



## NADH Oxidase from *Lactobacillus*

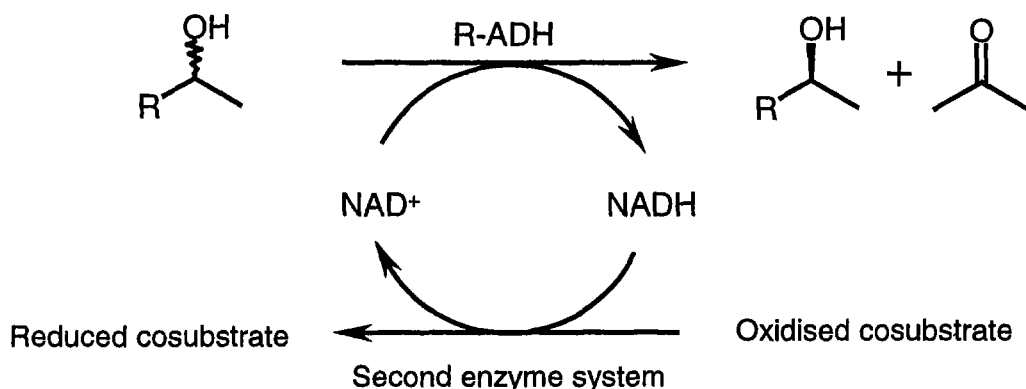


\*G00002\*

The present invention relates to an NADH oxidase from *Lactobacillus*, as well as the nucleic acids coding for this enzyme and vehicles containing these nucleic acids. In particular the invention relates to an oxidase from *Lactobacillus brevis* (DSM 20054).

Dehydrogenases, classified under E.C. 1.1, are enzymes that reversibly catalyse the oxidation or reduction of certain compounds such as for example alcohols and ketones. Some of the known dehydrogenases are cofactor-dependent, in other words they require further molecules to balance the electron transfer processes. NADH and NADPH for example are known to be such cofactors.

NADH is formed in the oxidation of, for example, alcohols to ketones according to the following reaction scheme. In order to avoid having to add stoichiometric amounts of expensive NAD<sup>+</sup>, the formation of NAD<sup>+</sup> can be initiated by a second enzyme system (Enzyme Catalysis in Organic Synthesis, Ed.: K. Drauz, H. Waldmann, VCH, 1<sup>st</sup> Edition, p. 721; reaction scheme 1).



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Systems known from the literature utilised for example for the regeneration of NAD<sup>+</sup> lactate dehydrogenase and pyruvate

with the formation of lactate, or glutamate dehydrogenase with ketoglutarate and ammonium with the formation of glutamate. The disadvantage of these systems is that auxiliary substrates such as pyruvate or ketoglutarate have to be used and products such as lactate or glutamate are formed, which for preparative utilisation have to be removed from the product that is actually formed. In addition equilibrium reactions are evolved that go to completion only with great technical difficulty.

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Alternatively such a second enzyme system may be an NADH oxidase that accepts atmospheric oxygen as oxidised cosubstrate and at the same time generates water or hydrogen peroxide with the formation of NAD<sup>+</sup>. NADH oxidases have the advantage in this respect that the reaction catalysed by them is irreversible, O<sub>2</sub> is used as regeneration substrate, and H<sub>2</sub>O or H<sub>2</sub>O<sub>2</sub> are formed as product. Enzymes of both groups (H<sub>2</sub>O-forming and H<sub>2</sub>O<sub>2</sub>-forming enzymes) are in principle known in the biochemical literature (for example, for H<sub>2</sub>O-forming enzymes see Lopez de Felipe, F. et al., J. Bacteriol. Vol. 180 (1998), 3804-08; for H<sub>2</sub>O<sub>2</sub>-forming enzymes see Nishiyama, Y. et al. J. Bacteriol. Vol. 183 (2001), 2431-2438 and the literature cited in each case). H<sub>2</sub>O<sub>2</sub>-forming enzymes are less suitable for use as regeneration enzyme since peroxide is known to be harmful to enzymes and should be decomposed as far as possible *in situ*, which requires further, disadvantageous but not impossible process steps.

