5 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 %, or more than 90 wt. %), in case an enzyme is used that retains sufficient activity in such a medium.

10 In a preferred embodiment the present invention relates to a biocatalytic process whereby as an N-protected precursor of DAB N⁵-protected ornithine is produced. For example, the preparation of N⁵-acetyl ornithine may comprise one or more of the following enzyme-catalyzed reactions:

- 1) glutamate to N-acetyl-glutamate
- 2) N-acetyl-glutamate to N-acetyl-glutamate 5-phosphate
- 15 3) N-acetyl-glutamate 5-phosphate to N-acetyl-glutamate semialdehyde
- 4) N-acetyl-glutamate semialdehyde to N²-acetyl-ornithine
- 5) N²-acetyl-ornithine to N⁵-acetyl-ornithine

Reaction 1) may be catalyzed by an enzyme selected from the group of acyltransferases (EC 2.3.1), preferably from the group of amino-acid N-acetyltransferases (EC 2.3.1.1). Preferably, the enzyme is specific for acetyl-CoA as the acetyl-group donor and glutamate as the acetyl-group acceptor. An amino-acid N-acetyltransferase may originate from prokaryotes or eukaryotes. Exemplary proteins that can catalyze reaction 1) is given in Table 1 with their accession number in the Uniprot database and their source (micro)organisms.

25 Reaction 2) may be catalyzed by an enzyme selected from the group of acetyl-glutamate kinases (EC 2.7.2.8). The enzyme may use ATP as a cofactor. An acetyl-glutamate kinase may originate from prokaryotes or eukaryotes. Exemplary proteins that can catalyze reaction 2) is given in Table 1 with their accession number in Uniprot and their source (micro)organisms.

30 Reaction 3) may be catalyzed by an enzyme selected from the group of oxidoreductases (EC 1.2.1), preferably from the group of N-acetyl-gamma-glutamyl-phosphate reductases (EC 1.2.1.38). The enzyme may use NADH or NADPH as cofactor. An N-acetyl-gamma-glutamyl-phosphate reductase may originate from prokaryotes or eukaryotes. Exemplary proteins that can catalyze reaction 3) is given in
35 Table 1 with their accession number in Uniprot and their source (micro)organisms.

Reaction 4) may be catalyzed by an enzyme selected from the group of transaminases (EC 2.6.1), preferably from the group of acetylornithine transaminases (EC 2.6.1.11). The enzyme may use glutamate as amino-group donor. An acetylornithine transaminase may originate from prokaryotes or eukaryotes.

- 5 Exemplary proteins that can catalyze reaction 4) is given in Table 1 with their accession number in Uniprot and their source (micro)organisms.

Reaction 5) may be catalysed by an N-acyltransferase such as glutamate N-acetyltransferase (EC 2.3.1.35).

- 10 Glutamate may be derived from a suitable carbon source via glutamate biosynthesis reactions well known in the art. Preferably, microorganisms accumulating high amount of glutamic acid are used, for example, *Corynebacterium glutamicum*. Methods to improve glutamic acid production, for example by genetic engineering are well known in the art (Kimura E., Adv Biochem Eng Biotechnol. 2003; 79: 37-57).

- 15 Alternatively, the preparation of N⁵-acetyl ornithine may comprise one or more of the following enzyme-catalyzed reactions:

- 6) glutamate to N-acetyl-glutamate
- 7) N-acetyl-glutamate plus ornithine to N²-acetyl-ornithine
- 8) N²-acetyl-ornithine to N⁵-acetyl-ornithine

Table 1: Enzymes for reaction steps 1 – 8

Reaction step	UniProt accession number	Enzyme	Microorganism
1/6	P0A6C5	Amino-acid acetyltransferase	<i>Escherichia coli</i>
1/6	P40360	Amino-acid acetyltransferase, mitochondrial	<i>Saccharomyces cerevisiae</i>
2	Q01217	Protein ARG5,6, mitochondrial	<i>Saccharomyces cerevisiae</i>
2	P0A6C8	Acetylglutamate kinase	<i>Escherichia coli</i>
3	Q01217	Protein ARG5,6, mitochondrial	<i>Saccharomyces cerevisiae</i>
3	Q8ZKL8	N-acetyl-gamma-glutamyl-phosphate reductase	<i>Salmonella typhimurium</i>
4	P18335	Acetylornithine/succinyl-diaminopimelate aminotransferase	<i>Escherichia coli</i>
4	P18544	Acetylornithine aminotransferase	<i>Saccharomyces cerevisiae</i>
5/7/8	Q04728	Arginine biosynthesis bifunctional protein ARG7, mitochondrial	<i>Saccharomyces cerevisiae</i>
5/7/8	Q9HW04	Glutamate N-acetyltransferase	<i>Pseudomonas aeruginosa</i>
5/7/8	Q59280	Glutamate N-acetyltransferase	<i>Corynebacterium glutamicum</i>

Reaction 6) is identical to reaction 1) and can be catalyzed by the same type of enzymes.

- 5 Reaction 7) may be catalyzed by an enzyme selected from the group of acyltransferases (EC 2.3.1), preferably glutamate N-acetyltransferases (EC 2.3.1.35). Preferably, the enzyme uses ornithine as acetyl-group acceptor, thereby generating glutamate and N-acetyl-ornithine as reaction product. Glutamate N-acetyltransferases might have hydrolytic activity towards N-acetyl-glutamate,
- 10 generating glutamate and acetate as hydrolysis products. Preferably, the enzyme used has no detectable hydrolytic activity; alternatively, a wild-type enzyme may be adapted

such that the hydrolytic activity is substantially lower as compared to the wild type enzyme. A glutamate N-acetyltransferase may originate from prokaryotes or eukaryotes. Exemplary proteins that can catalyze reaction 7) is given in Table 1 with their accession number in Uniprot and their source (micro)organisms.

5 Reaction 8) is identical to reaction 5 and may be catalysed by the same enzyme.

In a further preferred embodiment the present invention relates to a biocatalytic process whereby N-protected DAB is produced from N⁵-protected ornithine. In general, a suitable decarboxylase has N⁵-protected ornithine decarboxylase activity,
10 capable of catalysing the conversion of N⁵-protected ornithine into N-protected DAB.

An enzyme capable of decarboxylating N⁵-protected ornithine may in particular be selected from the group of decarboxylases (E.C. 4.1.1), preferably from the group of ornithine decarboxylases (EC 4.1.1.17), diaminopimelate decarboxylases (EC 4.1.1.20), branched chain alpha-keto acid decarboxylases (EC 4.1.1.72), alpha-
15 ketoisovalerate decarboxylases, alpha-ketoglutarate decarboxylases (EC 4.1.1.71).

One or more other suitable decarboxylases may be selected amongst the group of oxalate decarboxylases (EC 4.1.1.2), acetoacetate decarboxylases (EC 4.1.1.4), valine decarboxylases/leucine decarboxylases (EC 4.1.1.14), aspartate 1-decarboxylases (EC 4.1.1.11), 3-hydroxyglutamate decarboxylases (EC 4.1.1.16),
20 lysine decarboxylases (EC 4.1.1.18), arginine decarboxylases (EC 4.1.1.19), 2-oxoglutarate decarboxylases (EC 4.1.1.71), and diaminobutyrate decarboxylases (EC 4.1.1.86).

A decarboxylase may in particular be a decarboxylase of an organism selected from the group of squashes; cucumbers; yeasts; fungi, *e.g. Candida flareri*,
25 *Hansenula sp.*, *Kluyveromyces marxianus*, *Neurospora crassa*, *Rhizopus javanicus*, and *Saccharomyces cerevisiae*; mammals, in particular from mammalian brain; and bacteria, such as *Bacillus cadaveris*, *Escherichia coli*, *Lactococcus lactis*, *Mycobacterium tuberculosis*, *Pseudomonas sp.* and *Zymomonas mobilis*.

