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(54) Title: ENANTIOSELECTIVE PRODUCTION OF (R)-PANTOLACTONE

(57) Abstract: The present invention relates to formation of highly enriched (R)-pantolactone from conversion of keto-pantolactone in a bio-catalytic process using NADPH- dependent enzymes in the presence of NADH as co-factor.

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Enantioselective production of (R)-pantolactone

The present invention relates to formation of highly enriched (R)-pantolactone from conversion of keto-pantolactone in a bio-catalytic process using NADPH-dependent enzymes in the presence of NADH as co-factor.

- 5 Pantothenate or vitamin B5 is a member of the B complex of vitamins which is essential for mammals including humans. It has to be supplied as food or feed additive. In cells, pantothenate is used primarily for the biosynthesis of coenzyme A and acyl carrier protein. These essential coenzymes function in the metabolism of acyl moieties, which form thioesters with the sulfhydryl group of
10 the 4'-phosphopantethein portion of these molecules. Importantly, only (R)-pantothenate is biologically active as vitamin.

- Pantothenate has been synthesized conventionally via chemical synthesis from bulk chemicals. However, the substrates required for chemical synthesis are expensive and the racemic intermediates have to be optically resolved. Bacterial
15 or microbial systems, if available, that produce enzymes useful in pantothenate biosynthesis processes, are not yet economically viable for industrial production because of insufficient yield, poor selectivity of the used enzymes, undesired side-reactions in cheap crude enzyme preparations, or too high co-factor cost. For example, Si et al. (Appl Microbiol Biotechnol (2012) 93:1619–1625) describes
20 the use of *Stenotrophomonas maltophilia* and its application to stereospecific production of D-pantoic acid. The enzyme belongs to the enzyme family of pantoate 4-dehydrogenase (EC 1.1.1.106). While only few members of this family e.g. from *Pseudomonas* accept NADH as co-factor (Myöhänen, T.; Mäntsälä, P.; Biochim. Biophys. Acta 614, 266–273 (1980)) the enzyme from *Stenotrophomoeas*
25 was classified as NADPH dependent according to Si et al. (Appl Microbiol Biotechnol (2012) 93:1619–1625). The same co-factor dependency for NADPH is known for the pantoate 4-dehydrogenase from *Candida parapsilosis* described by Shimizu et al. (Appl Microbiol Biotechnol (2004) 64: 359–366). An obvious

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drawback of the described process is the use of expensive NADPH as co-factor.

Thus, there is a need for more effective and simpler methods towards enantioselective production of (R)-pantolactone with a high enantiomeric excess (e.e.) to be used in an industrial scale, with no need for further resolution
5 and/or purification steps and with the avoidance of expensive co-factors such as e.g. NADPH.

Surprisingly, we now identified a novel process for production of (R)-pantolactone via enantioselective catalytic reduction of keto-pantolactone (KPL), comprising the use of NADH as co-factors with known NADPH-dependent
10 reductases leading to a highly efficient enantioselective conversion of KPL.

Particularly, the present invention is directed to enantioselective reduction of KPL into (R)-pantolactone, wherein the reduction of KPL is catalyzed by enantioselective NADPH dependent alcohol dehydrogenase (ADH) enzymes (EC
15 1.1.1.2) or pantoate 4-dehydrogenase (EC 1.1.1.106) which surprisingly accept NADH as co-factor, wherein an e.e. in the range of about 80-99% could be achieved.

For the purpose of the present invention, i.e. enantioselective conversion of KPL into (R)-pantolactone as described herein, any NADPH dependent ADH isolated
20 from various sources can be used, particularly such enzymes isolated from archaea, bacteria, fungi including yeast, or higher eukaryotes. Preferably, the enzyme is selected from yeast, such as *Candida* or *Saccharomyces*, or from bacteria, such as *Bacillus* or *Escherichia*, e.g. *Stenotrophomonas*, preferably a NADPH-dependent reductase selected from *Candida parapsilosis*,
25 *Saccharomyces cerevisiae* or *Stenotrophomonas maltophilia*, more preferably Sma_KPR from *Stenotrophomonas maltophilia* NBRC 14161, Cpa_PKR_2 from *Candida parapsilosis* IFO 0708, ADH 440 (evo-1.1.440.S, evoXX technology GmbH, Germany; for specification see Table 3) originated from *E. coli*, most preferably Sma_KPR from *Stenotrophomonas maltophilia* NBRC 14161 or Cpa_PKR_2 from
30 *Candida parapsilosis* IFO 0708 or enzymes having equivalent activity of Sma_KPR from *Stenotrophomonas maltophilia* NBRC 14161 or Cpa_PKR_2 from *Candida parapsilosis* IFO 0708 or ADH 440 from *E. coli*.