30 The NADH oxidases from *Enterococcus* (and *Streptococcus*) *faecalis* (Schmidt, H.L. et al., Eur. J. Biochem. 156 (1986), 149-55), *Leuconostoc mesenteroides* (Koike, K. J. et

al., Biochem. (Tokyo) 97 (1985), 1279-88), *Streptococcus mutans* (Higuchi, M. et al., Biosci. Biotechnol. Biochem. 58 (1994), 1603-07), *Mycoplasma capricolum* (Klomkes, M., Altdorf, R., Ohlenbusch, H. D., Biol. Chem. Hoppe Seyler 5 366 (1985), 963-9), *Sulfolobus solfataricus* (Arcari, P. et al., J. Biol. Chem. 275 (2000), 895-900), *Thermus thermophilus* (Erdmann, H. et al., J. Mol. Biol. 230 (1993), 1086-8) and from *Thermus aquaticus* (Cocco, D. et al., Eur. J. Biochem. 174 (1988), 267-71) have been biochemically 10 characterised.

The object of the present invention was to provide an NADH oxidase with high activity that is suitable when coupled with dehydrogenases for NAD<sup>+</sup> regeneration. In particular 15 the NADH oxidase should be able to be produced easily and should be available in sufficient amount for advantageous use on an industrial scale.

This object is achieved according to the claims. Claim 1 20 relates to an NADH oxidase (NOX) from the genus *Lactobacillus*. Claim 2 is directed to preferred enzymes. Claim 3 claims the nucleic acids coding for these enzymes. Claim 4 in turn relates to vehicles that contain the nucleic acids according to the invention. Claim 5 is 25 directed to nucleic acids that hybridise with the nucleic acids according to the invention. Claim 6 protects preferred primers. Claim 7 is directed to a process for improving the NADH oxidases (NOX) starting from the nucleic acids according to the invention, while claim 8 covers 30 specific improved rec-NADH oxidases (NOX) and the nucleic acids coding for the latter. Claims 9 and 10 are directed to specific uses of the NADH oxidases (NOX) according to the invention and/or to nucleic acids according to the

invention. Claims 11 to 13 are directed to whole cell catalysts, while claim 14 covers a process for the production of the NADH oxidases from *Lactobacillus*.

5 Since an NADH oxidase (NOX) from *Lactobacillus* species is now available, it is possible advantageously to achieve the aforementioned object. Most particularly preferred in this connection is the use of an NADH oxidase (NOX) from *Lactobacillus brevis*, preferably DSM 20054 (Seq. 2) or  
10 *Lactobacillus kefir* (EP 91107067.0; DE 4014573).

The NADH oxidase is able to convert the NADH formed in the reaction into NAD<sup>+</sup>, according to the reaction scheme shown in the introduction. On account of the irreversibility of  
15 the reaction O<sub>2</sub> to H<sub>2</sub>O, there is a complete conversion of the employed racemic alcohol to ketone due to the equilibrium pressure, a highly enantiomer-enriched unreacted optical antipode of the employed alcohol remaining. Examples of the use of the NADH oxidase (NOX)  
20 include, *inter alia*, the production of R-alcohols when coupled with an S-alcohol dehydrogenase, of S-alcohols when coupled with an R-alcohol dehydrogenase, or the production of D-amino acids when coupled with an L-amino acid dehydrogenase. The coupling with hydroxyacid  
25 dehydrogenases and all other NAD-dependent dehydrogenases is also possible in this context. As further important products there may also be mentioned the oxidation products of the dehydrogenase reaction, for example ketones, keto acids or aldehydes.

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In another modification the invention is concerned with nucleic acids coding for an NADH oxidase according to the invention.

- 5 In spite of the relative ease of cultivation of the Lactobacillus strains and easy accessibility of the enzyme via chromatographic methods, it has become possible by specifying the nucleic acids coding for an NADH oxidase (NOX) according to the invention to obtain, in a
- 10 furthermore preferred way and manner, substances that enable the enzymes necessary for an enzymatic industrial process for the production of enantiomer-enriched compounds to be made available in sufficient amounts via recombinant techniques. It is possible with the nucleic acids to
- 15 obtain the enzymes in high yields from rapidly growing host organisms. Also, the gene sequences according to the invention have to be employed for the production of improved mutants.
- 20 In yet another modification the invention relates to plasmids or vectors containing one or more of the nucleic acids according to the invention.

