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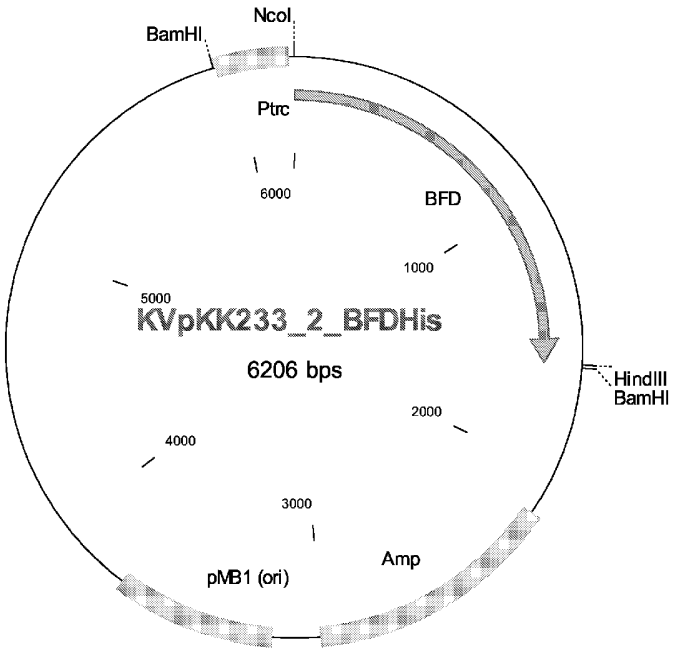
(54) Title: PROCESS FOR PREPARING ENANTIOMERICALLY ENRICHED α-HYDROXYKETONES



(57) Abstract: The present invention is directed towards a process for preparing alpha-hydroxyketones by reacting two aldehydes . The process under consideration operates enzymatically, preferably by use of benzaldehydelyase (BAL) or benzoylformate decarboxylase (BFD) , and makes it possible to obtain the desired compounds at high enantiomeric enrichments. The invention also relates to an appropriately modified whole-cell catalyst and its use in a process for preparing alpha-hydroxyketones.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Process for preparing enantiomerically enriched α-hydroxyketones

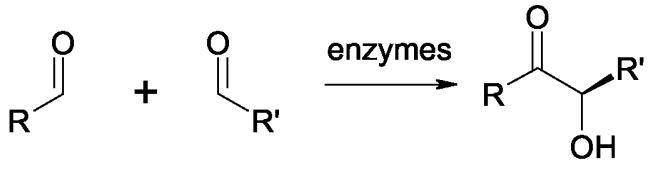
The present invention describes an enzymatic process for preparing enantiomerically enriched α-hydroxyketones. The process uses thiamine diphosphate (ThPP)-dependent enzymes which exhibit a CC ligase and/or CC lyase activity. The invention is furthermore directed towards a recombinant whole-cell catalyst and its use, with the catalyst comprising a cloned gene construct which encodes an enzyme as mentioned above.

Enantiomerically enriched α-hydroxyketones are valuable intermediates for preparing bioactive active compounds. They are frequently employed in the chemical industry, and in this context especially in organic synthesis, as starting materials for synthesizing pharmaceuticals or in the area of plant protection product synthesis. Because of the very mild conditions and high degree of specificity, the enzyme-catalyzed reaction represents a versatile alternative to the elaborated classical chemical methods for preparing such chiral compounds. The chemical methods can frequently only be used to synthesize racemic mixtures. The use of ThPP-dependent enzymes which exhibit CC ligase and/or CC lyase activity in stereoselective synthesis is consequently of great interest and has become ever more significant in recent years. An example of this is the use of pyruvate decarboxylase to prepare ephedrine precursors [Goetz, G.; Iwan, P.; Hauer, B.; Breuer, M.; Pohl, M. Biotechnology and Bioengineering 2001, 74(4), 317-325].

"CC ligase and/or CC lyase activity" is understood as meaning the formation of α-hydroxyketones from aldehydes or keto acids or their cleavage to give the aldehydes.

Thiamine diphosphate (ThPP)-dependent enzymes catalyze a whole number of biotransformations such as the enzymatic decarboxylation of keto acids to give aldehydes as well as the enzymatic C-N linkages (G. A. Sprenger, M. Pohl, J. Mol. Catal. B 1999, 6, 145-159). Furthermore, thiamine diphosphate (ThPP)-dependent enzymes such as aldehyde lyases (EC 4.1.2) can catalyze the asymmetric condensation of aldehydes to give α-hydroxyketones. The reaction under consideration here can be described as follows (Scheme 1).

Scheme 1



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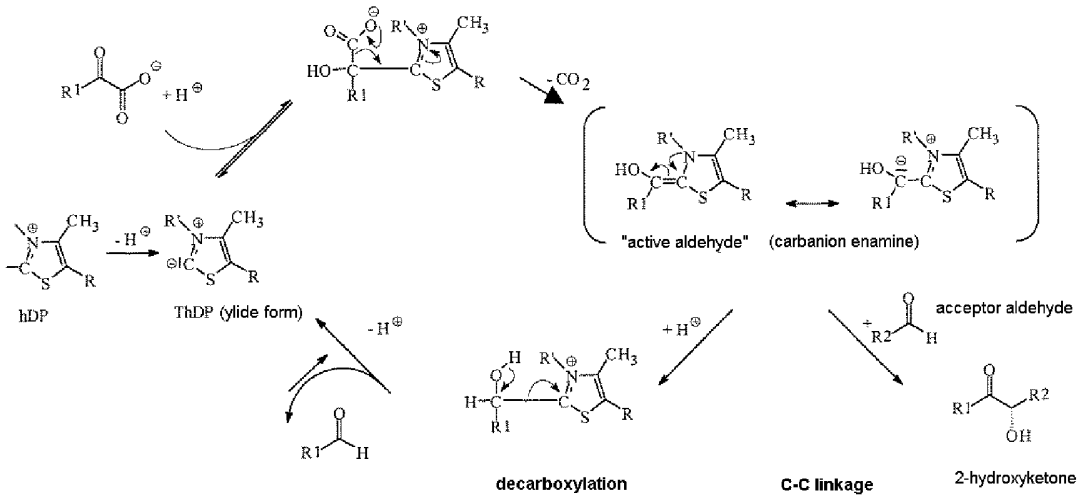
This enantioselective C-C bonding, which is termed carboligation, gives rise to optically active substances which, as has been said, can be found as structurally important subunits in biologically active natural products. Examples of enzymes of this nature are benzaldehyde lyase (BAL) [Gonzalez, B.; Vicuña, R. J. Bacteriol., 1989, 171, 2401-2405] and benzoylformate decarboxylase (BFD) [Wilcocks, R.; Ward, O. P.; Collins, S.; Dewdney, N. J.; Hong, Y.; Prosen, E., Applied and Environmental Microbiology 1992, 58, 1699-704].

ThPP is an essential cofactor which converts the substrates which are employed in the reaction into an activated form (Scheme 2).