In a further preferred embodiment the invention relates to a
30 biocatalytic process whereby N-protected DAB is produced through N-protected 4-aminobutyraldehyde. For example, the preparation of N-acetyl-DAB may comprise one or more of the following enzyme-catalyzed reactions:

- 9) glutamate to 4-aminobutyrate
- 10) 4-aminobutyrate to N-acetyl-4-aminobutyrate
- 35 11) N-acetyl-4-aminobutyrate to N-acetyl-4-aminobutyraldehyde

12) N-acetyl-4-aminobutyraldehyde to N-acetyl-DAB

Reaction 9) may be catalyzed by an enzyme selected from the group of decarboxylases (EC 4.1.1), preferably from the group of glutamate decarboxylases (EC 4.1.1.15). A glutamate decarboxylase may originate from prokaryotes or eukaryotes or archaea.

Reaction 10) may be catalyzed by an enzyme selected from the group of acyltransferases (EC 2.3.1), preferably from the group of amino-acid N-acetyltransferases (EC 2.3.1.1), glycine N-acyltransferases (EC 2.3.1.13), aspartate N-acetyltransferases (EC 2.3.1.17), glutamate N-acetyltransferases (EC 2.3.1.35), D-amino-acid N-acetyltransferases (EC 2.3.1.36) and diamine N-acetyltransferases (EC 2.3.1.57). Preferably, the enzyme used is selective towards the substrate 4-aminobutyrate. The wild-type enzyme might have low activity/selectivity towards 4-aminobutyrate as amino-group acceptor. Such wild-type enzymes may be adapted such that the activity / selectivity towards 4-aminobutyrate is substantially higher as compared to the wild type enzyme. The enzyme used may use acetyl-CoA as acetyl-group donor. Alternatively, the enzyme may also use an N-acetylated amino acid as acetyl-group donor, such as N-acetyl-glutamate. The enzyme may originate from prokaryotes or eukaryotes or archaea.

Alternatively, N-acetyl-4-aminobutyrate can be converted to N-acetyl-4-aminobutyraldehyde by the following enzyme-catalyzed reactions:

11a) N-acetyl-4-aminobutyrate to N-acetyl-4-aminobutyrate phosphate

11b) N-acetyl-4-aminobutyrate phosphate to N-acetyl-4-aminobutyraldehyde

Reaction 11a) may be catalyzed by an enzyme selected from the group of phosphotransferases (EC 2.7.2), preferably from the group of acetate kinases (EC 2.7.2.1), aspartate kinases (EC 2.7.2.4), butyrate kinases (EC 2.7.2.7), acetylglutamate kinases (2.7.2.8) and glutamate 5-kinases (2.7.2.11).

Reaction 11b) may be catalyzed by an enzyme selected from the group of oxidoreductases (EC 1.2.1), preferably from the group of N-acetyl-gamma-glutamyl-phosphate reductases (EC 1.2.1.38).

Exemplary proteins that can catalyze reaction steps 9) through 11) is given in Table 2 with their accession number in Uniprot and their source (micro)organisms.

Table 2: Enzymes for reaction steps 9 – 11(a/b)

Reaction step	Uniprot accession	Enzyme	Microorganism
9	P69908	Glutamate decarboxylase	<i>E. coli</i>
9	Q04792	Glutamate decarboxylase	<i>S. cerevisiae</i>
10	P0A951	Diamine acetyltransferase	<i>E. coli</i>
10	P21673	Diamine acetyltransferase	<i>H. sapiens</i>
10	P41929	Lysine acetyltransferase	<i>Yarrowia lipolytica</i>
11	P77674	gamma-aminobutyraldehyde dehydrogenase	<i>E. coli</i>
11a	P0A6C8	acetylglutamate kinase	<i>E. coli</i>
11b	P11446	N-acetyl-gamma-glutamyl-phosphate reductase	<i>E. coli</i>
11b	Q01217	N-acetyl-gamma-glutamyl-phosphate reductase	<i>S. cerevisiae</i>

Reaction 12) relates to a biocatalytic process whereby N-protected DAB is produced from N-protected 4-aminobutyraldehyde.

In general, a suitable aminotransferase has N-protected 4-aminobutyraldehyde aminotransferase activity, capable of catalysing the conversion of N-protected 4-aminobutyraldehyde to N-protected DAB.

The aminotransferase may in particular be selected amongst the group of aspartate aminotransferases, omega-aminotransferase (EC 2.6.1), classIII-aminotransferase (EC 2.6.1), 4-amino-butyrate aminotransferases (EC 2.6.1.19), L-lysine 6-aminotransferase (EC 2.6.1.36), 5-aminovalerate aminotransferases (EC 2.6.1.48), lysine:pyruvate 6-aminotransferases (EC 2.6.1.71) and putrescine-aminotransferase (EC 2.6.1.82).

In an embodiment an aminotransferase may be selected amongst the group of alanine aminotransferases (EC 2.6.1.2), leucine aminotransferases (EC 2.6.1.6), alanine-oxo-acid aminotransferases (EC 2.6.1.12), β -alanine-pyruvate aminotransferases (EC 2.6.1.18), (S)-3-amino-2-methylpropionate aminotransferases (EC 2.6.1.22), L,L-diaminopimelate aminotransferase (EC 2.6.1.83).

The aminotransferase may in particular be selected amongst aminotransferases from a mammal, plant or micro-organism. More in particular, the aminotransferase may be derived from *Asplenium*, more in particular *Asplenium unilaterale* or *Asplenium septentrionale*, *Bacillus*, in particular *Bacillus*

weihenstephanensis, *Bacillus cereus* and *Bacillus subtilis*, *Ceratonia*, more in particular *Ceratonia siliqua*, *Enterobacter*, *Erwinia*, more in particular *E. carotovora*, *Escherichia*, more in particular *E. coli*, *Legionella*, *Mercurialis*, in particular *Mercurialis perennis*, more in particular shoots of *Mercurialis perennis*, *Neisseria*, *Nitrosomonas*,

5 *Pseudomonas*, in particular *Pseudomonas aeruginosa*, *Rhodobacter*, in particular *Rhodobacter sphaeroides*, *Rhodopseudomonas*, *Salmonella*, more in particular *S. typhi*, *S. paratyphi*, *Shigella*, more in particular *Sh. boydii*, *Sh. flexneri*, *S. sonnei*, *Staphylococcus*, in particular *Staphylococcus aureus*, *Vibrio*, in particular *Vibrio fluvialis*, or yeast, in particular *Saccharomyces cerevisiae*.

10 In case the enzyme is of a mammal, it may in particular originate from mammalian kidney, from mammalian liver, from mammalian heart or from mammalian brain. For instance a suitable enzyme may be selected amongst the group of 4-amino-butyrate aminotransferase from mammalian liver, in particular 4-amino-butyrate aminotransferase from pig liver; 4-amino-butyrate aminotransferase from mammalian
15 brain, in particular 4-aminobutyrate aminotransferase from human, pig, or rat brain; omega-aminotransferase of *Vibrio fluvialis*, 4-amino-butyrate aminotransferase from *E. coli*, and 5-aminovalerate aminotransferase from *Clostridium* in particular from *Clostridium aminovalericum*.