- As plasmids or vectors there may in principle be used all
- 25 embodiments for this purpose available to the person skilled in the art. Such plasmids and vectors may be found in the article by Studier et al., Methods Enzymol. 1990, 185, 61-69 or in the brochures published by the companies Novagen, Promega, New England Biolabs, Clontech or Gibco
- 30 BRL. Further preferred plasmids and vectors may be found in: DNA cloning: a practical approach. Volume I-III, edited by D. M. Glover, IRL Press Ltd., Oxford, Washington DC,

1985, 1987; Denhardt, D. T. and Colasanti, J.: A survey of vectors for regulating expression of cloned DNA in *E. coli*. In: Rodriguez, R.L. and Denhardt, D. T (eds), Vectors, Butterworth, Stoneham, MA, 1987, pp179-204; Gene expression technology. In: Goeddel, D. V. (eds), Methods in Enzymology, Volume 185, Academic Press, Inc., San Diego, 1990; Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

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Plasmids with which the gene construct containing the nucleic acid according to the invention can be cloned in a most particularly preferred manner in the host organism include: pKK-177-3H (Roche Biochemicals), pBTac (Roche Biochemicals), pKK-233 (Stratagene) or pET (Novagen). With the exception of the TOPO series, which has integrated a resistance to kanamycin, all other plasmids should contain a  $\beta$ -lactamase for ampicillin resistance.

20 The invention is similarly aimed at microorganisms containing the nucleic acids according to the invention.

The microorganism in which the nucleic acids are cloned serves for the propagation and production of a sufficient amount of the recombinant enzyme. The processes for this are well known to the person skilled in the art (see below). As microorganisms there may in principle be used all organisms for this purpose available to the person skilled in the art, such as for example prokaryotes or eukaryotes, such as *Pseudomonas*, *Streptomyces*, *Arthrobacter*, *Bacillus*, *Staphylococcus*, *E. coli*, *Candida*, *Hansenula*, *Pichia* and baculoviruses. *E. coli* strains are

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preferably to be used for this purpose. Most particularly preferred are: E. coli NM 522, JM109, JM105, RR1, DH5 $\alpha$ , TOP 10- or HB101. Plasmids with which the gene construct containing the nucleic acid according to the invention is preferably cloned in the host organism are specified further above.

The invention also includes nucleic acids that hybridise under stringent conditions with the single-strand nucleic acids according to the invention or with their complementary single-strand nucleic acids. The expression "under stringent conditions" is understood to be the conditions described by Sambrook et al. (Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989), 1.101-1.104). Preferably a stringent hybridisation takes place according to the present invention if, after washing for one hour with 1 x SSC and 0.1 % SDS (sodium dodecylsulfonate) at 50 °C, preferably at 55 °C, more preferably at 62 °C and most preferably at 68 °C, and more preferably for 1 hour with 0.2 x SSC and 0.1 % SDS at 50 °C, preferably at 55 °C, more preferably at 62 °C and most preferably at 68 °C, a positive hybridisation signal is still observed.

A following aspect of the invention is aimed at primers for the production of the gene sequences according to the invention by means of all types of polymerase chain reaction. The primers include the sense and antisense primers coding for the corresponding amino acid sequences. Suitable primers may in principle be obtained by methods known to the person skilled in the art. The discovery of

primers according to the invention is carried out by comparison with known DNA sequences or by translation of the visually detected amino acid sequences into the codon of the organism under consideration (e.g. for Streptomyces: Wright et al., Gene 1992, 113, 55-65). Common features in the amino acid sequence of proteins of so-called superfamilies are also of use for this purpose (Firestone et al., Chemistry & Biology 1996, 3, 779-783). Further information relating to the above may be found in "Oligonucleotide synthesis: a practical approach", edited by M.J. Gait, IRL Press Ltd, Oxford Washington DC, 1984; PCR Protocols: A guide to methods and applications, edited by M.A. Innis, D.H. Gelfound, J.J. Sninsky and T.J. White. Academic Press, Inc., San Diego, 1990. The following primers are most preferred:

DIFAAGDSA Seq. 3; AS-Primer

MKVTVVGCT Seq. 4; S-Primer

In a furthermore further development the present invention relates to a process for the production of improved rec-NADH oxidases and rec-NADH oxidases obtained thereby or nucleic acids coding for the latter, in which connection, starting from the nucleic acids according to the invention coding for an NADH oxidase (NOX) according to the invention,

a) the nucleic acids are subjected to a mutagenesis,

b) the nucleic acids obtainable from a) are cloned in a suitable vector and this is transferred to a suitable expression system, and

c) the proteins formed having an improved activity and/or selectivity are detected and isolated. This process may be executed once or any desired number of times in succession.

The procedure for improving the enzymes according to the invention by mutagenesis methods has long been known to the person skilled in the art. As mutagenesis methods there  
 5 may be used all methods for this purpose available to the person skilled in the art. In particular these include saturation mutagenesis, random mutagenesis, shuffling methods as well as site-directed mutagenesis (Eigen M. and Gardinger W. (1984) Evolutionary molecular engineering  
 10 based on RNA replication. Pure & Appl. Chem. 56(8), 967-978; Chen & Arnold (1991) Enzyme engineering for nonaqueous solvents: random mutagenesis to enhance activity of subtilisin E in polar organic media. Bio/Technology 9, 1073-1077; Horwitz, M. and L. Loeb (1986) "Promoters  
 15 Selected From Random DNA-Sequences" Proceedings Of The National Academy Of Sciences Of The United States Of America 83(19): 7405-7409; Dube, D. and L. Loeb (1989) "Mutants Generated By The Insertion Of Random Oligonucleotides Into The Active-Site Of The Beta-Lactamase  
 20 Gene" Biochemistry 28(14): 5703-5707; Stemmer PC (1994). Rapid evolution of a protein *in vitro* by DNA shuffling. Nature. 370; 389-391 and Stemmer PC (1994) DNA shuffling by random fragmentation and reassembly: *In vitro* recombination for molecular evolution. Proc Natl Acad Sci USA. 91; 10747-  
 25 10751).

The new nucleic acid sequences that are obtained are cloned according to the methods specified hereinafter in a host organism (literature, see below) and the expressed enzymes  
 30 are detected and then isolated using suitable screening methods (Roberts J., Stella V.J. and Decedue C.J. (1985) A colorimetric assay of pancreatic lipase: rapid detection of

lipase and colipase separated by gel filtration. Lipids 20(1): 42-45; Pratt R.F., Faraci W.S. and Govardhan C.P. (1985) A direct spectrophotometric assay for D-alanine carboxypeptidases and for the esterase activity of beta-lactamases. Anal. Biochem. 144(1): 204-206; Brückner, H., R. Wittner, and H. Godel (1991) Fully automated high-performance liquid chromatographic separation of DL-amino acids derivatized with o-Phthaldialdehyde together with N-isopropyl-cysteine. Application to food samples).

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The present invention is also concerned with the use of the NADH oxidase (NOX) according to the invention, optionally improved by mutation, for the production of chiral enantiomer-enriched organic compounds such as for example alcohols or amino acids. Such compounds are used in a similar manner to the already known processes (DE10037101, Enzyme Catalysis in Organic Synthesis, Ed.: K. Drauz, H. Waldmann, VCH, 1<sup>st</sup> Edition).

Moreover the nucleic acids according to the invention and improved still further that code for the NADH oxidase (NOX) under consideration, are preferably suitable for the production of whole-cell catalysts (DE10037115.9 as well as the literature cited therein).

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The invention likewise provides a whole-cell catalyst containing a cloned gene for a dehydrogenase and a cloned gene for an NADH oxidase. Preferably the whole-cell catalyst according to the invention should contain an NADH oxidase (NOX) from *Lactobacillus*. It is extremely preferred if this is an NADH oxidase from *Lactobacillus brevis*, preferably DSM 20054 or *Lactobacillus kefir* DSM

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20587. The production of such an organism is known to the person skilled in the art (PCT/EP00/08473; PCT/US00/08159; relevant literature, see below).