Scheme 2



The following substrates have thus far been used in the reaction under consideration:

- condensation of aromatic aldehydes (substituted benzylaldehydes) to give benzoins [(a) Demir, A.S., Dünnwald, T., Iding, H., Pohl, M., and Müller, M. "Asymmetric benzoin reaction catalyzed by benzoylformate decarboxylase." Tetrahedron: Asymmetry, 1999, 10, 4769-4774; (b) Demir, A.S.; Sesenoglu, O.; Eren, E.; Hosrik, B.; Pohl, M.; Janzen, E.; Kolter, D.; Feldmann, R.; Dünkelmann, P.; Müller, M. Adv. Synth. Catal. 2002, 344, 96-103].

- mixed condensation of aromatic aldehydes and acetaldehyde to give derivatives of 2-HPP (2-hydroxy-propiophenone) [Dünnwald, T., Demir, A.S., Siegert, P., Pohl, M., and Müller, M. Eur. J. Org. Chem., 2000, 2161-2170].

- condensation of benzaldehyde with formaldehyde [Demir, A.S., Ayhan, P., Cigdem Igdir, A., Duygu, A.N. Tetrahedron, 2004, 60, 6509-6512].

- condensation of aromatic aldehydes with methoxy-derivatized acetaldehydes: [Demir, A.S.; Sesenoglu, O.;

Dünkelmann, P.; Müller, M. Org. Letters. 2003, 5, 2047-2050].

The conditions which have been employed to date for the reaction under consideration can be summarized as follows:

- the substrate concentration is normally 20 mM to at most 65 mM for the donor substrate when using BAL
- the pH has regularly been set in the range 6.0-7.5
- a single-phase reaction system consisting of phosphate buffer containing 20-30% DMSO is used
- the biocatalysts employed are the isolated enzymes
- the cofactor ThPP is added externally (0.15 mM)
- the Mg^{2+} metal addition is added externally (2.5 mM)

The aspects concerning the present reaction which have thus far become known do not provide support for using this reaction for the industrial-scale synthesis of enantiomerically enriched α-hydroxyketones.

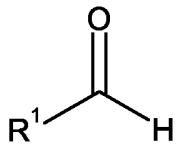
The object of the present invention was therefore to specify another process which helps to bring about the synthesis of enantiomerically enriched α-hydroxyketones. In particular, the process according to the invention should be able to implement the synthesis of these compounds on an industrial scale in a manner which is advantageous from the economic and/or ecological point of view. In this connection, attention is very particularly to be given to the matter of economizing on expensive synthesis aids, such as the cofactor ThPP, and/or of the efficiency of the reaction under consideration.

These objects, and other objects which are not mentioned in more detail but which ensue in an obvious manner from the prior art are achieved by means of a process having the

features of Claim 1. Other preferred embodiments of the process according to the invention are protected in the following subclaims two to five. The concluding Claims 6 to 15 are directed towards a whole-cell catalyst according to the invention and its use in synthesis for preparing α-hydroxyketones.

The set object is, completely surprisingly and extremely advantageously, achieved by, in a process for preparing enantiomerically enriched α-hydroxyketones by reacting two aldehydes in the presence of a thiamine diphosphate (ThPP)-dependent enzyme possessing CC ligase and/or CC lyase activity, operating in an aqueous medium at a pH of 8-10. Previously, a pH of 6.0 to 7.5 for the reaction in question has always been favoured in the literature. The CC bond-forming enzymes, such as PDC, which act in a manner which is comparatively similar, likewise also have their pH optimum in the neutral pH range. Furthermore, there was the danger that, as C,H-acid compounds, the α-hydroxyketones which were formed in the reaction according to the invention would, in the given pH range, be subject to racemization and aldol condensation. Both considerations would have kept the skilled person from using the described reaction conditions. In this respect, it is not possible to assume that the reaction according to the invention is obvious.

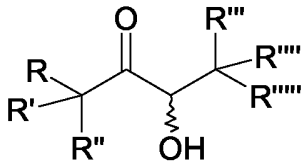
In principle, any aldehydes which the skilled person takes into consideration for the present purpose can be used in the reaction. Preferably, two identical or two different linear or branched-chain aliphatic or aromatic aldehydes can be employed in the reaction according to the invention. Particular preference is given to using compounds of the general formula II in this connection,



(II)

in which
R^1 can be H, (C_1-C_{15})-alkyl, (C_2-C_{15})-alkenyl, (C_2-C_{15})-alkynyl, (C_2-C_{15})-alkoxyalkyl, (C_6-C_{18})-aryl, (C_7-C_{19})-aralkyl, (C_3-C_{18})-heteroaryl, (C_4-C_{19})-heteroalkyl, (C_1-C_8)-alkyl-(C_6-C_{18})-aryl, (C_1-C_8)-alkyl-(C_3-C_{19})-heteroaryl, (C_3-C_8)-cycloalkyl, (C_1-C_8)-alkyl-(C_3-C_8)-cycloalkyl or (C_3-C_8)-cycloalkyl-(C_1-C_8)-alkyl.

Astonishingly, it has been possible to show that using BAL or BFD and proceeding from appropriate aldehydes makes it possible to synthesize saturated or unsaturated, highly enantiomerically enriched aliphatic α-hydroxyketones of the formula 3(see Table 3),



3

in which

R, R′, R″ , R‴, R⁗ , R ⁗⁗ can be, independently of each other, H, (C_1-C_{15})-alkyl, (C_2-C_{15})-alkenyl, (C_2-C_{15})-alkynyl or (C_2-C_{15})-alkoxyalkyl. (C_1-C_8)-Alkyl and (C_2-C_8)-alkoxy-alkyl are preferred radicals. Radicals R = R⁗ = *iso*-propyl, R = R″ = R⁗ = R⁗⁗ = methyloxy and R = R⁗ = n-butyl are particularly preferred. It was not readily possible to deduce this from the data which were known from

the literature. The acceptance of such branched radicals is surprising for steric reasons, in particular.

The skilled person is free to choose the enzymes which he uses for the reaction according to the invention. The enzymes are those possessing CC ligase and/or CC lyase activity (EC 4.1.2). Within the context of the invention, "CC ligase or CC lyase activity" is understood as meaning the ability to create CC bonds between two carbonyl functions such that α-hydroxyketones are obtained, or to cleave the CC bond between a carbonyl function and a hydroxyl group which is in the α position relative to it. In the ligase reaction, the CC bond is synthesized in accordance with what has been said above, such that an α-hydroxyketone which is highly enantiomerically enriched is formed. In the lyase reaction, racemic or slightly enantiomerically enriched α-hydroxyketones are enantioselectively cleaved, with this leading in turn to α-hydroxyketones which are highly enantiomerically enriched in the unreacted isomer.