In particular, the amino donor can be selected from the group of
20 ammonia, ammonium ions, amines and amino acids. Suitable amines are primary amines and secondary amines. The amino acid may have a D- or L-configuration. Examples of amino donors are alanine, glutamate, isopropylamine, 2-aminobutane, 2-aminoheptane, phenylmethanamine, 1-phenyl-1-aminoethane, glutamine, tyrosine, phenylalanine, aspartate, alpha-aminoisobutyrate, beta-alanine, 4-aminobutyrate, and
25 alpha-aminoadipate.

In a further preferred embodiment, the method for preparing N-protected DAB comprises a biocatalytic reaction in the presence of an enzyme capable of catalysing a reductive amination reaction in the presence of an ammonia source, selected from the group of oxidoreductases acting on the CH-NH₂ group of donors (EC
30 1.4), in particular from the group of amino acid dehydrogenases (E.C. 1.4.1). In general, a suitable amino acid dehydrogenase has 6-aminocaproic acid 6-dehydrogenase activity, catalysing the conversion of N-protected 4-aminobutyraldehyde to N-protected DAB. In particular a suitable amino acid dehydrogenase be selected amongst the group of diaminopimelate dehydrogenases
35 (EC 1.4.1.16), lysine 6-dehydrogenases (EC 1.4.1.18), glutamate dehydrogenases (EC

1.4.1.3; EC 1.4.1.4), and leucine dehydrogenases (EC 1.4.1.9).

In an embodiment, an amino acid dehydrogenase may be selected amongst an amino acid dehydrogenases classified as glutamate dehydrogenases acting with NAD or NADP as acceptor (EC 1.4.1.3), glutamate dehydrogenases acting with NADP as acceptor (EC 1.4.1.4), leucine dehydrogenases (EC 1.4.1.9),
 5 diaminopimelate dehydrogenases (EC 1.4.1.16), and lysine 6-dehydrogenases (EC 1.4.1.18).

An amino acid dehydrogenase may in particular originate from an organism selected from the group of *Corynebacterium*, in particular *Corynebacterium glutamicum*; *Proteus*, in particular *Proteus vulgaris*; *Agrobacterium*, in particular
 10 *Agrobacterium tumefaciens*; *Geobacillus*, in particular *Geobacillus stearothermophilus*; *Acinetobacter*, in particular *Acinetobacter* sp. ADP1; *Ralstonia*, in particular *Ralstonia solanacearum*; *Salmonella*, in particular *Salmonella typhimurium*; *Saccharomyces*, in particular *Saccharomyces cerevisiae*; *Brevibacterium*, in particular *Brevibacterium flavum*; and *Bacillus*, in particular *Bacillus sphaericus*, *Bacillus cereus* or *Bacillus subtilis*. For instance a suitable amino acid dehydrogenase may be selected amongst diaminopimelate dehydrogenases from *Bacillus*, in particular *Bacillus sphaericus*; diaminopimelate dehydrogenases from *Brevibacterium* sp.; diaminopimelate dehydrogenases from *Corynebacterium*, in particular diaminopimelate dehydrogenases
 20 from *Corynebacterium glutamicum*; diaminopimelate dehydrogenases from *Proteus*, in particular diaminopimelate dehydrogenase from *Proteus vulgaris*; lysine 6-dehydrogenases from *Agrobacterium*, in particular *Agrobacterium tumefaciens*, lysine 6-dehydrogenases from *Geobacillus*, in particular from *Geobacillus stearothermophilus*; glutamate dehydrogenases acting with NADH or NADPH as cofactor (EC 1.4.1.3) from
 25 *Acinetobacter*, in particular glutamate dehydrogenases from *Acinetobacter* sp. ADP1; glutamate dehydrogenases (EC 1.4.1.3) from *Ralstonia*, in particular glutamate dehydrogenases from *Ralstonia solanacearum*; glutamate dehydrogenases acting with NADPH as cofactor (EC 1.4.1.4) from *Salmonella*, in particular glutamate dehydrogenases from *Salmonella typhimurium*; glutamate dehydrogenases (EC
 30 1.4.1.4) from *Saccharomyces*, in particular glutamate dehydrogenases from *Saccharomyces cerevisiae*; glutamate dehydrogenases (EC 1.4.1.4) from *Brevibacterium*, in particular glutamate dehydrogenases from *Brevibacterium flavum*; and leucine dehydrogenases from *Bacillus*, in particular leucine dehydrogenases from *Bacillus cereus* or *Bacillus subtilis*.

35 The biocatalytic enzyme may be used in any form. For example, the

biocatalytic enzyme may be used - for example in the form of a dispersion, emulsion, a solution or in immobilized form (for instance loaded on a support, e.g. a particulate or monolithic carrier material) - as crude enzyme, as a commercially available enzyme, as an enzyme further purified from a commercially available preparation, as an enzyme
5 obtained from its source by a combination of known purification methods, in whole (optionally permeabilised and/or immobilised) cells that naturally or through genetic modification possess hydrolytic activity, or in a lysate of cells with such activity.

The biocatalytic enzyme may be obtained or derived from any organism, in particular from an animal, plant, bacterium, a mould, a yeast or fungus.

10 It will be clear to the average person skilled in the art that use can also be made of mutants of naturally occurring (wild type) enzymes with biocatalytic activity in the process according to the invention. Mutants of wild-type enzymes can for example be made by modifying the DNA encoding the wild-type enzymes using mutagenesis techniques known to the person skilled in the art (random mutagenesis,
15 site-directed mutagenesis, directed evolution, gene shuffling, etc.) so that the DNA encodes an enzyme that differs by at least one amino acid from the wild-type enzyme or so that it encodes an enzyme that is shorter compared to the wild-type and by effecting the expression of the thus modified DNA in a suitable (host) cell. Mutants of the biocatalytic enzyme may have improved properties, for instance with respect to one
20 or more of the following aspects: selectivity towards the substrate, activity, stability, solvent resistance, pH profile, temperature profile, substrate profile.

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

A cell, in particular a recombinant cell, comprising one or more enzymes for catalysing one or several reaction step in a method of the invention can be constructed using molecular biological techniques, which are known in the art *per se* (Maniatis et al. 1982 "Molecular cloning: a laboratory manual". Cold Spring Harbor
30 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 Press; F. Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York 1987). For
35 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 (such as a recombinant vector) which comprises one or more genes encoding one or more of said biocatalysts. One or more vectors may be used, each 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 an biocatalyst.

As used herein, the term "operably linked" refers to a linkage of 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.

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 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 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.

The promoter that could be used to achieve the expression of the nucleic acid sequences coding for an enzyme for use in a method of the invention, in particular an aminotransferase, an amino acid dehydrogenase or a decarboxylase, such as described herein above may be native to the nucleic acid sequence coding for the enzyme to be expressed, or may be heterologous to the nucleic acid 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 for

the enzyme of interest) is used, the heterologous promoter is preferably capable of 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 well known to the person skilled in the art.

A "strong constitutive promoter" is one 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 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 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*, *xlnC*, *xlnD*, cellulase promoters such as *eglA*, *eglB*, *cbhA*, promoters of transcriptional regulators such as *areA*, *creA*, *xlnR*, *pacC*, *prfT*, or another promoter, 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 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, 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 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.

5 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 host cell comprising one or more enzymes capable of catalysing at least one reaction step in a method of the invention.

10 In a specific embodiment, a host cell according to the invention is a recombinant cell comprising a nucleic acid sequence encoding an enzyme capable of catalysing a transamination reaction or a reductive amination reaction to form N²-protected ornithine from N-protected glutamate semialdehyde, or encoding an enzyme capable of catalyzing the N-acyltransferase reaction to form N⁵-protected ornithine from N²-protected ornithine, or encoding an enzyme capable of catalyzing the
15 aminotransferase reaction to form N-protected DAB from N-protected 4-aminobutyraldehyde. Said sequence may be part of a vector or may have been inserted into the chromosomal DNA.