5 The advantage of such an organism is the simultaneous expression of both enzyme systems, whereby only a re-organism still has to be used for the reaction. In order to match the expression of the enzymes having regard to their reaction rates, the correspondingly coding nucleic  
10 acid fragments may be exposed to various plasmids having different numbers of copies, and/or promoters of different strengths may be used for a variously strong expression of the genes. With enzyme systems matched in this way there is advantageously no accumulation of a possibly inhibiting  
15 intermediate compound, and the reaction under consideration may proceed at an optimal overall rate. This has however been known for a long time to the person skilled in the art (PCT/EP00/08473; Gellissen et al., Appl. Microbiol. Biotechnol. 1996, 46, 46-54).

20 The invention also provides a process for the production of the NADH oxidase from *Lactobacillus*, in which for the advantageous expression of the NADH oxidase (NOX) the microorganism has to be cultivated under aerobic  
25 conditions.

It has been found that *Lactobacillus* grows significantly better in a standard medium under aerobic conditions than under the otherwise conventional anaerobic conditions. It  
30 is preferred to use a *Lactobacillus brevis*, preferably DSM 20054, or *Lactobacillus kefir* DSM 20587. According to the invention those conditions in which the microorganism, when cultivated in flasks as a shaken culture or when cultivated

in a fermenter, is grown in the presence of measurable concentrations of oxygen, are according to the invention advantageously regarded as aerobic conditions. The expression "measurable oxygen concentration" is understood  
5 to mean that the dissolved oxygen is 1% of the saturation value for oxygen, the oxygen being measured under otherwise normal conditions with an oxygen electrode.

The nucleic acids according to the invention can thus  
10 preferably be used for the production of rec-NADH oxidase (NOX). By means of recombinant techniques that have long been known to the person skilled in the art (see below), organisms have been obtained that are able to produce the enzyme under consideration in an amount sufficient for an  
15 industrial process. The production of the rec-enzymes according to the invention is carried out by genetic engineering processes known to the person skilled in the art (Sambrook et al. 1989, Molecular cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press,  
20 Balbas P & Bolivar F. 1990; Design and construction of expression plasmid vectors in E.coli, Methods Enzymology 185, 14-37; Vectors: A Survey of Molecular Cloning Vectors and Their Uses. R.L. Rodriguez & D.T. Denhardt, Eds: 205-225). With regard to the general procedure (PCR and fusion  
25 PCR, inverse PCR, cloning, expression etc.), reference may be made to the following literature and the references cited therein: Riley J, Butler R, Finniear R, Jenner D, Powell S, Anand R, Smith JC, Markham AF (1990). A novel, rapid method for the isolation of terminal sequences from  
30 yeast artificial chromosome (YAC) clones. Nucl Acids Res. 18, 8186; Triglia T, Peterson MG, Kemp DJ (1988). A procedure for in vitro amplification of DNA segments that

lie outside the boundaries of known sequences. Nucleic  
Acids Res. 16, 8186; Sambrook J, Fritsch EF, Maniatis T  
(1989). Molecular Cloning. Cold Spring Harbour Laboratory  
Press; Vectors: A Survey of Molecular Cloning Vectors and  
5 Their Uses. R.L. Rodriguez & D.T. Denhardt, II.

The enzyme under consideration may be used in the free form  
as homogeneously purified compounds, or as enzyme produced  
by recombinant technology. Furthermore the enzyme may also  
10 be employed as a constituent of an intact host organism or  
in conjunction with the macerated cell mass of the host  
organism purified to an arbitrarily high degree. It is  
also possible to use the enzymes in immobilised form  
(Bhavender P. Sharma, Lorraine F. Bailey and Ralph A.  
15 Messing, "Immobilisierte Biomaterialien - Techniken and  
Anwendungen", Angew. Chem. 1982, 94, 836-852). The  
immobilisation is preferably carried out by lyophilisation  
(Dordick et al. J. Am. Chem. Soc. 194, 116, 5009-5010;  
Okahata et al. Tetrahedron Lett. 1977, 38, 1971-1974;  
20 Adlercreutz et al. Biocatalysis 1992, 6, 291-305). It is  
most particularly preferred to carry out the lyophilisation  
in the presence of surfactants such as aerosol OT or  
polyvinylpyrrolidone or polyethylene glycol (PEG) or Brij  
52 (diethyleneglycolmonocetyl ether) (Goto et al.  
25 Biotechnol. Techniques 1997, 11, 375-378). The use as  
CLECs is also possible (St Clair et al. Angew Chem Int Ed  
Engl 2000 Jan, 39(2), 380-383).