Enzymes which can be employed in this manner are described in Enzyme Catalysis in Organic Synthesis, 1995, Vol. II, p. 575 and Pohl, M.; Sprenger, G. A.; Müller, M. Current Opinion in Biotechnology 2004, 15(4), 335-342. Other source organisms for such enzymes can be *Streptomyces, S. cerevisiae, Acinetobacter, Archaea, E. coli, yeasts, Zymomonas, Bacteroides fragilis and Klebsiella*. Preference is given to using a benzaldehyde lyase(BAL) or benzoylformate decarboxylase(BFD), or the mutants which are derived from these enzymes, in this connection. These enzymes are preferably derived from the organism *Pseudomonas*, very particularly preferably *Pseudomonas fluorescens* (Seq. ID NO: 2) or *Pseudomonas putida* (Seq. ID NO: 1). With regard to generating suitable mutants, the reader may be referred to methods in the prior art (S. Chusacultanachai, Y. Yuthavong, Methods in Molecular

Biology, Vol. 270, Ed. S. E. Melville, Humana Press, page 319-333; B. Lingen, J. Grötziner, D. Kolter, M.-R.Kula, M. Pohl, Protein Engineering, 2002, 15, 858-593).

The reaction under consideration can be carried out in water as solvent. However, for reasons of the solubility of the substrates employed, it may be advantageous to carry out the underlying reaction in an aqueous medium in which at least one further organic solvent which is soluble or insoluble in water and which is selected from the group consisting of ethers, alcohols, esters, ketones, acid amides, alkanes, aromatic compounds and sulphoxides is added to the water phase. Very particular preference is given to additionally using solvents which are selected from the group consisting of ethers and sulphoxides.

In a number of applications, it has proved to be advantageous to switch to a two-phase system composed of water and an organic phase, preferably ether or ester, which is immiscible with water. Particular preference is given to methyl *tert*-butyl ether and ethyl acetate in this connection.
Using the two-phase system makes it possible to achieve markedly higher substrate and product concentrations in the medium, with this resulting in a higher space/time yield. Whereas molarities of at most 65 mM in the reaction have been described in the literature to date, molarities of 1 M of substrate can be achieved in this way using the process which is described herein. Whereas a markedly elevated excess (up to a 6-fold excess) of acceptor aldehyde frequently has to be added in single-phase systems, this is no longer necessary when using the two-phase system. This helps to save consumption costs and is therefore particularly advantageous against the economic background. The reader is referred to Tables 1 and 2 for the results of the reactions in a two-phase system.

As has already been intimated above, it has proved, in contrast to the teaching disclosed in the prior art, to be advantageous for the reaction according to the invention to carry it out in a pH range of from 8.0 to 10. As Figure 1 shows, the pH optimum for the reaction under consideration is pH 9.5. The reaction according to the invention is therefore preferably carried out in a pH range of 9.5 ± 0.5, particularly preferably 9.5 ± 0.2.

The present invention also relates to a recombinant whole-cell catalyst which comprises a cloned gene construct which encodes a thiamine diphosphate (ThPP)-dependent enzyme possessing CC ligase and/or CC lyase activity, with the host organism being DSM 14459.

For the use of particular enzymes having the desired activity, the reader is referred to what has been stated above with regard to the process. Very particular preference is also given here to using a gene construct which encodes a benzaldehyde lyase (BAL) or a benzoylformate decarboxylase (BFD) or the mutants which are derived from these enzymes.

The whole-cell catalyst is prepared using methods of the prior art (Sambrook, J.; Fritsch, E. F. und Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York). Plasmids which are preferably used for cloning the gene construct, which exhibits the nucleic acid according to the invention, into the host organism are likewise known to the skilled person (see also WO 04005517; see below). Suitable plasmids or vectors are in principle any embodiments which are available to the skilled person for this purpose. These plasmids and vectors are listed, for example, in Studier and coworkers (Studier, W. F.; Rosenberg A. H.; Dunn J. J.; Dubendroff J. W.; (1990), Use of the T7 RNA polymerase to direct expression of cloned genes, Methods Enzymol. 185, 61-89) or in the brochures supplied by the companies

Novagen, Promega, New England Biolabs, Clontech or Gibco BRL. Other preferred plasmids and vectors can be found in: Glover, D. M. (1985), DNA cloning: a practical approach, Vol. I-III, IRL Press Ltd., Oxford; Rodriguez, R.L. and Denhardt, D. T (eds) (1988), Vectors: a survey of molecular cloning vectors and their uses, 179-204, Butterworth, Stoneham; Goeddel, D. V. (1990), Systems for heterologous gene expression, Methods Enzymol. 185, 3-7; Sambrook, J.; Fritsch, E. F. und Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York.

Plasmids which can very preferably be used to clone the gene constructs exhibiting the nucleic acid sequences under consideration into the host organism are, or are based on: pUC18/19 (Roche Biochemicals), pKK-177-3H (Roche Biochemicals), pBTac2 (Roche Biochemicals), pKK223-3 (Amersham Pharmacia Biotech), pKK-233-3 (Stratagene) or pET (Novagen). Extreme preference is given, in this connection, to pBAL, $pBAL_{his}$, PKK233_2_BALhis; pBFD/trc, pBDF and PKK233_2_BFDhis (Fig. 3 and Fig. 4).

The use of a recombinant whole-cell catalyst which comprises a cloned gene construct which encodes a thiamine diphosphate (ThPP)-dependent enzyme possessing CC ligase and/or CC lyase activity in a process for preparing enantiomerically enriched α-hydroxyketones forms a concluding part of the subject-matter of the present invention.

With regard to the preferred embodiments of the use, according to the invention, of the whole-cell catalyst, the reader is referred to what has been said above in connection with using the enzymes in the process according to the invention. The embodiments which were preferred there can be used in an equivalent manner when employing the whole-cell catalyst.

Any known host cells are suitable for being used as the whole-cell catalyst according to the invention. Examples of organisms which may be mentioned as such in this regard are yeasts such as *Hansenula polymorpha*, *Pichia sp. and Saccharomyces cerevisiae*, prokaryotes, such as *E. coli* and *Bacillus subtilis*, or eukaryotes, such as mammalian cells, insect cells or plant cells. The methods for cloning are well known to the skilled person (Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York). Preference is given to using *E. coli* strains for this purpose. Those which are very particularly preferred are: E. coli XL1 Blue, NM 522, JM101, JM109, JM105, RR1, DH5α, TOP 10-, HB101, BL21 codon plus, BL21 (DE3) codon plus, BL21, BL21 (DE3) and MM294. Extreme preference is given to using, in this connection, the organism (DSM 14459) which is described in EP 1444367.

In a preferred embodiment of the use according to the invention, the whole-cell catalyst is preferably used in the reaction without any prior lysis treatment, i.e. coming directly from the fermentation. However, it can also be advantageous to pretreat it before its use such that the permeability of the cell membrane for the substrates and products is increased as compared with that of the intact system. Particular preference is given, in this connection, to a process in which the whole-cell catalyst is, for example, pretreated by being frozen and/or treated with an organic component, e.g. toluene.

According to the invention, the whole-cell catalyst can surprisingly be used without the addition of an "external" cofactor (Fig. 2). This means that no additional cofactor has to be added to the whole-cell mixture since the cells themselves already contain a cofactor which is suitable for the conversion reaction and can evidently use this cofactor. Cofactors which are suitable for the reaction are

to be understood, in particular, as meaning ThPP. This is, however, particularly surprising in the light of the fact that the intracellular quantity of ThPP is actually too low for a preparative conversion. The cofactor can no longer be formed subsequently in the cells which have died, as is the case in fermentation processes, for example when pyruvate decarboxylase is used for preparing ephedrine precursors. The high conversion rates and productivities observed when using the whole-cell catalyst under the described limiting conditions were therefore in no way to be expected. The high yields of product are also all the more surprising when consideration is taken of the fact that the cell contains other enzymes, such as alcohol dehydrogenases, which can involve the substrates in side reactions. The ready diffusion of the substrates through the cell walls and membranes, and also the diffusion of the products back into the medium, were not to be foreseen, either.