As used herein, the term "keto-pantolactone" also includes the respective ring-opened form, i.e. ketopantoic acid.

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As used herein, terms like "alcohol dehydrogenase", "pantoate 4-dehydrogenase", "reductase" or "enantioselective alcohol dehydrogenase (ADH) enzymes" are used interchangeably herein and define suitable enzymes to be used for the performance of the present invention, i.e. conversion of KPL into
5 pantolactone with a high percentage, i.e. at least about 80%, of (R)-pantolactone.

The terms "conversion", "enantioselective reduction", "biocatalytic process", "enantioselective catalytic reduction" or "enzymatic conversion" are used interchangeably herein and describe the reduction of KPL into (R)-pantolactone
10 catalyzed by the ADHs as defined herein.

As used herein, the term "used in the presence of NADH" or "together with NADH as cofactor" is used interchangeably herein and refers to a reaction wherein NAD (NAD⁺) is added but the reduced form, i.e. NADH, is actually used by the enzyme as co-factor.

15 The enzymatic conversion of KPL as defined herein into (R)-pantolactone, as described herein can be performed with isolated enzymes, such as e.g. dehydrogenase enzymes as defined herein, particularly ADHs, such as e.g. ADHs as defined herein originating from *Stenotrophomonas maltophilia*, or *Candida parapsilosis*, particularly Sma_KPR or Cpa_PKR_2, that might be expressed in a
20 suitable host cell, such as e.g. yeast or bacteria, preferably Pichia or Escherichia, such as e.g. as heterologous enzymes. Particularly, the enantioselective enzymes as described herein are heterologous expressed in *Escherichia coli*. Isolation of suitable enzymes can be done by known methods, the respective enzymes might be further used in isolated form or in the form of a cell free extract (cfe), powder
25 or liquid formulations, i.e. in immobilized or non-immobilized form. Preferably, the enzymes as defined herein, such as e.g. the reductases from *Stenotrophomonas*, *Candida*, as defined herein, are used as cfe. If the enzymes are used in isolated form, such as e.g. in liquid or immobilized form, they might be either covalently bound or adsorbed, e.g. on epoxy carrier, depending on the
30 supplier. When using immobilized forms, the enzymes might be used several times with more or less the same performance (so-called recycling). Suitable enzymes to be used for the purpose of the present invention might be sourced from commercially available sources, such as e.g. ADH 440 (evo-1.1.440.S; evoXX technology GmbH, Germany).

35 The enzymatic conversion of KPL as defined herein into (R)-pantolactone, as described herein can be performed in a process wherein a suitable host cell

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expressing said enzymes, such as e.g. dehydrogenase enzymes as defined herein, particularly recombinant host cell expressing heterologous enzymes, is cultivated under suitable conditions in the presence of a substrate allowing the conversion of KPL into (R)-pantolactone as described herein via catalytic action of said expressed enzymes. Such expression system wherein recombinant host cell expressing the respective enzymes as defined herein is acting on the substrate, i.e. KPL, is also called a "recombinant whole cell biocatalyst" to be used for conversion of KPL into (R)-pantolactone.

The enzymes to be used for the present invention are known as NADPH-dependent alcohol dehydrogenases, i.e., using NADPH as co-factor for their catalytic activity and have not been disclosed to use other co-factors such as e.g. NADH, NAD or NAD⁺ as co-factor. As used herein, the term "co-factor" and "co-enzyme" are used interchangeably herein. As it is known in biocatalysis, the performance of an enzyme is normally very much related/restricted to the corresponding co-factor.

The process according to the present invention comprises incubation of KPL in the presence of a suitable enzyme as defined herein said process being furthermore carried out in the presence of NADH as co-factor. Such process might be conducted at a pH in the range of about 7.5 to about 4.5, such as e.g. a pH of about 7.4, 7.3, 7.2, 7.0, 6.9, 6.8, 6.7, 6.6, 6.5, 6.0, 5.9, 5.8, 5.7, 5.6, 5.5, 5.0, 4.5.