The expression "optically enriched (enantiomer-enriched)  
30 compounds" within the scope of the invention is understood  
to mean the presence of an optical antipode in a mixture  
with the other antipode, in an amount of >50 mole %.

The organism *Lactobacillus brevis* DSM 20054 or  
*Lactobacillus kefir* DSM 20587 has been filed at the German  
Collection for Microorganisms and Cell Cultures under the  
5 corresponding number and is accessible to the public.

The expression "nucleic acids" covers all types of single-  
strand or double-strand DNA as well as RNA or mixtures  
thereof.

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The expression "improved rec-enzymes" is understood  
according to the claims to mean in particular those that  
enzymes that have a modified substrate spectrum, are more  
active and/or more selective, or are more stable under the  
15 employed reaction conditions.

Among the claimed protein sequences and nucleic acid  
sequences there are also covered according to the invention  
those sequences that exhibit an homology (exclusive of the  
20 natural degeneration) greater than 80 %, preferably greater  
than 90 %, 91 %, 92 %, 93 % or 94 %, more preferably  
greater than 95 % or 96 %, and particularly preferably  
greater than 97 %, 98 % or 99 % with respect to one of  
these sequences, provided that the mode of action and/or  
25 purpose of such a sequence is retained. The expression  
"homology" (or identity) as used herein may be defined by  
the equation  $H (\%) = [1 - V/X] \times 100$ , where H denotes  
homology, X is the total number of nucleotide bases/amino  
acids of the comparison sequence, and V is the number of  
30 different nucleotide bases/amino acids of the sequence  
under consideration, referred to the comparison sequence.

In any case the term "nucleic acids coding for amino acid sequences" covers all sequences that appear possible according to the degeneration of the genetic code.

- 5 The literature references cited in this specification are counted as part of the disclosure.

## SEQUENCE PROTOCOL

&lt;110&gt; Degussa AG

5 &lt;120&gt; NADH oxidase from Lactobacillus

&lt;130&gt; 010281 AM

&lt;140&gt;

10 &lt;141&gt;

&lt;160&gt; 5

&lt;170&gt; PatentIn Ver. 2.1

15

&lt;210&gt; 1

&lt;211&gt; 1353

&lt;212&gt; DNA

&lt;213&gt; Lactobacillus brevis

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&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(1353)

25 &lt;400&gt; 1

atg aaa gtc aca gtt gtt ggt tgt aca cat gcc gga acc ttt gcg att 48  
 Met Lys Val Thr Val Val Gly Cys Thr His Ala Gly Thr Phe Ala Ile  
           1                  5                  10                  15

30 aaa caa atc ttg gcc gaa cac cct gat gcc gaa gtg acc gtc tac gaa 96  
 Lys Gln Ile Leu Ala Glu His Pro Asp Ala Glu Val Thr Val Tyr Glu  
                   20                  25                  30

          cgt aac gat gtc att tca ttt ctc tct tgt gga atc gcc ctt tac ctg 144  
 35 Arg Asn Asp Val Ile Ser Phe Leu Ser Cys Gly Ile Ala Leu Tyr Leu  
                   35                  40                  45

          ggg gga aaa gtc gct gat ccg caa ggc ctc ttt tat tca agt cct gaa 192  
           Gly Gly Lys Val Ala Asp Pro Gln Gly Leu Phe Tyr Ser Ser Pro Glu