As already described in the process above, the whole-cell catalyst can be used both for synthesizing the α-hydroxyketones enantioselectively and for resolving racemic or slightly enantiomerically enriched α-hydroxyketones enantioselectively. Products which are highly enantiomerically enriched are obtained in both cases.

The enzyme under consideration can be used for the process according to the invention in free form, as a homogeneously purified compound or as a recombinantly prepared enzyme. As already intimated above, the enzyme can also be employed as a component of an intact recombinantly prepared host organism or in combination with the host organism cell mass which has been disrupted and purified to as high a degree as desired.

It is likewise possible to use the enzymes in immobilized form (Sharma B. P.; Bailey L. F. and Messing R. A. (1982), Immobilisierte Biomaterialien - Techniken und Anwendungen

[Immobilized biomaterials - techniques and applications], Angew. Chem. 94, 836-852). The immobilization is advantageously effected by means of lyophilization (Paradkar, V. M.; Dordick, J. S. (1994), Aqueous-Like Activity of α-Chymotrypsin Dissolved in Nearly Anhydrous Organic Solvents, J. Am. Chem. Soc. 116, 5009-5010; Mori, T.; Okahata, Y. (1997), A variety of lipi-coated glycoside hydrolases as effective glycosyl transfer catalysts in homogeneous organic solvents, Tetrahedron Lett. 38, 1971-1974; Otamiri, M.; Adlercreutz, P.; Matthiasson, B. (1992), Complex formation between chymotrypsin and ethyl cellulose as a means to solubilize the enzyme in active form in toluene, Biocatalysis 6, 291-305). Very particular preference is given to lyophilization in the presence of surface-active substances such as Aerosol OT or polyvinylpyrrolidone or polyethylene glycol (PEG) or Brij 52 (diethylene glycol monocetyl ether) (Kamiya, N.; Okazaki, S.-Y.; Goto, M. (1997), Surfactant-horseradish peroxidase complex catalytically active in anhydrous benzene, Biotechnol. Tech. 11, 375-378). It is likewise possible to conceive of using the enzymes as CLECs (St. Clair, N.; Wang, Y.-F.; Margolin, A. L. (2000), Cofactor-bound cross-linked enzyme crystals (CLEC) of alcohol dehydrogenase, Angew. Chem. Int. Ed. 39, 380-383).

In a general embodiment when using the abovementioned enzymes in the process according to the invention, the skilled person proceeds by suspending the substrate and enzymes, which have the appropriate activity and which are in the intended form (homogeneously purified, as CLECs, immobilized, etc.), in an appropriate medium, preferably an aqueous two-phase mixture, and leaving them at an appropriate temperature which is in the range from 0 to 100 degrees celsius, preferably in the range from 20 to 80 degrees celsius and very particularly preferably in the range from 30 to 50 degrees celsius.

With regard to using the enzymes and using the whole-cell

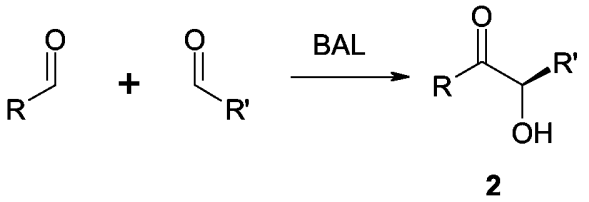
catalyst, the process according to the invention can take place as a batch process. The biomass can readily be separated from the product by means of filtration or centrifugation. The resulting α-hydroxyketones can then be isolated using customary methods (chromatography, crystallization, etc.).

However, the process under consideration can be carried out continuously and, in particular, the whole-cell catalysts can be used continuously. For this, the reaction is effected in what is termed an enzyme-membrane reactor, in which high molecular weight substances, i.e. the biomass, are retained behind an ultrafiltration membrane and low molecular weight substances, such as the products which are produced, are able to pass through the membrane. A procedure of this nature has already been described many times in the prior art (Wandrey et al. in Jahrbuch 1998, Verfahrenstechnik und Chemieingenieurwesen [1998 Yearbook, Process Technology and Chemical Engineering], VDI, pp. 151ff; Kragl et al., Angew. Chem. 1996, 6, 684).

As already intimated, the conversion of the substrate employed into the desired product can take place in cell culture using a suitable whole-cell catalyst. A nutrient medium which is suitable for the host organism employed is used in this connection. The media which are suitable for the host cells are well known and commercially available. In addition, customary additives, such as antibiotics, growth-promoting agents, such as sera (fetal calf serum, etc.) and similar known additives, can be added to the cell cultures.

As is evident from the following results of the experimental studies, the whole-cell catalyst under consideration, or the free enzymes, can be used to achieve outstanding yields and enantiomeric excesses with regard to the α-hydroxyketone which is in each case obtained (Tables 1 to 3).

Table 1.- BAL-catalyzed carboligation of different substrates in a two-phase system. [a]



2

2a: R = R' = phenyl
2b: R = phenyl, R' = CH_3
2c R = phenyl, R' = $CH(OCH_3)_2$
2d R = furyl, R' = $CH(OCH_3)_2$
2e R = phenyl, R' = $CHCH_2$

Donor (M) [b]	Acceptor (M) [b]	Conversion (time)	Yield (%)	e.e. (%)
benzaldehyde (0.5)	benzaldehyde (0.5)	90% (24 h)	80 (**2a**)	-
benzaldehyde (0.5)	acetaldehyde (0.65)	90% (24 h)	70 (**2b**)	98
benzaldehyde (1)	acetaldehyde (1.25)	90% (24 h)	70 (**2b**)	98
benzaldehyde (0.4)	2,2-dimethoxy-acetaldehyde (1.2)	95% (20 h)	78 (**2c**)	98
furfural (0.4)	2,2-dimethoxy-acetaldehyde (1.2)	95% (16 h)	90 (**2d**)	98
benzaldehyde (0.05)	acrolein (0.15)	-	30 (**2e**)	98
benzaldehyde (0.1)	acrolein (0.15)	60% (20 h)	50 (**2e**)	98
2,2-dimethoxy-acetaldehyde (0.5)	2,2-dimethoxy-acetaldehyde (0.5)	60% (40 h)	50 (2f)	95

[a]: MTBE 25 mL. 25 ml of buffer (100 mM pH 7-9). 1 g of BAL whole-cell catalyst without any addition of cofactor.