Recovery of N-protected precursor of DAB

20 Prior to conversion of the N-protected precursor into DAB, the N-protected precursor is to be recovered from the biocatalytic reaction mixture.

The recovery of the N-protected precursor from the biocatalytic reaction mixture can be carried out by methods known in the art for the recovery of similar chemical entities from a biocatalytic reaction mixture. In particular for a
25 fermentative production process such a recovery process may comprise at least one step selected from the group consisting of cell separation (filtration, membrane separation (MF), sedimentation (as gravity and centrifugal), crystallization, to remove the cells. A further concentration and purification of N-protected precursor may be required for economical favorable purification. For further concentration, techniques as
30 evaporation and membrane separation (RO, NF and UF) can be applied. Also techniques as (eutectic) freeze concentration can be used.

Further isolation may be necessary either by ion-exchange (chromatography) or by crystallization/precipitation

35 This process should not necessarily result in meticulous purification of the N-protected precursor, but the N-protected precursor should be purified to at least

such an extent that subsequent conversion of the N-protected precursor into DAB will not substantially be hampered by contaminants and side products originating from the biocatalytic reaction mixture. Optionally, the N-protected precursor also may be concentrated.

5 Furthermore, the N-protected precursor may be transferred to a medium which is optimized for the at least one subsequent transformation step.

Conversion of N-protected precursor of DAB to DAB

The direct or indirect conversion of the N-protected precursor into DAB according to present invention may involve at least one biocatalytic (in particular enzymatic) or chemical conversion step. It may also involve a combination of
10 biocatalytic and chemical conversion steps.

For example, the conversion of biocatalytically produced N-protected DAB into unprotected DAB may be carried out by a biocatalytic or a chemical hydrolytic process. For the biocatalytic process use can be made of a suitable hydrolase. In an advantageous method of the invention, the deacylation is biocatalysed. In particular
15 use may be made of a hydrolytic enzyme capable of catalysing the deacylation of N-Ac-DAB, more in particular capable of catalysing the deacetylation of N-acetyl-DAB.

When N-acetyl-DAB is converted to DAB by either chemical or biocatalytic hydrolysis, this generally leads to the formation of both DAB and acetate.
20 After recovery of DAB, the acetate containing part is preferably reused in the process. In case of a fermentative process the acetate may be reused as a carbon source to grow the microorganism, or, as a carbon source to produce the N-protected-DAB or a compound which can be converted into N-protected DAB in the fermentation process.

The term 'hydrolytic enzyme' is used herein for enzymes from the classification group E.C. 3. Preferably, one or more hydrolytic enzymes are used
25 selected from the group of carboxylic ester hydrolases (E.C. 3.1.1), thiolester hydrolases (E.C. 3.1.2) and peptidases (E.C. 3.4).

In particular a peptidase (E.C. 3.4) may be used. Preferred peptidases are peptidases selected from the group of serine-type carboxypeptidases
30 (E.C. 3.4.16), metallocarboxypeptidases (E.C. 3.4.17), cysteine-type carboxypeptidases (E.C. 3.4.18), serine endopeptidases (E.C. 3.4.21), cysteine endopeptidases (E.C. 3.4.22), aspartic endopeptidases (E.C. 3.4.23) and metalloendopeptidases (E.C. 3.4.24), in particular from serine endopeptidases (E.C. 3.4.21). In particular good results have been achieved with a serine endopeptidase,
35 preferably subtilisin (E.C. 3.4.21.62), such as subtilisin Carlsberg.

Examples of organisms from which the hydrolytic enzyme may be derived include *Trichoderma* sp, such as from *Trichoderma reesei*; *Rhizopus* sp., such as from *Rhizopus oryzae*; *Bacillus* sp, such as from *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus clausii*, *Bacillus lentus*, *Bacillus alkalophilus*, *Bacillus halodurans* ; *Aspergillus* sp., such as from *Aspergillus oryzae* or *Aspergillus niger*; *Streptomyces* sp., such as from *caespitosus Streptomyces* or *Streptomyces griseus*; *Candida* sp.; fungi; *Humicola* sp; *Rhizoctonia* sp.; *Cytophagia*; *Mucor* sp.; and animal tissue, in particular from pancreas, such as from porcine pancreas, bovine pancreas or sheep pancreas.

As indicated above, a preferred enzyme is subtilisin. Various subtilisins are known in the art, see e.g. US 5,316,935 and the references cited therein. Subtilisin A is a commercially available subtilisin from Novozymes. Particularly preferred is subtilisin Carlsberg. Alcalase[®] has been found to be particularly suitable for use in a method of the invention. This product is available from Novozymes (Bagsvaerd, Denmark). Alcalase[®] is a cheap and industrially available proteolytic enzyme mixture produced by *Bacillus licheniformis* (containing subtilisin Carlsberg as a major enzyme component). Experiments with purified subtilisin confirmed that subtilisin catalyses transesterification, activation and peptidic bond formation.

Novozymes (Bagsvaerd, Denmark) offers ovozyme, liquanase, Alcalase[®], Alcalase-ultra[®] (in particular effective at alkaline pH), duramyl, esperase, kannase, savinase, savinase ultra, termamyl, termamyl ultra, novobate, polarzyme, neutrase, novoline, pyrase, novocor (bacterial alkaline proteases).

Proteinase-K is available from New England Biolabs, Ipswich (MA), USA).

Novo Nordisk Biochem North America Inc (Franklinton NC, USA) offers Protease *Bacillus species* (Esperase 6.0 T; Savinase 6.0 T), Protease *Bacillus subtilis* (Neutrase 1.5 MG), Protease *Bacillus licheniformis* (Alcalase 3.0 T).

Amano International Enzyme Co (Troy, Va, USA) offers Protease *Bacillus subtilis* (Proleather; Protease N) and Protease *Aspergillus oryzae* (Prozyme 6).

Suitable examples of this class of enzymes are e.g. *Rhizopus japonicus* lipase, lipase AP-6 of *Aspergillus niger*, lipase QL of *Alcaligenes* sp, protease B of *Bacillus amyloliquefaciens* (SEQ ID NO 19), Delvolase of *Bacillus licheniformis* (SEQ ID NO 20), *Rhizopus oryzae* lipase, Esperase, Alcalase, *Aspergillus species* acylase, Prozyme, Protease M, Protease N. The hydrolase preferably is selected from the group of hydrolases acting on ester bonds (lipases, esterases) (EC 3.1), peptide

hydrolases acting on peptide bonds (peptidase, proteinases) (EC 3.4), and hydrolases acting on C-N bonds other than peptide bonds (EC 3.5).

In particular a hydrolase acting on C-N bonds other than peptide bonds may be selected from the group of carboxylic ester hydrolases (EC 3.1.1) and
5 amidases acting on linear amides (EC 3.5.1), specifically from the group of amino amidases, more specifically from the group of amino amidases from *Mycobacterium*, more specifically amino amidases from *Mycobacterium neoaurum*.

The chemical hydrolysis of the N-protected DAB may comprise a process known in the art for similar reactions. A suitable method involves deacylation
10 by a $(\text{PhO})_3\text{P} \cdot \text{Cl}_2$ reagent prepared in situ by titrating a solution of triphenyl phosphate with chlorine. This method is generally described by Saggiari et al (Organic Letters (2004), 6 (22), pp. 3885-3888).

The conversion of N⁵-protected ornithine into unprotected DAB may proceed by first specifically decarboxylating the N⁵-protected ornithine to result into N-
15 protected DAB and subsequently hydrolyzing N-protected DAB to yield unprotected DAB as described before.