[illegible]

|    |                                                                 |                 |
|----|-----------------------------------------------------------------|-----------------|
|    | Ser Glu Thr Val Glu Ser Phe Thr Asp Ser Ala Thr Gly Leu Thr Ile |                 |
|    | 210                                                             | 215 220         |
|    | aag act gat aag aac agc tat gaa acg gat ctc gct att tta tgc att | 720             |
| 5  | Lys Thr Asp Lys Asn Ser Tyr Glu Thr Asp Leu Ala Ile Leu Cys Ile |                 |
|    | 225                                                             | 230 235 240     |
|    | ggc ttt aga cca aat acc gac ctg ctg aaa ggc aaa gtc gat atg gca | 768             |
| 10 | Gly Phe Arg Pro Asn Thr Asp Leu Leu Lys Gly Lys Val Asp Met Ala |                 |
|    |                                                                 | 245 250 255     |
|    | cca aac ggc gcg att att acg gat gac tac atg cgt tct tct aac cct | 816             |
|    | Pro Asn Gly Ala Ile Ile Thr Asp Asp Tyr Met Arg Ser Ser Asn Pro |                 |
|    |                                                                 | 260 265 270     |
| 15 |                                                                 |                 |
|    | gat att ttc gcc gct ggt gac agt gct gct gtg cac tac aac cca acc | 864             |
|    | Asp Ile Phe Ala Ala Gly Asp Ser Ala Ala Val His Tyr Asn Pro Thr |                 |
|    |                                                                 | 275 280 285     |
| 20 |                                                                 |                 |
|    | cat cag aat gct tac att ccc tta gca act aac gcg gtg cgt caa ggt | 912             |
|    | His Gln Asn Ala Tyr Ile Pro Leu Ala Thr Asn Ala Val Arg Gln Gly |                 |
|    |                                                                 | 290 295 300     |
|    | atc cta gtc ggt aaa aac cta gtt aag ccg acc gtc aag tat atg gga | 960             |
| 25 | Ile Leu Val Gly Lys Asn Leu Val Lys Pro Thr Val Lys Tyr Met Gly |                 |
|    |                                                                 | 305 310 315 320 |
|    | aca caa tca tct tct ggt ttg gca ctc tat gat cgg acg atc gtc tca | 1008            |
|    | Thr Gln Ser Ser Ser Gly Leu Ala Leu Tyr Asp Arg Thr Ile Val Ser |                 |
| 30 |                                                                 | 325 330 335     |
|    | act ggt tta acg cta gca gct gca aaa caa caa ggg gtg aac gct gaa | 1056            |
|    | Thr Gly Leu Thr Leu Ala Ala Ala Lys Gln Gln Gly Val Asn Ala Glu |                 |
|    |                                                                 | 340 345 350     |
| 35 |                                                                 |                 |
|    | caa gtg att gtt gaa gat aat tat cgc cca gag ttt atg ccg tca act | 1104            |
|    | Gln Val Ile Val Glu Asp Asn Tyr Arg Pro Glu Phe Met Pro Ser Thr |                 |
|    |                                                                 | 355 360 365     |

gaa ccc gtt ttg atg tca tta gtc ttt gac ccc gac aca cac cgg atc 1152  
 Glu Pro Val Leu Met Ser Leu Val Phe Asp Pro Asp Thr His Arg Ile  
 370 375 380

5 tta ggt ggt gcg tta atg agt aaa tac gat gtt tca caa tcg gcc aac 1200  
 Leu Gly Gly Ala Leu Met Ser Lys Tyr Asp Val Ser Gln Ser Ala Asn  
 385 390 395 400

10 acc ctt tct gtt tgc atc caa aac gaa aat aca att gat gac tta gcg 1248  
 Thr Leu Ser Val Cys Ile Gln Asn Glu Asn Thr Ile Asp Asp Leu Ala  
 405 410 415

15 atg gtt gat atg ctc ttc cag cct aac ttt gat cga cca ttc aac tac 1296  
 Met Val Asp Met Leu Phe Gln Pro Asn Phe Asp Arg Pro Phe Asn Tyr  
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20 cta aac atc tta gcg caa gct gct cag gca aag gtt gcc caa tca gtt 1344  
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25  
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 <213> Lactobacillus brevis

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 35 40 45  
 Gly Gly Lys Val Ala Asp Pro Gln Gly Leu Phe Tyr Ser Ser Pro Glu  
 50 55 60