The process according to the present invention wherein KPL is enantioselectively converted via action of a reductase together with NADH as defined herein and performed at a pH between about 7.5 and 4.5 might be combined with an in-situ co-factor regeneration system, such as e.g. co-expression of co-factor regeneration enzymes, including but not limited to glucose dehydrogenase (GDH) converting D-glucose to D-gluconic acid and glucose as substrate, such as e.g. co-expression of *E. coli* GDH. Alternatively, GDH (CAS Number 9028-53-9) is available from various suppliers as known in the art.

In one embodiment, the process according to the present invention is performed with Sma_KPR from *Stenotrophomonas maltophilia* NBRC 14161, preferably a polypeptide with at least about 80%, such as e.g. 85, 90, 95, 98 or 100% identity to SEQ ID NO:1, including polynucleotides encoding said polypeptide such as e.g. the polynucleotide of SEQ ID NO:2, in the presence of NADH, resulting in (R)-pantolactone with an e.e. of at least about 85%, particularly with the process being carried out at pH of about 5.5. The e.e. might

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be further increased to about 100% with a process carried out at pH of about 7.5. Using such process, the yield of converted (R)-pantolactone might be in the range of at least about 87%.

In one embodiment, the process according to the present invention is
5 performed with Cpa_PKR_2 from *Candida parapsilosis* IFO 0708, preferably a polypeptide with at least about 80%, such as e.g. 85, 90, 95, 98 or 100% identity to SEQ ID NO:3 including polynucleotides encoding said polypeptide such as e.g. the polynucleotide of SEQ ID NO:4, in the presence of NADH, resulting in (R)-pantolactone with an e.e. of at least about 84%, particularly with the process
10 being carried out at pH of about 5.5. The e.e. might be further increased to at least about 99% with a process carried out at pH of about 7.5. Using such process, the yield of converted (R)-pantolactone might be in the range of at least about 39%.

The "enantiomeric excess" (e.e.) as used herein refers to the degree to which a
15 sample contains one enantiomer (such as e.g. (R)-pantolactone) in higher amount than the other enantiomer (such as e.g. (S)-pantolactone), see Helpfile on selectivity, Uni Graz, 2012: <http://biocatalysis.uni-graz.at/enantio/DataFiles/Selectivity-Help.pdf>.

The terms "sequence identity", "% identity" are used interchangeable herein. For
20 the purpose of this invention, it is defined here that in order to determine the percentage of sequence identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes. In order to optimize the alignment between the two sequences gaps may be introduced in any of the two sequences that are compared. Such alignment can
25 be carried out over the full length of the sequences being compared.

Alternatively, the alignment may be carried out over a shorter length, for example over about 20, about 50, about 100 or more nucleic acids/bases or amino acids. The sequence identity is the percentage of identical matches between the two sequences over the reported aligned region. The percent
30 sequence identity between two amino acid sequences or between two nucleotide sequences may be determined using the Needleman and Wunsch algorithm for the alignment of two sequences (Needleman, S. B. and Wunsch, C. D. (1970) J. Mol. Biol. 48, 443-453). Both amino acid sequences and nucleotide sequences can be aligned by the algorithm. The Needleman-Wunsch algorithm
35 has been implemented in the computer program NEEDLE. For the purpose of this invention the NEEDLE program from the EMBOSS package was used (version