For the specific decarboxylation of N⁵-protected ornithine to result into N-protected DAB use may be made of a suitable biocatalyst, such as an enzyme with lyase activity. Suitable enzymes with lyase activity belong to class EC 4. More in
20 particular use can be made of carbon-carbon lyases (EC 4.1) such as carboxylases (EC 4.1.1) as exemplified by ornithine decarboxylase (SpeC) of *Escherichia coli* (EC 4.1.1.17), branched chain alpha-ketoacid decarboxylase (KdcA; SEQ ID 8) and alpha-ketoisovalerate decarboxylase (KivD; SEQ ID 10) of *Lactococcus lactis* and lysine decarboxylase (LysA; SEQ ID 12) of *Escherichia coli*.

25 Alternatively, the first mentioned specific decarboxylation of N⁵-protected ornithine may be carried out by a chemical conversion known in the art for similar chemical entities. Suitable chemical decarboxylation reactions for this purpose can be done by heating the compound in a high boiling solvent, such as diphenylmethane, optionally in the presence of a catalytic amount of an organic
30 peroxide, or may be done by heating the compound with one or more equivalents of a ketone or aldehyde.

The subsequent hydrolysis of N-protected DAB may be performed by a biocatalytic or a chemical process as described above for the biocatalytically produced N-protected DAB.

35 As an alternative for the two-step conversion described above, use

can be made of a one-pot process to produce DAB from N⁵-protected ornithine. This process may proceed by either first deacylating the N⁵-protected ornithine and subsequent decarboxylation, or by first decarboxylating N⁵-protected ornithine and subsequent deacylation according to methods known in the art for similar compounds.

- 5 Decarboxylation can be done as described above. Deacylation can be done by the method described above for N-protected DAB.

The conversion of N-protected 4-aminobutyraldehyde into unprotected DAB may proceed by first specifically replacing the aldehyde oxygen by an amino group, thereby forming N-protected DAB, and subsequently de-protecting the latter. For the first conversion use may be made of a suitable biocatalyst, such as an enzyme with transferase activity (EC 2) as described before. Suitable enzymes with transferase activity for this particular purpose are exemplified by the transferases transferring nitrogenous groups (EC 2.6), more in particular aminotransferases (transaminases) (EC 2.6.1), further in particular 4-amino-butyrate aminotransferase from mammalian liver, more in particular 4-amino-butyrate aminotransferase from pig liver; 4-amino-butyrate aminotransferase from mammalian brain, more in particular 4-aminobutyrate aminotransferase from human, pig, or rat brain; omega-aminotransferase of *Vibrio fluvialis*, 4-amino-butyrate aminotransferase from *E. coli*, and 5-aminovalerate aminotransferase from *Clostridium*, more in particular from *Clostridium aminovalericum*, ornithinetransaminase (EC 2.6.1.11), L-alanine:3-oxopropionate aminotransferase (EC 2.6.2.18) and putrescine amino transferase of e.g. *Shigella* or *Salmonella*. Particularly suitable aminotransferases are e.g. omega-aminotransferase of *Vibrio fluvialis* (SEQ ID 5), or aminotransferases of *Pseudomonas aeruginosa* (gi9946143 (SEQ ID 1) or gi9951072 (SEQ ID 3)), *Paracoccus denitrificans* (ZP00628577; SEQ ID 14), *Bacillus weihenstephanensis* (ZP01186960 (SEQ ID 16)).

Other suitable biocatalysts for the conversion of N-protected 4-aminobutyraldehyde into N-protected DAB are enzymes with oxidoreductases (EC 1), more in particular oxidoreductases acting on CH-NH₂ groups (EC 1.4) or CH-NH groups (EC 1.5) of donors, and more in particular enzymes of the classes EC 1.4.1, 1.4.3 (preferably 1.4.3.4) and 1.4.99.

Alternatively the first conversion of N-protected 4-aminobutyraldehyde may be carried out by a chemical conversion known in the art for similar chemical entities. Suitable chemical reactions for this purpose can be executed by a reductive amination of N-protected 4-aminobutyraldehyde according to methods known in the art for similar compounds (see e.g. DE 4322065). A suitable method is for example

reaction with ammonia and hydrogen over a heterogeneous catalyst (such as RaNi , Ni/SiO_2 and or Al_2O_3 , Ru/C , Rh/C) or a homogeneous catalyst (such as a homogeneous Rh catalyst).

The subsequent hydrolysis of N-protected DAB may be performed by
 5 a biocatalytic or a chemical process as described above for the biocatalytically produced N-protected DAB.

Description of the figures

Fig. 1. TLC of the end time sample of the bioconversion of N-Ac-
 10 Ornithine towards N-Ac- DAB. 1) Glutamate DC; 2) Aspartate DC; 3) LysA; 4) KdcA; 5) KivD; 6) Kgd; 7) Lysin DC; 8) ODC LJ110; 9) ODC DH5 α ; 10) enzyme blank; 11) Chemical blank; 12) N-Ac-Ornithine reference sample; 13) N-Ac-DAB reference sample; 14) N-Ac-Ornithine & N-Ac-DAB reference sample.

15 EXAMPLES

General Methods

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 Press; F.
 25 Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York 1987).

Plasmids and Strains

pBAD/Myc-His C was obtained from Invitrogen (Carlsbad, CA, USA).
 30 Plasmid pBAD/Myc-His-DEST constructed as described in WO2005/068643, was used for protein expression. *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used for all cloning procedures and for expression of target genes.

Media

LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) was used for growth of *E. coli*. Antibiotics (50 µg/ml carbenicillin) were supplemented to maintain plasmids. For induction of gene expression under control of the P_{BAD} promoter in
5 pBAD/Myc-His-DEST derived plasmids, L-arabinose was added to a final concentration of 0.2% (w/v).

Production of amino amidase of *M. neoaurum*

Amino amidase was obtained by growth of *Mycobacterium neoaurum*
10 strain ATCC 25795 under following conditions. One litre of Mycomed medium containing 4,8 g/l nitrilotriacetic acid (NTA), 4 g/l urea, 6 g/l glucose, 20 g/l yeast carbon base (YCB from Difco), 1.55 g/l K₂HPO₄ and 0.85 g/l NaH₂PO₄.H₂O were adjusted to pH 7 and inoculated with a glycerol stock culture of *Mycobacterium neoaurum* strain ATCC 25795. The preculture was shaken on a New Brunswick Scientific G53 shaker
15 (150 rpm, amplitude 4 cm) at 37°C for 168 hours. When an optical density (OD_{620 nm}) of 3.45 was reached, 500 ml of the preculture was used to inoculate 9 l of Mycomed medium. Amidase expression was induced by NTA present in the Mycomed medium. The fermentation culture was stirred at 375-750 rpm at an aeration rate of 0.5-2 l/min. The pH was kept constant at 7 by addition of H₃PO₄ and NaOH. The cultivation
20 temperature was 37°C. After 44 hours of cultivation, the culture was feeded by addition of 10 g/l YCB. After 68 hours of cultivation, the culture was feeded by addition of 10 g/l glucose. After 94 hours of cultivation, the culture was harvested by centrifugation at 12,000 g for 10 minutes. The cell pellet was washed in 20 mM HEPES/NaOH buffer, pH 7 and subsequently freeze-dried for storage.

25

Identification of plasmids

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

Cloning of target genes

Design of expression constructs

attB sites were added to all genes upstream of the ribosomal binding site and start codon and downstream of the stop codon to facilitate cloning using the
5 Gateway technology (Invitrogen, Carlsbad, CA, USA).