[b] the concentration is based on the volume employed in the aqueous phase

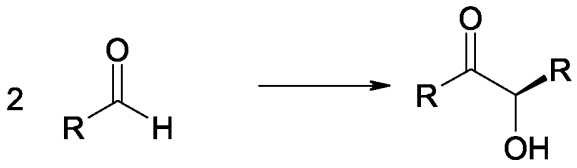
Table 2. BFD-catalyzed carboligation of benzaldehyde and acetaldehyde in a two-phase system.[a]

Benzaldehyde	Acetaldehyde	Conversion (time)	Yield (% 2-HPP)[a]	ee (S)-2-HPP
100 mM	900 mM	90% (16 h)	60	94%
300 mM	900 mM	80% (20 h)	60	94%
400 mM	1200 mM	70% [b] (60 h)	n.d.	94%
800 mM	2400 mM	40% (40 h)	n.d.	94%

[a]: MTBE 25 mL. 25 ml of buffer, 100 mM pH 7-9. 1 g BFD whole-cell catalyst without any addition of cofactor.

[b] 60 g of whole-cell catalyst/l

Table 3.- Carboligation of aliphatic aldehydes catalyzed by BAL and BFD.[a]



2f: R = $CH(OCH_3)_2$
3a: R = CH_3
3b: R = CH_2CH_3
3c R = $(CH_2)_2CH_3$
3d $(CH_2)_3CH_3$
3e $CH_2CH(CH_3)_2$

Aldehyde (mM)	Cosolvent	BAL		BFD	
		Conversion (%) (5-20 h)	ee	Conversion (5-20 h)	ee
acetaldehyde (50)	-	> 90 (**3a**)[a)]	20	>90 (**3a**)[a)]	18 % (S)
propanal (50)	-	> 90-(**3b**)[a)]	40 (R)	>90 (**3b**) [a)]	60 (S)
butanal (50)	-	> 90 (**3c**)[a)]	50 (S)	>90 (**3c**) [a)]	80 (S)
butanal (50)	DMSO (20%)	> 90 (**3c**)[a)]	55 (S)	>90 (**3c**) [a)]	80 (S)
butanal (50)	iPr-OH (20%)	> 90 (**3c**)[a)]	75-80 (S)	>90 (**3c**) [a)]	80 (S)
butanal (100)	iPr-OH (20%)	>95 (**3c**)[a)]	76 (S)		
butanal (100)	iPr-OH (20%)	>95 (**3c**)[b)]	76 (S)		
butanal (400)	iPr-OH (20%)	80 (**3c**)[b)]	76 (S)		
pentanal (50)	-	> 90(**3d**)[a)]	30 (S)	>90 (**3d**)[a)]	65 (S)
pentanal (50)	DMSO (20%)	> 90 (**3d**)[a)]	30 (S)	>90 (**3d**)[a)]	65 (S)
pentanal (50)	IPr-OH (20%)	> 90 (**3d**)[a)]	60 (S)	>90 (**3d**)[a)]	65 (S)
3-methylbutanal (50)	-	> 90 (**3e**)[a)]	90		
3-methylbutanal (100)	-	90 (**3e**)[b)]	ca. 90		
2,2-methoxy-acetaldehyde (50)	-	95 (**2f**) [a)]	96		
2,2-methoxy-acetaldehyde (200)	-	90 (**2f**) [b)]	90 - 95		

[a)] free enzyme: 1000 U

[b)] whole-cell catalyst, 40 g/l

The advantages which are achieved when the process according to the invention and/or the whole-cell catalyst according to the invention are used for asymmetric enzymatic carboligation and its reverse reaction are not rendered obvious by the prior art. The implementation of the process according to the invention in the appropriate pH range and/or in a two-phase system enables the enzymes under consideration to be used in a much more efficient manner. Expensive cofactors are preferably not added when the whole-cell catalyst is used, with this helping to economize on the cost of substances employed.

According to the present invention, a "whole-cell catalyst" is to be understood as meaning an intact cell in which at least one gene which can catalyze the conversion, according to the invention, of a substrate into a product is expressed. According to the invention, the intact cell is able to express a ThPP-dependent CC lyase and/or CC ligase. Preference is given to the whole-cell catalyst being a recombinantly altered microorganism which is adapted to the requirements of the desired reaction. The two whole-cell catalysts which are described in the experimental section are preferred as being particularly suitable whole-cell catalysts.

(C_1-C_8)-Alkyl is to be regarded as being methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl, *tert*-butyl, pentyl, hexyl, heptyl or octyl and all bond isomers. The (C_1-C_8)-alkyl can be singly or multiply substituted by halogen-containing radicals and/or radicals which contain other heteroatoms. (C_1-C_{15})-Alkyl is to be understood correspondingly except that in this case up to 15 C atoms can be present in the radical.

(C_2-C_8)-Alkenyl is to be understood as being a (C_1-C_8)-alkyl radical as described above, with the exception of methyl, which possesses at least one double bond. (C_2-C_{15})-Alkenyl

is to be understood correspondingly except that in this case up to 15 C atoms can be present in the radical.

(C_2-C_8)-Alkynyl is to be understood as meaning a (C_1-C_8)-alkyl radical as described above, with the exception of methyl, which possesses at least one triple bond. (C_2-C_{15})-Alkynyl is to be understood correspondingly except that in this case up to 15 C atoms can be present in the radical.

(C_1-C_8)-Acyl is understood as meaning a (C_1-C_8)-alkyl radical which is bonded to the molecule by way of a C=O function.

(C_3-C_8)-Cycloalkyl is understood as meaning cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl or cycloheptyl radicals, etc.

A (C_6-C_{18})-aryl radical is understood as meaning an aromatic radical which possesses 6 to 18 C atoms. This includes, in particular, compounds such as phenyl, naphthyl, anthryl, phenanthryl and biphenylyl radicals. The (C_6-C_{18})-aryl radical can be substituted singly or multiply by (C_1-C_8)-alkyl, (C_1-C_8)-alkoxy, (C_1-C_8)-haloalkyl, halogen, OH, $N((C_1-C_8)\text{-alkyl})_2$, NO_2, (C_1-C_8)-acyl, $NH(C_1-C_8)$-acyl or $N((C_1-C_8)\text{-acyl})_2$.

A (C_7-C_{19})-aralkyl radical is a (C_6-C_{18})-aryl radical which is bonded to the molecule by way of a (C_1-C_8)-alkyl radical.

(C_1-C_8)-Alkoxy is a (C_1-C_8)-alkyl radical which is bonded to the molecule under consideration by way of an oxygen atom.

(C_2-C_8)-Alkoxyalkyl is a (C_1-C_8)-alkyl radical which has an oxygen atom in the C chain.

(C_1-C_8)-Haloalkyl is a (C_1-C_8)-alkyl radical which is substituted by one or more halogen atoms.