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- 2.8.0 or higher, EMBOSS: The European Molecular Biology Open Software Suite (2000) Rice, Longden and Bleasby, Trends in Genetics 16, (6) pp276—277, <http://emboss.bioinformatics.nl/>). For protein sequences EBLOSUM62 is used for the substitution matrix. For nucleotide sequence, EDNAFULL is used. The
- 5 optional parameters used are a gap-open penalty of 10 and a gap extension penalty of 0.5. The skilled person will appreciate that all these different parameters will yield slightly different results but that the overall percentage identity of two sequences is not significantly altered when using different algorithms.
- 10 After alignment by the program NEEDLE as described above the percentage of sequence identity between a query sequence and a sequence of the invention is calculated as follows: number of corresponding positions in the alignment showing an identical amino acid or identical nucleotide in both sequences divided by the total length of the alignment after subtraction of the total
- 15 number of gaps in the alignment. The identity as defined herein can be obtained from NEEDLE by using the NOBRIEF option and is labeled in the output of the program as "longest identity". If both amino acid sequences which are compared do not differ in any of their amino acids, they are identical or have 100% identity.
- 20 As used herein, the term "specific activity" or "activity" with regards to enzymes means its catalytic activity, i.e. its ability to catalyze formation of a product from a given substrate. The specific activity defines the amount of substrate consumed and/or product produced in a given time period and per defined amount of protein at a defined temperature. Typically, specific activity is
- 25 expressed in μmol substrate consumed or product formed per min per mg of protein. Typically, activity is given per mg of the specific enzyme; if specified otherwise, activity can also be given on mg enzyme preparation (e.g. total protein, total soluble protein etc.). Typically, $\mu\text{mol}/\text{min}$ is abbreviated by U (= unit). Therefore, the unit definitions for specific activity of $\mu\text{mol}/\text{min}/(\text{mg}$ of
- 30 protein) or $\text{U}/(\text{mg}$ of protein) are used interchangeably throughout this document. An enzyme is active, if it performs its catalytic activity in vivo, i.e. within the host cell as defined herein or within an in vitro system in the presence of a suitable substrate. The skilled person knows how to measure enzyme activity, in particular activity of ADHs as defined herein, such as in
- 35 particular KPL-reductase activity. Analytical methods to evaluate the capability of a suitable enzyme as defined herein for formation of (R)-pantolactone from conversion of KPL as defined herein are known in the art.

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The present invention is directed to a process as defined herein for enantioselective reduction of KPL, wherein the KPL is enzymatically converted into (R)-pantolactone in the presence of NADH as co-factor, said process being carried out at a pH between about 7.5 and 4.5, with an e.e. in the range of at least about 84-100%, and a yield of (R)-pantolactone of at least about 39%,
5 depending on the used enzyme and the pH conditions.

In one aspect of the present invention, a host cell comprising and expressing an enantioselective enzyme as defined herein, is used in a process for production of (R)-pantolactone from conversion of KPL in the presence of NADH as co-factor
10 as defined herein. The host cell comprising the enzyme may be cultured in an aqueous medium supplemented with appropriate nutrients under aerobic or anaerobic conditions and as known by the skilled person for the respective host cells. Optionally, such cultivation is carried out at a pH of about 7.5 to about 4.5. The cultivation/growth of the host cell may be conducted in batch, fed-batch,
15 semi-continuous or continuous mode. (R)-pantolactone might be isolated and/or optionally further purified.

In one embodiment of the invention the conversion as described herein is carried out using an immobilized enzyme for enantioselective conversion of KPL into (R)-pantolactone. Such process might be performed under conditions
20 known in the art, e.g. such as in an aqueous medium or under conditions mentioned herein. Particularly, the process might be carried out at pH between about 7.5 and 4.5, using NADH and GDH/glucose as *in situ* co-factor regeneration system.

With regards to the present invention, it is understood that organisms, such as
25 e.g. animals, microorganisms, fungi, algae or plants also include synonyms or basonyms of such species having the same physiological properties, as defined by the International Code of Zoological Nomenclature, International Code of Nomenclature of Prokaryotes or the International Code of Nomenclature for algae, fungi, and plants (Melbourne Code).

30 The process as described herein might be carried out at temperatures of about 21°C to about 45°C, such as e.g. between about 21°C to about 40°C, particularly between about 21°C to about 35°C, preferably between about 25°C to about 31°C, more preferably about 28°C.

In particular, the present invention is directed to the following embodiments (1)
35 to (9):