Gene synthesis and construction of plasmids

Synthetic genes were obtained from DNA2.0 and codon optimised for expression in *E. coli* according to standard procedures of DNA2.0. The
10 aminotransferase genes from *Vibrio fluvialis* JS17 [SEQ ID No. 5] and *Bacillus weihenstephanensis* KBAB4 [SEQ ID No. 16] encoding the amino acid sequences of the *V. fluvialis* JS17 omega-aminotransferase [SEQ ID No. 6] and the *B. weihenstephanensis* KBAB4 aminotransferase (ZP_01186960) [SEQ ID No. 17], respectively, were codon optimised and the resulting sequences [SEQ ID No. 7] and
15 [SEQ ID No. 18] were obtained by DNA synthesis.

The gene constructs were cloned into pBAD/Myc-His-DEST expression vectors using the Gateway technology (Invitrogen) via the introduced *attB* sites and pDONR201 (Invitrogen) as entry vector as described in the manufacturer's protocols (www.invitrogen.com). This way the expression vectors pBAD-*Vfl*_AT and
20 pBAD-*Bwe*_AT were obtained, respectively. The corresponding expression strains were obtained by transformation of chemically competent *E. coli* TOP10 (Invitrogen) with the respective pBAD-expression vectors.

In a similar way, an expression vector was made with the aminotransferase gene from *Paracoccus denitrificans* [SEQ ID No 14] encoding the
25 amino acid sequence of SEQ ID No 15.

Cloning by PCR

The aminotransferase genes were amplified from genomic DNA by PCR using PCR Supermix High Fidelity (Invitrogen) according to the manufacturer's
30 specifications, using primers as listed in Table 3.

Table 3

Origin of gene	Gene SEQ ID No	Enzyme SEQ ID No	Forward primer SEQ ID No	Reverse primer SEQ ID No
<i>Pseudomonas aeruginosa</i> gi9946143	1	2	23	24
<i>Pseudomonas aeruginosa</i> gi9951072	3	4	25	26

PCR reactions were analyzed by agarose gel electrophoresis and PCR products of the correct size were eluted from the gel using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Purified PCR products were cloned into pBAD/*Myc*-His-DEST expression vectors using the Gateway technology (Invitrogen) via the introduced *attB* sites and pDONR-zeo (Invitrogen) as entry vector as described in the manufacturer's protocols. The sequence of genes cloned by PCR was verified by DNA sequencing. This way the expression vectors pBAD-*Pae*-_gi9946143_AT, pBAD-*Pae*_gi9951072_AT and pBAD-*Pde*_AT_ZP00628577 were obtained. The corresponding expression strains were obtained by transformation of chemically competent *E. coli* TOP10 (Invitrogen) with the pBAD constructs.

Growth of *E. coli* for protein expression

Small scale growth was carried out in 96-deep-well plates with 940 µl media containing 0.02% (w/v) L-arabinose. Inoculation was performed by transferring cells from frozen stock cultures with a 96-well stamp (Kühner, Birsfelden, Switzerland). Plates were incubated on an orbital shaker (300 rpm, 5 cm amplitude) at 25°C for 48 h. Typically an OD_{620nm} of 2 – 4 was reached.

Preparation of cell lysates*Preparation of lysis buffer*

The lysis buffer contained the ingredients listed in table 4:

Table 4

1M MOPS pH 7.5	5 ml
DNAse I grade II (Roche)	10 mg
Lysozyme	200 mg
MgSO ₄ .7H ₂ O	123.2 mg
dithiothreitol (DTT)	154.2 mg
H ₂ O (MilliQ)	Balance to 100 ml

5

The solution was freshly prepared directly before use.

Preparation of cell free extract by lysis

Cells from small scales growth (see previous paragraph) were harvested by centrifugation and the supernatant was discarded. The cell pellets formed during centrifugation were frozen at -20 °C for at least 16 h and then thawed on ice. 500 µl of freshly prepared lysis buffer were added to each well and cells were resuspended by vigorously vortexing the plate for 2-5 min. To achieve lysis, the plate was incubated at room temperature for 30 min. To remove cell debris, the plate was centrifuged at 4 °C and 6000 g for 20 min. The supernatant was transferred to a fresh plate and kept on ice until further use.

15

Preparation of cell free extract by sonification

Cells from medium scales growth (see previous paragraph) were harvested by centrifugation and the supernatant was discarded. 1 ml of potassium phosphate buffer pH7 was added to 0.5 g of wet cell pellet and cells were resuspended by vigorously vortexing. To achieve lysis, the cells were sonicated for 20 min. To remove cell debris, the lysates were centrifuged at 4 °C and 6000 g for 20 min. The supernatant was transferred to a fresh tube and frozen at -20°C until further use.

20

25 Bioconversion of N-acetyl-4-aminobutyraldehyde to N-acetyl-DAB*Screening conditions*

All enzymes were suspended in 100 mM potassium phosphate buffer pH 7.5 to a final volume of 100 µl. The enzymatic reactions were started by addition of 150 µl of a stock solution containing the amine donor (L-alanine or (S)-alpha

methylebenzylamine) and cofactor (PLP). The final concentrations in the 250 µl reaction mixture were: N-acetyl-aminobutanal (70 mM), amine donor (140 mM), PLP (12.5 mM). The two amine donors were tested separately. The reaction mixtures were incubated overnight (16.5 and 16 hours) at 28 °C, while shaking at 560 rpm on an IKA orbital shaker. After incubation, the reaction mixtures were stopped and diluted by addition of 750 µl 60% dilution of acetonitril with 0.2 % formic acid. The microtiterplates were centrifuged at 3500 rpm for 20 minutes. Analysis was performed by means of LC-MS analysis (see Resolve Job 2009-02-00649).

Analytical Methods:

In total, 148 samples were analyzed by means of LC-MS, using the analytical method described in job 2009-01-00306. Detection limit; linear range: for amine donor L-alanine 0 µmol/L – 550 µmol/L and for amine donor (S)-alpha methylebenzylamine 0 µmol/L – 280 µmol/L.

Results of bioconversion of N-acetyl-4-aminobutyraldehyde into N-acetyl-DAB

In total 148 transaminase enzymes were screened for the route of N-acetyl-4-aminobutyraldehyde towards N-acetyl-DAB. All samples have been analyzed by means of LC-MS for conversion. The conversions were calculated based on the formation of N-acetyl-DAB. In total, 31 aminotransferase hits (>2% conv.) were found when using L-alanine as amine donor. 20 of these were also positive when using (S)-α-methyl benzylamine as amine donor. Five of these positive hits are exemplified in Table 5.

Table 5:

Hits showing >2% conversion in the bioconversion of N-acetyl-4-aminobutyraldehyde into N-acetyl-DAB

Enzyme / origin	Orf/insert	platform
<i>Vibrio fluvialis</i> JS17	clone	SdW/RS AT3
<i>monas aeruginosa</i>	Pae_AT_gi9946143	SPEED TA 1
<i>Pseudomonas aeruginosa</i>	Pae_AT_gi9951072	SPEED TA 1
<i>Paracoccus denitrificans</i>	Pde_AT_ZP00628577	SdW/RS AT3
<i>Bacillus weihenstephanesis</i>	Bwe_AT_ZP01186960	SdW/RS AT3

Bioconversion of N-Acetyl-DAB into DAB.Screening conditions

All enzymes were suspended in a final volume of 100 µl of 100 mM potassium phosphate at pH 7.5. The enzymatic reactions were started by addition of 150 µl of a 13.33 mg/ml N-acetyl-DAB.HCl in potassium phosphate buffer 100 mM (final reaction concentration of 8 mg/ml $\approx$ 48 mM N-acetyl-DAB). The reaction mixtures were incubated overnight at 37 °C, while shaking at 460 rpm on an IKA orbital shaker. After incubation, the reaction mixtures were stopped and diluted by addition of 750 µl 100 mM HClO₄ in H₂O, pH 1.0. The microtiter plates were centrifuged at 3500 rpm for 20 minutes and analyzed for DAB by means of HPLC-PCR-FL analysis as described below.