Within the context of the invention, a (C_3-C_{18})-heteroaryl radical designates a 5-membered, 6-membered or 7-membered aromatic ring system which is composed of from 3 to 18 C atoms and which possesses heteroatoms, such as nitrogen, oxygen or sulphur, in the ring. These heteroaromatic radicals are regarded as being, in particular, radicals such as 1-, 2- or 3-furyl, 1-, 2- or 3-pyrrolyl, 1-,2- or 3-thienyl, 2-, 3- or 4-pyridyl, 2-, 3-, 4-, 5-, 6- or 7-indolyl, 3-, 4- or 5-pyrazolyl, 2-,4- or 5-imidazolyl, acridinyl, quinolinyl, phenanthridinyl or 2-, 4-, 5- or 6-pyrimidinyl. This radical may be substituted singly or multiply by (C_1-C_8)-alkyl, (C_1-C_8)-alkoxy, (C_1-C_8)-haloalkyl, halogen, OH, NO_2, N((C_1-C_8)-alkyl)$_2$, (C_1-C_8)-acyl, NH(C_1-C_8)-acyl or N((C_1-C_8)-acyl)$_2$.

A (C_4-C_{19})-heteroaralkyl is understood as meaning a heteroaromatic system which corresponds to the (C_7-C_{19})-aralkyl radical.

Suitable halogens (Hal, halogen atom) are fluorine, chlorine, bromine and iodine. Chlorine and bromine are preferred. This applies in a corresponding manner to the halide ions.

The described chemical structures relate to all the possible stereoisomers which can be attained by altering the configuration of the individual chiral centres, axes or planes, that is to all the possible diastereomers and to all the optical isomers, or their mixtures, which form a part thereof.

Within the context of the invention, the term enantiomerically enriched is understood as meaning the proportion, in a range of > 50% and < 100%, of an enantiomer when present in a mixture together with its optical antipode.

Within the context of the invention, the term diastereomeric enrichment is understood as meaning the proportion of a diastereomer, in a range of > 50% and < 100%, when present in a mixture together with its other diastereomers.

Explanation of the Figures:

Fig. 1. pH profile of BAL in connection with the conversion of benzaldehyde to benzoin and of benzaldehyde and acetaldehyde to 2-HPP.

Fig. 2. Conversion of benzaldehyde (400 mM) and dimethoxyaldehyde (1.2 mM) in 25 ml of MTBE and 25 ml of phosphate buffer (pH 9.5). When using the BAL whole-cell catalyst, 40 g of cells/litre; when adding cofactor: 0.3 mM ThPP, 2.5 mM $MgSO_4$ (the concentrations are based on the water phase).

Examples:

Chemicals and biocatalysts. - all the reagents were obtained commercially and used without further purification.

The preparation of the BAL whole-cell catalyst and of the BFD whole-cell catalyst, as well as the isolation of the BAL and BFD enzyme, were carried out in accordance with Iding, H.; Dünnwald, T.; Greiner, L.; Liese, A.; Müller, M.; Saegert, P.; Grötzinger, J.; Demir, A.S.; Pohl, M. Chem. Eur. J. 2000, 6, 1483-1495, M. Sanchez-Gonzales, J. P. N. Rosazza, Adv. Synth. Catal. 2003, 345, 819-824 and the literature references described therein.

The plasmid used for BFD (Seq. ID NO: 1) is pKK233_2_BFD_His (Fig. 4).

The plasmids used for BAL (Seq. ID NO: 2) are pKK233_2_BAL_His (Fig. 3) and pBAL [E. Janzen, Dissertation, Heinrich Heine University, Düsseldorf, 2002, A. Demir, M. Pohl, E. Janzen, M. Müller, J. Chem. Soc. Perkin Transactions 1, 2001, 633-635].

Analysis: the conversion and the ee were determined by means of chiral GC using a Chirasil-DEX CB (Varian) column, 25 M × 0.32 mm, fitted with an FID detector.

The CC ligase activity was determined in accordance with Demir, A. S.; Sesenoglu, O.; Eren, E.; Hosrik, B.; Pohl, M.; Janzen, E.; Kolter, D.; Feldmann, R.; Dunkelmann, P.; Müller, M. Advanced Synthesis & Catalysis 2002, 344(1), 96-103.

Example 1: Reactions using the BAL whole-cell catalyst in a two-phase system for preparing α-hydroxyketones

2 g of the BAL whole-cell catalyst cells are taken up in 25 ml of phosphate buffer (100 mM, pH 9) and treated with 25 ml of methyl tert-butyl ether. The substrates are then added. The reaction mixture is stirred at room temperature for 24 h. (Results, see Table 1.)

Example 2: Reactions using the BFD whole-cell catalyst in a two-phase system for preparing α-hydroxyketones

2 g of the BFD whole-cell catalyst cells are taken up in 25 ml of phosphate buffer (100 mM, pH 9) and treated with 25 ml of methyl tert-butyl ether. The substrates are then added. The reaction mixture is stirred at room temperature for 24 h. (Results, see Table 2.)

Example 3: Reactions using free BFD or BAL for preparing α-hydroxyketones

Free BAL or BFD (approx. 100-1000 U) is added to 50 ml of phosphate buffer (50 mM, 2.5 mM $MgSO_4$ and 0.3 mM ThdP; pH 8) with or without cosolvent (none, DMSO, iPr-OH) after which the substrates are added. The reaction is stirred at room temperature. The reaction mixture is extracted with dichloromethane (4 × 50 ml) and the organic phase is washed 3 times with water. The solvent is stripped off after drying over $MgSO_4$ (Results, see Table 3).

Example 4: Synthesizing different aliphatic α-hydroxyketones

Different aliphatic α-hydroxyketones are synthesized as described in Example 3 (Results, see Table 3)

1,1,4,4-Tetramethoxy-3-hydroxy-2-butanone (**2f**): yellow liquid. ^{1}H-NMR: δ (ppm) 3.3 (6H, ss), 3.5 (6H, ss), 4.5 (1H d), 4.65 (1H d), 4.9 (1H, s). ^{13}C-NMR: δ (ppm) 54.56 (O$\underline{C}$H$_3$), 55.06 (O$\underline{C}$H$_3$), 55.90 (O$\underline{C}$H$_3$), 57.18 (O$\underline{C}$H$_3$), 74.98 ($\underline{C}$H-OH), 102.27 (O$\underline{C}$HO), 105.72 (O$\underline{C}$HO), 203.30 ($\underline{C}$=O).

3-Hydroxy-2-butanone (**3a**): white solid. ^{1}H-NMR: δ (ppm) 1.4 (3H, d), 2.2 (3H, s), 3.5 (1H CH-O$\underline{H}$, s), 4.25 (1H, C$\underline{H}$-OH, m).

4-Hydroxy-3-hexanone (**3b**): colourless liquid. ^{1}H-NMR: δ (ppm) 0.9 (3H, t), 1.15 (3H, t), 1.6 (2H m), 2.5 (2H, m), 3.4 (1H CH-O$\underline{H}$, d), 4.2 (1H, C$\underline{H}$-OH, d).

5-Hydroxy-4-octanone (**3c**): yellow liquid. ^{1}H-NMR: δ (ppm) 0.9 (6H, t), 1.2-1.8 (8H, mm), 3.5 (1H CH-O$\underline{H}$, d), 4.2 (1H, C$\underline{H}$-OH, d). ^{13}C-NMR: δ (ppm) 15.07 (CH$_3$), 15.23 (CH$_3$), 17.8 (CH$_3$-$\underline{C}$H$_2$), 19.42 (CH$_3$-$\underline{C}$H$_2$), 37.21 ($\underline{C}$H$_2$-CHOH, s), 40.96 ($\underline{C}$H$_2$-CO), 77.51 ($\underline{C}$H-OH), 213.68 ($\underline{C}$=O).