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- (1) Process for enantioselective conversion of keto-pantolactone into R-pantolactone, comprising incubation of keto-pantolactone in the presence of a NADPH dependent reducing enzyme and NADH as co-factor.
- (2) Process according to embodiment (1), wherein the enzyme is selected from alcohol dehydrogenase EC enzymes (EC 1.1.1.2) or pantoate 4-dehydrogenase (EC 1.1.1.106).
- (3) Process according to embodiment (1) or (2), wherein the pH is in the range of about 7.5 to about 4.5.
- (4) Process according to embodiment (1), (2) or (3), wherein the reducing enzyme is an alcohol dehydrogenase originating from *Candida* or *Stenotrophomonas*, particularly selected from *Candida parapsilosis* or *Stenotrophomonas maltophilia*, preferably Sma_KPR from *Stenotrophomonas maltophilia* NBRC 14161, Cpa_PKR_2 from *Candida parapsilosis* IFO 0708, most preferably Sma_KPR from *Stenotrophomonas maltophilia* NBRC 14161 with at least about 80% identity to SEQ ID NO:1 or Cpa_PKR_2 from *Candida parapsilosis* IFO 0708 with at least about 80% identity to SEQ ID NO:3.
- (5) Process according to embodiment (4), wherein the reducing enzyme is a commercially available NADPH-dependent alcohol dehydrogenase, particularly ADH 440 (catalogue # evo 440, evoXX GmbH, Germany).
- (6) Process according to embodiment (1), (2), (3), (4) or (5), wherein the enzyme is used as a recombinant whole cell biocatalyst or as cell free extract.
- (7) Process according to embodiment (1), (2), (3), (4), (5) or (6), further comprising an in-situ co-factor regeneration system, particularly comprising glucose dehydrogenase and glucose.
- (8) Process for production of (R)-pantolactone with e.e. of at least about 99%, comprising enantioselective reduction of keto-pantolactone in the presence of an enantioselective alcohol dehydrogenase (ADH), particularly an ADH enzyme selected from *Candida parapsilosis* or *Stenotrophomonas maltophilia*, preferably Sma_KPR from *Stenotrophomonas maltophilia* NBRC 14161 or Cpa_PKR_2 from *Candida parapsilosis* IFO 0708, most preferably an enzyme with at least about 80% identity to SEQ ID NOs: 1 or 3, at a pH of about 7.5.
- (9) Process for production of (R)-pantolactone with e.e. of at least about 94%, comprising enantioselective reduction of keto-pantolactone in the presence of the enantioselective alcohol dehydrogenase ADH 440 (catalogue # evo 1.1.440,

evoXX GmbH, Germany) at a pH of about 5.5, preferably wherein the yield of (R)-pantolactone is at least about 59%.

The following examples are illustrative only and are not intended to limit the scope of the invention in any way.

5 Examples

Example 1: General methods and media

All basic molecular biology and DNA manipulation procedures described herein were generally performed according to Sambrook et al. (1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press: New York) or Ausubel
10 et al. (1998. Current Protocols in Molecular Biology. Wiley: New York). Media used for protein production are listed in Table 1.

Table 1: Media composition.

Medium	Components
LB	5 g/l yeast extract 10 g/l tryptone 5 g/l NaCl
2xTY	10 g/l yeast extract (final) 16 g/l tryptone 5 g/l NaCl

Example 2: Expression of enzymes in Escherichia coli

15 Gene encoding sequences 1, 3, 5, 7, were ordered codon-optimized for expression in E. coli as synthetic DNA at ATUM (Newark, California, US). All constructs were used for transformation of E. coli RV308ΔrhaBAD. Transformations were done as follows:

Competent cells were thawed on ice. Subsequently, 1 μl plasmid DNA was added
20 to the cells and the mix was incubated for 5 minutes on ice. After a heat shock treatment (60 sec, 42°C) cells were immediately placed on ice again for 5 minutes. 300 μl SOC medium (Catalogue # 15544034, Thermo Fisher Scientific, USA) were added and the cells were incubated for 1-2 hours at 37°C and gentle shaking (300 rpm). Subsequently, 50 μl of the reaction mix was plated on LB
25 medium containing 50 μg/ml neomycin. Alternatively, the full reaction mix was centrifuged (2 min, 6000 rpm) and the cell pellet was resuspended in 100 μl SOC

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medium and completely plated on the selective agar plates. Selection plates were incubated overnight at 37°C.

Recombinant strains were cultivated for ADH expression as follows: 20 ml of LB medium containing 100 mg/l neomycin were inoculated with the glycerol stock of the respective clone. The culture was incubated over-night at 28°C and with shaking at 110 rpm. The next day, 100 ml 2xTY medium containing 100 mg/l neomycin in a 500 ml baffled Erlenmeyer flask were inoculated with 1 ml of the preculture (initial OD₆₂₀ ~0.05). The culture was incubated on an orbital shaker (180 rpm) at 28°C. When the OD₆₂₀ reached ~0.5, the protein expression was induced by addition of 100 µl 20% (v/v) L-rhamnose (final concentration 0.02%). The cultivation of the strains was continued over-night (180 rpm, 28°C). On the next day, cells were harvested by centrifugation at 4°C for 15 min and 5000 rpm. Cell pellets were stored at -20°C.