HPLC-MS analysis method for the determination of DABSample preparation:

A mixture of acetonitrile and water with 0.2% of formic acid is used to dilute the sample. The percentage of acetonitrile must be at least 50%

LC conditions:

Column	50 x 4.6 mm, HP HILIC, 3 µm (Alltech)	
Column temperature	room temperature (24°C)	
Eluent	A: acetonitrile containing 0.2% formic acid	
	B: water containing 0.2% formic acid	
Gradient	time (min)	% eluent B
	0	5
	2.5	20
	10	20
	11.1	5
	15	5
Flow	1 ml/min, before entering the MS the flow is split 1:5	
Injection volume	2 µl	

MS conditions:

Ionisation	positive ion turbo ionspray
source conditions	ionspray voltage : 5kV
	temperature : 400°C
	defragmentation potential: 51V
	focusing potential: 180V
Scan mode (DAB)	selective ion mode m/z 72 (dwell time 200 msec)

Under the applied conditions DAB elutes at 6.3 minutes

5 *Results of bioconversion of N-acetyl-DAB to DAB*

A selection from the enzymes tested, which showed hydrolytic activity in the bioconversion of N-acetyl-DAB to DAB are listed in table 6. A few of these enzymes are also characterized by their sequence information incorporated into this patent application.

Table 6: Hydrolysis of N-acetyl-DAB to DAB

Biocatalyst	Supplier	Concentration DAB (μ M)	SEQ ID No	Uniprot No
<i>Rhizopus japonicus</i> lipase	Biocatalysts LTD	690		
<i>Aspergillus niger</i> lipase (lipase AP-6)	Amano	278		
<i>Alcaligenes sp.</i> lipase (lipase QL)	Meito Sangyo	186		
<i>Bacillus amyloliquefaciens</i> protease (protease B)	DSM-Gist	142	19	
<i>Bacillus licheniformis</i> protease (Delvolase)	DSM-Gist	162	20	
<i>Rhizopus oryzae</i> lipase	DSM-Gist	1846		P61872
Esperase	NOVO	158		
Alcalase	NOVO	130		
<i>Aspergillus</i> species (Acylase)	Sigma	270		
Prozyme 6	Amano	346		
Protease M	Amano	206		
<i>Bacillus subtilis</i> (Protease N)	Amano	514		
<i>Mycobacterium neoaurum</i> L- amino amidase	DSM	1174		
Cerdase	Novozymes	530	21	
chem blank		108		
chem blank		104		

Conclusion

A large number of hydrolytic enzymes were found to be useful as
5 biocatalyst for the conversion of N-acetyl-DAB to DAB.

N-protected DAB precursors with other acyl-protecting groups can be
prepared by acylation of for example 4-aminobutyraldehyde or ornithine. For examples,
by acylation with acetic acid anhydride in formic acid to introduce a formyl protecting
group or by reaction of C2-C6 carboxylic acid anhydride or acyl chloride to introduce a
10 N-acetyl, N-propionyl, N-butyryl, N-valeryl or N-caproyl protecting group, respectively.

It is anticipated that these N-protected DAB precursors such as N-formyl –DAB, and the higher homologues with C3-C6 acyl protecting groups, can be converted analogously by the enzymes described above.

5 Bio conversion of N⁵-acetyl-ornithine into N-acetyl-DAB

Cell cultivation and expression

This bioconversion was made with decarboxylases. Most of the decarboxylases were expressed in *E.coli* under standard condition.

10 Precultures were made by inoculation of 5 ml LB^{carb} medium with *E. coli* Top10 harbouring pBAD-DEST_lysA, pBAD-DEST_kdcA, pBAD-DEST_kivD or pBAD-DEST_kgd from glycerol stocks. The precultures were incubated overnight at 28 °C. 0.5 ml of each preculture was diluted out in 50 ml LB^{carb} medium. The cultures were incubated at 28 °C until an OD₆₀₀ of 0.6 was reached (on average after 3-4 hrs). Protein expression was induced by adding arabinose to a final concentration of 0.02%. After
15 overnight incubation at 28 °C the cells were harvested (10 min, 5000 rpm, 4 °C). For analysis with SDS-PAGE 1 ml samples were taken before induction, 3 hours after induction and overnight. The cells were pelleted (5 min, 13,000 rpm) and the pellet was stored at -20 °C.

20 The two ornithine decarboxylases pBAD2_ODC *E.coli* DH5α/LJ110 were grown and expressed under slightly different conditions. Here the main culture was grown to an OD₆₂₀ of 1.5 before inducing with 50μM IPTG. All other conditions were the same as described above.

CFE preparation by sonification

25 Cell pellets were thawed on ice and resuspended in 2 volumes 50 mM kalium-phosphate (KPi) buffer pH 7.5. The cell suspensions were sonificated for 10 minutes with pulses for 10 seconds on and off. After sonification cell debris was pelleted by centrifugation (20 min, 13,200 rpm, 4 °C). SDS-PAGE analysis was used to determine the expression levels and the CFE's were stored at -20°C

Table 7 Bioconversion conditions for the conversion of N-Ac-ornithine towards N-Ac-DAB\

Reaction	200mM K- acetate pH4.6	200 mM KP pH 6.9	200mM KP pH 7.5	200mM KP pH 6.5	100mM K- acetate pH5.7	200mM N-Ac- Ornithine	10mM PLP	100mM ThD	4M NaCl	50mM EDTA	1M MgCl2	Enzyme	Water	Total
Glutamate DC A	1,875					1,25	0,05		0,25			1	0,575	5
Aspartate DC A			1,875			1,25	0,05			0,5		1	0,325	5
LysA				1,875		1,25	0,05				0,025	1	0,8	5
KdcA				1,875		1,25		0,05			0,025	1	0,8	5
KivD				1,875		1,25		0,05			0,025	1	0,8	5
Kgd				1,875		1,25		0,05			0,025	1	0,8	5
Lysin DC					1,875	1,25	0,05				0,025	1	0,8	5
Ornithine DC LJ110		1,875				1,25	0,05				0,025	1	0,8	5
Ornithine DC DH5a		1,875				1,25	0,05				0,025	1	0,8	5
Blanco					1,875		0,05				0,025	1	2,05	5
Blanco					1,875	1,25	0,05				0,025		1,8	5

All N-Ac-Ornithine towards N-Ac-DAB reactions were stirred and incubated at 37°C. Samples were taken at 0; 2; 18; 28 and 44 hours and stored at -20°C. For analysis 500µl of each sample was added to 500µl of acetonitril and spinned at maximum speed for 10 minutes. Samples are analysed on TLC and run with an eluent of ammonia: methanol (1:1) and stained with a ninhydrine spray. For quantitative analysis the samples are measured by LC-MS-MS according to the method described below.

HPLC-MS analysis method for the determination of N-acetyl-DAB

10 Sample preparation:

A mixture of acetonitrile and water with 0.2% of formic acid is used to dilute the sample. The percentage of acetonitrile must be at least 50%

Experiments were performed on the PE SCIEX API2000 LC-MS/MS from Applied Biosystems.