6-Hydroxy-5-decanone (**3d**): colourless liquid. ^{1}H-NMR: δ (ppm) 0.9 (6H, dd), 1.3-1.8 (12H, mm), 2.45 (2H C$\underline{H}_2$-CO, m), 3.4 (1H, CH-O$\underline{H}$, d), 4.2 (1H, C$\underline{H}$-OH, d).

Patent Claims:

1. Process for preparing enantiomerically enriched α-hydroxyketones by reacting two aldehydes in the presence of a thiamine diphosphate (ThPP)-dependent enzyme possessing CC ligase and/or CC lyase activity in an aqueous medium at a pH of 8-10.

2. Process according to Claim 1, characterized in that the aldehydes employed are two identical or two different linear or branched-chain aliphatic or aromatic aldehydes.

3. Process according to Claim 1 and/or 2, characterized in that the enzymes employed are a benzaldehyde lyase (BAL) or a benzoylformate decarboxylase (BFD).

4. Process according to one or more of Claims 1 to 3, characterized in that use is made of an aqueous medium in which at least one further organic solvent which is soluble or insoluble in water and which is selected from the group consisting of ethers, alcohols, esters, ketones, acid amides, alkanes, aromatic compounds and sulphoxides is added to the water phase.

5. Process according to one or more of Claims 1 to 4, characterized in that the reaction is carried out at a pH of 9.5 ± 0.5.

6. Recombinant whole-cell catalyst which comprises a cloned gene construct which encodes a thiamine diphosphate (ThPP)-dependent enzyme possessing CC ligase and/or CC lyase activity, with the host organism being DSM 14459.

7. Whole-cell catalyst according to Claim 6, characterized in that the gene construct encodes a benzaldehyde lyase (BAL) or a benzoylformate decarboxylase (BFD).

8. Use of a recombinant whole-cell catalyst which comprises a cloned gene construct which encodes a thiamine diphosphate (ThPP)-dependent enzyme possessing CC ligase and/or CC lyase activity in a process for preparing enantiomerically enriched α-hydroxyketones.

9. Use according to Claim 8, characterized in that the whole-cell catalyst is used without any prior lysis treatment.

10. Use according to Claim 8 and/or 9, characterized in that no additional cofactor is added.

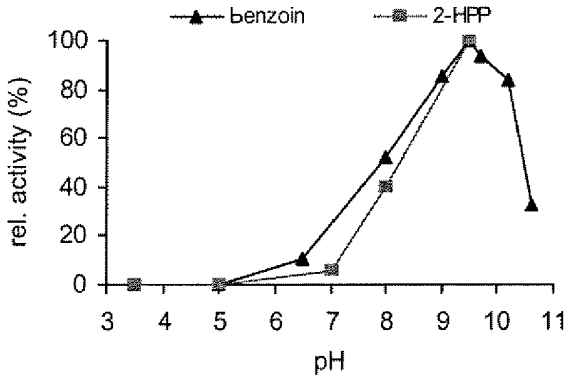
11. Use according to one or more of Claims 8 to 10, characterized in that the process is carried out in an aqueous medium at a pH of 8-10.

12. Use according to one or more of Claims 8 to 11, characterized in that the aldehydes employed are two identical or two different linear or branched-chain aliphatic or aromatic aldehydes.

13. Use according to one or more of Claims 8 to 12, characterized in that use is made of an aqueous medium in which at least one further organic solvent which is soluble or insoluble in water and which is selected from the group consisting of ethers, alcohols, esters, ketones, acid

amides, alkanes, aromatic compounds and sulphoxides is added to the water phase.

Fig. 1


benzoin
2-HPP
rel. activity (%)
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80
60
40
20
0
3
4
5
6
7
8
9
10
11
pH

Fig. 2

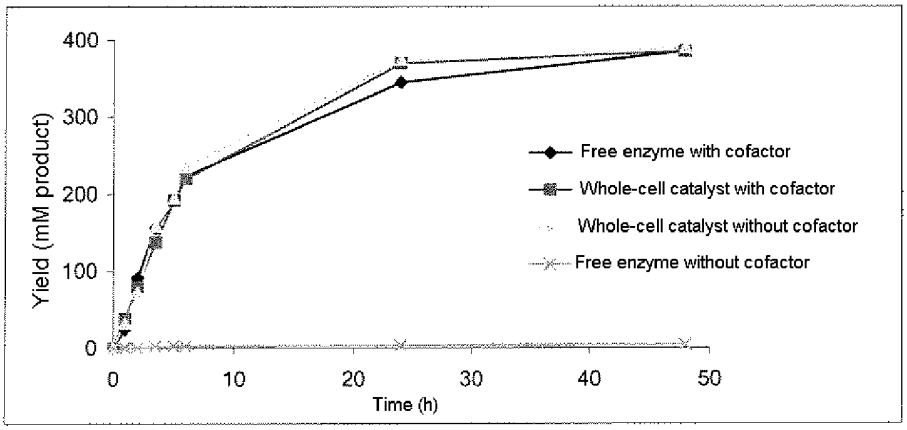

Yield (mM product)
400
300
200
100
0
0
10
20
30
40
50
Time (h)
Free enzyme with cofactor
Whole-cell catalyst with cofactor
Whole-cell catalyst without cofactor
Free enzyme without cofactor