For further studies cell free extract was prepared. Wet cells were resuspended in 200 vol% of 100 mM potassium phosphate buffer pH 7.0 with respect to their wet weight. The cell suspension was sonicated on ice until the OD₆₂₀ of the suspension was below 10% of the starting value. Cell debris was subsequently removed by centrifugation (4°C, 5000 rpm for 10 min). The clear supernatant (cell free extract, CFE) was stored until further use at -20°C.

Example 3: Enantioselective reduction of KPL into (R)-pantolactone in the presence of NADH

Formation of (R)-pantolactone was tested with different enzymes using NAD⁺ as co-factor at constant pH 5.5 or 7.5. Reactions on a 10 ml scale were performed with 100 mM KPL, MgCl₂ (1mM final conc.) ZnSO₄ (1mM final concentration), 5% (vol/vol) enzyme (CFE; see Ex. 2), 150 mM (final conc.) D-glucose & 20 U/ml (final conc.) of GDH (Codexis, GDH 105). NAD⁺ (0.7 mM final conc.) was added. Incubations were done at 28°C, shaken (stirrer speed 10) for 1 -3 hours. 250 µl sample was acidified with 25 µl of HCl (37%), heated at 80°C for 15 min and extracted with 900 µl ethyl acetate with internal standard (anisole, ca. 1 mg/ml). After 10 min of shaking, the phases separated within 10 min and a sample of the ethyl acetate layer was taken for analysis.

Measurements were carried out on an Agilent HP 6890 gas chromatograph equipped with hot-split injector, autosampler and FID detector. Column used was Chirasil DEX CB, dimensions 25 m x 250 µm x 0.25 µm film thickness. The isothermal temperature was 125°C during 12 minutes. As a carrier gas Helium

with a flow of 1.5 ml/min was used. Injection volume was 1 µl, with a split ratio of 1 : 30 at a hot split injection at 220°C. A flame ionisation detector was used at 250°C, with a hydrogen flow of 40 mL/min, an air flow of 450 mL/min and a make-up flow of 30 mL/min. The results for ketopantolactone reductase from *S. maltophilia* or polyketone reductase from *C. parapsilosis* (Cpa_PKR_2) compared to ADH 440 (evo-1.1.440.S, evoXX technology GmbH, Germany; see Table 3) are shown in Table 2 below.

Table 2A: (R)-selectivity of selected enzymes at pH 7.5. "ee R-PL" means ee of (R)-pantolactone measured at the end, "yield R-PL" means the percentage of (R)-pantolactone based on mol. All enzymes are used as CFE. The bio blank contains CFE of *E. coli* RV308ΔrhaBAD (no plasmid). The chemical blank contains all components of the reaction but no CFE. For more details, see text.

Enzyme	Mass balance (mol based)	ee R-PL	Yield R-PL
Sma_KPR	96	100	87
Cpa_PKR_2	107	99	59
Bio blank	107	62	2
Chemical blank	n.a.	n.a.	n.a.

Table 2B: (R)-selectivity of selected enzymes at pH 5.5. "ee R-PL" means ee of (R)-pantolactone measured at the end, "yield R-PL" means the percentage of (R)-pantolactone based on mol. All enzymes are used as CFE, except for ADH 440 which is in powder form. For more details, see text or legend to Table 2A.

Enzyme	Mass balance (mol based)	ee R-PL	Yield R-PL
Sma_KPR	97	85	87
Cpa_PKR_2	98	84	59
ADH 440	96	94	39
Bio blank	98	-35	5
Chemical blank	n.a.	n.a.	n.a.

Table 3: enzyme specification of ADH 440 (evo-1.1.440.S) according to the supplier (evoXX technology GmbH, Germany). "U" is defined as 1 unit reducing 1 µmol cyclohexanone per minute at pH 7.0 and 30°C.