15 LC conditions:

Column	50 x 4.6 mm, HP HILIC, 3 µm (Alltech)	
Column temperature	room temperature (24°C)	
Eluent	A: acetonitrile containing 0.2% formic acid B: water containing 0.2% formic acid	
Gradient	time (min)	% eluent B
	0	5
	2.5	20
	8	20
	8.1	5
	12	5
Flow	1 ml/min, before entering the MS the flow is split 1:5	
Injection volume	2 µl	

MS conditions:

Ionisation	positive ion turbo ionspray	
	source conditions	ionspray voltage : 5kV temperature : 400°C defragmentation potential: 11V focusing potential: 350V
Scan mode (N-Ac-DAB)	selective ion mode	m/z 72 & 114 (dwell time 200 msec)

Under the applied conditions N-Ac-DAB elutes at 4.2 minutes

Results of bioconversion of N⁵-Acetyl-ornithine towards N-Acetyl-DAB

For the conversion of ornithine towards DAB the end time samples
5 (44 hrs.) are analysed on TLC (Figure 1).

All end time samples were analyzed with LC-MS-MS. Those showing a value of at least 3 micromol above the level of the control samples are shown in table 8.

10 Table 8 LC-MS-MS results of bioconversion

N-Acetyl DAB		
	m/z 131 → 72	m/z 131 → 114
sample	micromol/l	micromol/l
1	9	-
3	3	-
4	4	3
5	10	8
6	5	4
	mmol/l	mmol/l
CB200109	370	344

CLAIMS

1. A method for the preparation of 1,4-diaminobutane [DAB] involving at least one biocatalytic step which comprises the biocatalytic production of at least one N-protected precursor of DAB and subsequent *in vitro* conversion of the N-protected precursor into DAB.
2. A method for the preparation of DAB involving at least one biocatalytic step, and comprising the steps of
 - a) biocatalytically preparing an N-protected precursor of DAB yielding a biocatalytic reaction mixture containing the N-protected precursor of DAB,
 - b) recovering the N-protected precursor from the biocatalytic reaction mixture
 - c) converting the N-protected precursor into DAB.
3. A method according to any of the claims 1 or 2, wherein the N-protected precursor of DAB is selected from the group consisting of N⁵-protected ornithine, N-protected DAB, and N-protected 4-aminobutyraldehyde.
4. A method according to any of the claims 1 or 2, wherein the at least one N-protected precursor of DAB is selected from the group consisting of N⁵-acetyl ornithine, N-acetyl DAB, and N-acetyl 4-aminobutyraldehyde.
5. A method according to any of the claims 1 or 2, wherein the biocatalytic step is a fermentation step
6. A method according to claims 5, wherein the fermentation step takes place in a unicellular host.
7. A method according to claim 6, wherein the fermentation step takes place in a host cell selected from the group consisting of animal cells, plant cells, bacteria, archaea, yeasts and fungi.
8. A method according to claim 2, wherein in the recovery of the N-protected precursor from the biocatalytic reaction mixture is carried out by at least one step selected from the group consisting of filtration, sedimentation, crystallization, affinity chromatography, size exclusion chromatography, membrane separation and evaporation
9. A method according to claim 2, wherein conversion step c) of the N-protected precursor into DAB involves at least one enzymatic or chemical treatment step.
10. A method for the preparation of DAB according to any of the claims 1 to 9, wherein

the N-protected DAB is converted into DAB using a hydrolytic enzyme.

11. Method according to claim 10, wherein a hydrolytic enzyme is used selected from the group consisting of carboxylic ester hydrolases, thiolester hydrolysases, lipases and peptidases, in particular from lipases and peptidases.
- 5 12. Method according to claim 10, wherein the hydrolytic enzyme is a peptidase selected from the group of serine-type carboxypeptidases, metallocarboxypeptidases, cysteine-type carboxypeptidases, serine endopeptidases, cysteine endopeptidases, aspartic endopeptidases and metalloendopeptidases, in particular from serine endopeptidases.
- 10 13. Method according to claim 12, wherein the serine endopeptidase is a subtilisin, preferably subtilisin Carlsberg.

FIGURE 1/1

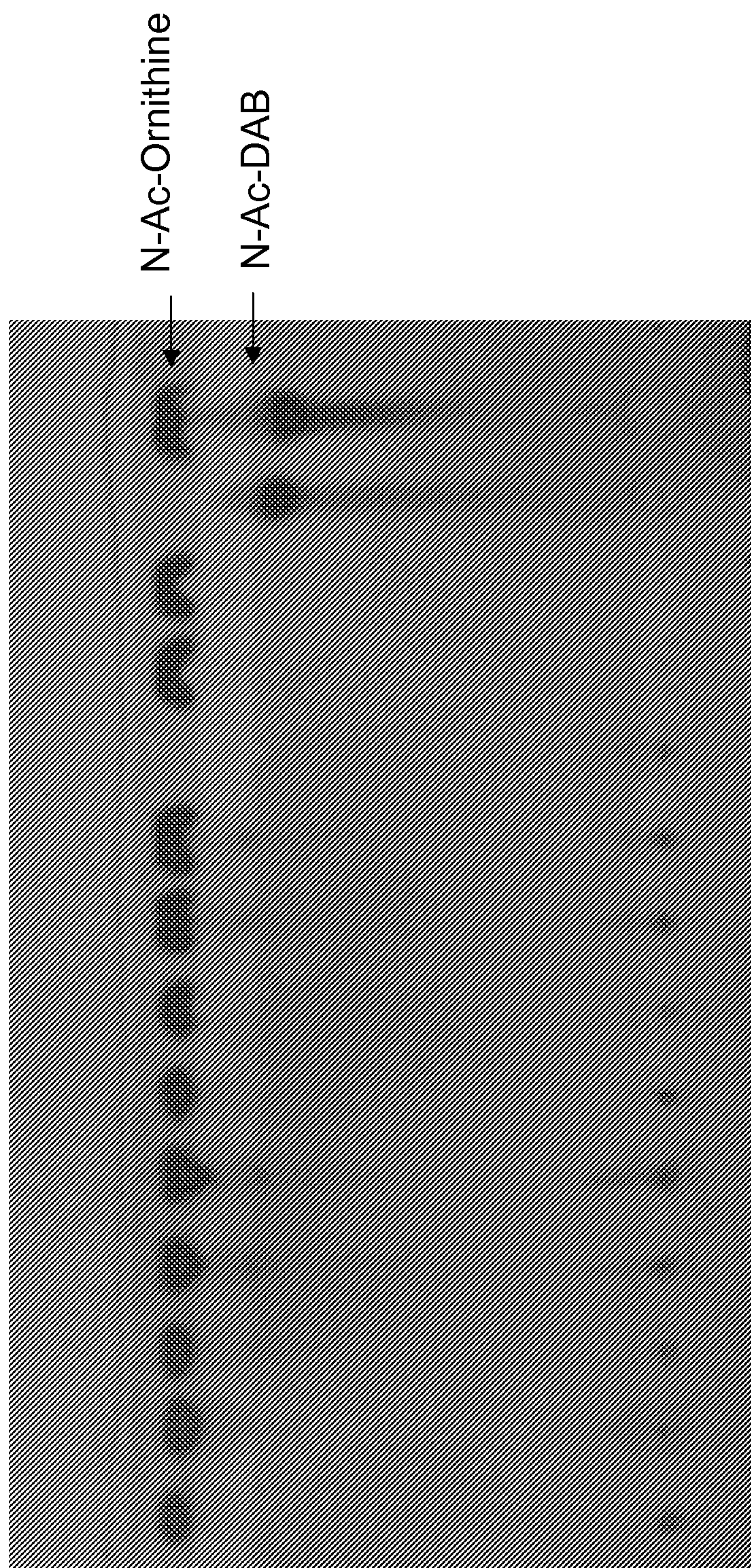


FIGURE 1/1