Fig. 3: PKK233_2_BALhis

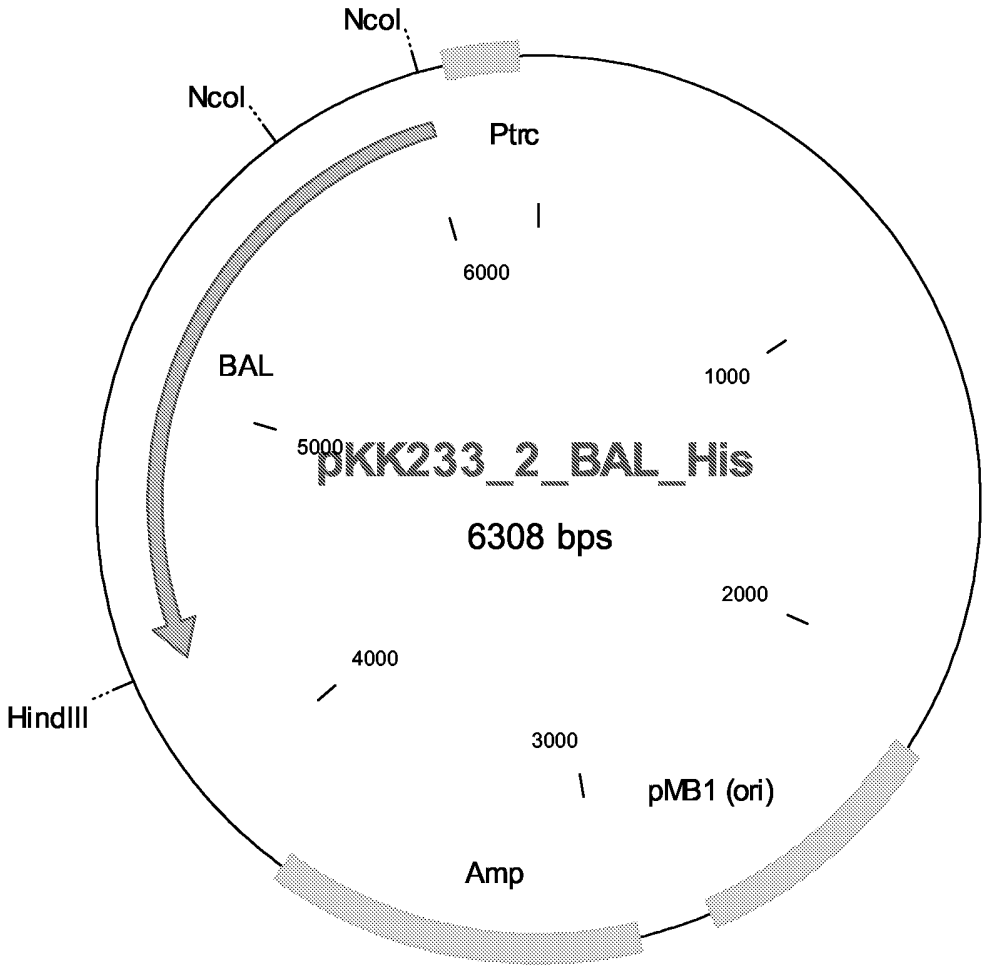
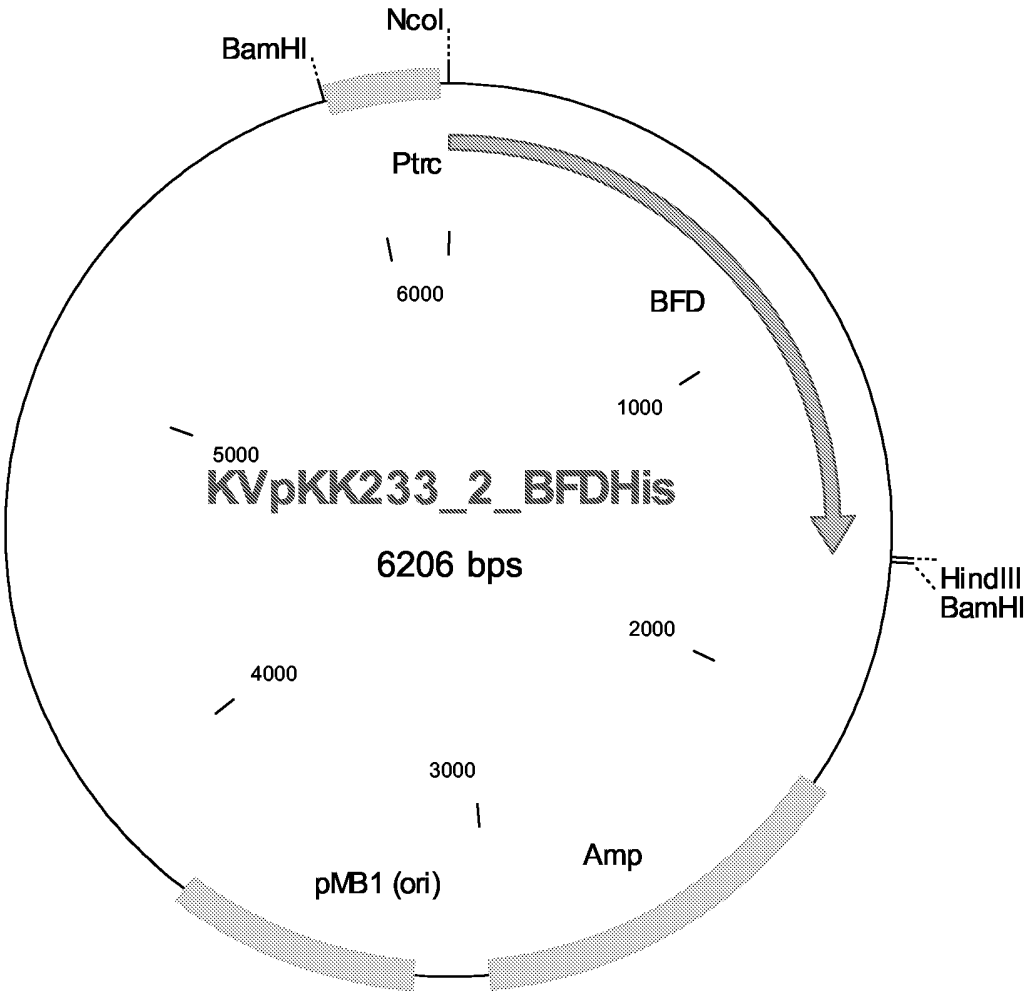


Fig.4 PKK233_2_BFDhis



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/050624

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/26 C12P41/00 C12N1/20

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

B. FIELDS SEARCHED

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

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, FSTA, PAJ, 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	IDING H ET AL: "Benzoylformate decarboxylase from Pseudomonas putida as stable catalyst for the synthesis of chiral 2-hydroxy ketones" CHEMISTRY, VCH VERLAGSGESELLSCHAFT, WEINHEIM, DE, vol. 6, no. 8, 14 April 2000 (2000-04-14), pages 1483-1495, XP002147924 ISSN: 0947-6539 page 1489, column 1, line 12 - line 14; figure 2 -----	1-13
X	WO 02/02753 A (FORSCHUNGSZENTRUM JUELICH GMBH; POHL, MARTINA; MUELLER, MICHAEL; DEMIR) 10 January 2002 (2002-01-10) page 2, line 24 - page 11, line 13 ----- -/--	8-10,12, 13

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 April 2006	10/05/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Schönwasser, D

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GONZALEZ B ET AL: "BENZALDEHYDE LYASE, A NOVEL THIAMINE PPI-REQUIRING ENZYME, FROM PSEUDOMONAS FLUORESCENS BIOVAR I" JOURNAL OF BACTERIOLOGY, WASHINGTON, DC, US, vol. 171, no. 5, May 1989 (1989-05), pages 2401-2405, XP001042139 ISSN: 0021-9193 cited in the application the whole document	1-13
A	WO 03/042412 A (DEGUSSA AG; MAY, OLIVER; LIEBETON, KLAUS; ECK, JUERGEN) 22 May 2003 (2003-05-22) page 8, line 22 - line 28	6,7
T	DE MARIA ET AL: "Preparative enantioselective synthesis of benzoins and (R)-2-hydroxy-1-phenylpropanone using benzaldehyde lyase" JOURNAL OF MOLECULAR CATALYSIS. B, ENZYMATIC, ELSEVIER, AMSTERDAM,, NL, vol. 38, no. 1, 2 January 2006 (2006-01-02), pages 43-47, XP005232022 ISSN: 1381-1177 the whole document	1-13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2006/050624

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0202753 A	10-01-2002	DE 10032254 A1	24-01-2002
		EP 1297159 A2	02-04-2003
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		EP 1444367 A1	11-08-2004