CAS number	9028-12-0
EC number	1.1.1.2
Parent strain	Escherichia coli

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Biological origin	bacterial
Optimal pH range	reduction: 6.0-9.0 oxidation: 9.5-10.5
Cofactor	prefers NADP over NAD
Enzyme for cofactor regeneration	glucose dehydrogenase
Enzyme activity (U/mg)	≥1.0

Claims

1. Process for enantioselective conversion of keto-pantolactone into R-pantolactone, comprising incubation of keto-pantolactone in the presence of NADH or NAD⁺ and an NADPH dependent enzyme.
- 5 2. Process according to claim 1, wherein the enzyme is selected from alcohol dehydrogenase EC enzymes (EC 1.1.1.2) or pantoate 4-dehydrogenase (EC 1.1.1.106).
3. Process according to claim 1 or 2, wherein the pH is in the range of about 7.5 to about 4.5.
- 10 4. Process according to any one of claims 1 to 3, wherein the reducing enzyme is an alcohol dehydrogenase originating from *Candida*, *Escherichia* or *Stenotrophomonas*, particularly selected from *Candida parapsilosis* or *Stenotrophomonas maltophilia*, preferably Sma_KPR from *Stenotrophomonas maltophilia* NBRC 14161, Cpa_PKR_2 from *Candida parapsilosis* IFO 0708, most
15 preferably Sma_KPR from *Stenotrophomonas maltophilia* NBRC 14161 with at least about 80% identity to SEQ ID NO:1 or Cpa_PKR_2 from *Candida parapsilosis* IFO 0708 with at least about 80% identity to SEQ ID NO:3.
5. Process according to claim 4, wherein the reducing enzyme is a commercially available NADPH-dependent alcohol dehydrogenase, particularly
20 alcohol dehydrogenase (ADH) originating from *Escherichia coli*, such as ADH 440 (evo-1.1.440.S, evoXX technology GmbH, Germany).
6. Process according to any one of claims 1 to 5, wherein the enzyme is used as a recombinant whole cell biocatalyst or as cell free extract.
7. Process according to any one of claims 1 to 6, further comprising an in-situ
25 co-factor regeneration system, particularly comprising glucose dehydrogenase and glucose.
8. Process for production of (R)-pantolactone with an enantiomeric excess (e.e.) of at least about 99%, comprising enantioselective reduction of keto-pantolactone in the presence of an enantioselective ADH, particularly an ADH
30 enzyme selected from *Candida parapsilosis* or *Stenotrophomonas maltophilia*, preferably Sma_KPR from *Stenotrophomonas maltophilia* NBRC 14161 or Cpa_PKR_2 from *Candida parapsilosis* IFO 0708, most preferably an enzyme with at least about 80% identity to SEQ ID NOs: 1 or 3, at a pH of about 7.5.

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9. Process for production of (R)-pantolactone with e.e. of at least about 94%, comprising enantioselective reduction of keto-pantolactone in the presence of the enantioselective ADH originated from *Escherichia coli*, particularly ADH 440 (evo-1.1.440.S, evoXX technology GmbH, Germany) at a pH of about 5.5.
- 5 10. Process according to claim 9, wherein the yield of (R)-pantolactone is at least about 59%.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2021/078384

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/078384

A. CLASSIFICATION OF SUBJECT MATTER INV. C12P17/04 C12P41/00 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHAO MAN ET AL: "Asymmetric reduction of ketopantolactone using a strictly (R)-stereoselective carbonyl reductase through efficient NADPH regeneration and the substrate constant-feeding strategy", BIOTECHNOLOGY LETTERS, KLUWER ACADEMIC PUBLISHERS, DORDRECHT, vol. 39, no. 11, 21 August 2017 (2017-08-21), pages 1741-1746, XP036336790, ISSN: 0141-5492, DOI: 10.1007/S10529-017-2415-1 [retrieved on 2017-08-21] abstract; page 1742, paragraph 3 - column 2, paragraph 3; figure 1 ----- -/-	1, 3, 6, 7
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 22 December 2021		Date of mailing of the international search report 10/01/2022
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Schönwasser, D

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2021/078384

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KATAOKA M ET AL: "Gene cloning and overexpression of two conjugated polyketone reductases, novel aldo-keto reductase family enzymes, of Candida parapsilosis", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 64, no. 3, 31 October 2003 (2003-10-31), pages 359-366, XP019623550, ISSN: 1432-0614 page 359, column 2, paragraph 2 page 364, column 1, line 6 - column 2, line 10; figure 4b -----	1, 2, 4, 6, 8
A	JP 3 135740 B2 (OSAKA ORGANIC CHEM IND) 19 February 2001 (2001-02-19) [0006], [0010]; example 1 -----	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2021/078384

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 3135740 B2	19-02-2001	JP 3135740 B2	19-02-2001
		JP H06311889 A	08-11-1994