Glu Leu Gln Lys Leu Gly Ala Asn Val Gln Met Asn His Asn Val Leu  
 65 70 75 80  
 Ala Ile Asp Pro Asp Gln Lys Thr Val Thr Val Glu Asp Leu Thr Ser  
 85 90 95  
 5 His Ala Gln Thr Thr Glu Ser Tyr Asp Lys Leu Val Met Thr Ser Gly  
 100 105 110  
 Ser Trp Pro Ile Val Pro Lys Ile Pro Gly Ile Asp Ser Asp Arg Val  
 115 120 125  
 Lys Leu Cys Lys Asn Trp Ala His Ala Gln Ala Leu Ile Glu Asp Ala  
 10 130 135 140  
 Lys Glu Ala Lys Arg Ile Thr Val Ile Gly Ala Gly Tyr Ile Gly Ala  
 145 150 155 160  
 Glu Leu Ala Glu Ala Tyr Ser Thr Thr Gly His Asp Val Thr Leu Ile  
 165 170 175  
 15 Asp Ala Met Asp Arg Val Met Pro Lys Tyr Phe Asp Ala Asp Phe Thr  
 180 185 190  
 Asp Val Ile Glu Gln Asp Tyr Arg Asp His Gly Val Gln Leu Ala Leu  
 195 200 205  
 Ser Glu Thr Val Glu Ser Phe Thr Asp Ser Ala Thr Gly Leu Thr Ile  
 20 210 215 220  
 Lys Thr Asp Lys Asn Ser Tyr Glu Thr Asp Leu Ala Ile Leu Cys Ile  
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 260 265 270  
 Asp Ile Phe Ala Ala Gly Asp Ser Ala Ala Val His Tyr Asn Pro Thr  
 275 280 285  
 His Gln Asn Ala Tyr Ile Pro Leu Ala Thr Asn Ala Val Arg Gln Gly  
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 Ile Leu Val Gly Lys Asn Leu Val Lys Pro Thr Val Lys Tyr Met Gly  
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 325 330 335  
 35 Thr Gly Leu Thr Leu Ala Ala Ala Lys Gln Gln Gly Val Asn Ala Glu  
 340 345 350  
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 Glu Pro Val Leu Met Ser Leu Val Phe Asp Pro Asp Thr His Arg Ile

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      370              375              380
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1

5

10

15

10 Lys Gln Ile Leu Ala Glu Glu Pro Asp Ala Asp Xaa Xaa Val Tyr

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25

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## Examples:

1) Cultivation of *Lactobacillus brevis* for obtaining NADH oxidase

5

*Lactobacillus brevis* DSM 20054 was cultivated in a standard medium for lactobacillae. In this connection it was found that the strain grew significantly better under the addition of oxygen than under the anaerobic conditions that are in fact normally used for lactobacillus strains. An enzyme test on the activity of an NADH oxidase showed that the strain particularly under these unusual cultivation conditions exhibits a high activity of an NADH oxidase (NOX).

15

For the enzyme purification and characterisation cells were therefore cultivated under the following conditions:

Medium: Pro 1 L: 10 g caseine peptone, tryptically digested; 10 g meat extract; 5 g yeast extract; 20 g glucose; 1 g Tween 80; 2 g  $K_2HPO_4$ ; 5 g Na acetate; 2 g diammonium citrate; 0.2 g  $MgSO_4 \times 7 H_2O$ ; 0.05 g  $MnSO_4 \times H_2O$ ; pH = 6.2-6.5.

25 Cultivation is carried out in a shaken flask (equipped with 2 baffles) at 30 °C for 2 days while stirring. The cell mass is then obtained by centrifugation and may be stored at -20 °C for a relatively long time.

30 2) Enzyme isolation

a) Crude extract production

The cells were macerated by wet grinding with glass beads. For this, the moist bacterial mass (10 g) was suspended in 0.1 M sodium acetate buffer, pH 4.5 (30 ml) and macerated by grinding in a stirred cell glass bead mill (SCP  
5 disintegrator, Innomed-Konsult AB, Sweden) at 4000 rpm for 20 minutes. The macerated cells were centrifuged off and the supernatant was identified as crude extract.

The selected pH value of 4.5 has a decisive influence on  
10 the enzyme yield in the crude extract; under these conditions a crude extract preparation is obtained having a specific activity of 17.8 U/mg and a yield of 700 units, whereas when using a buffer with a pH of 7.4 the specific activity was only 2.6 U/mg and the yield was 580 units.

15

The enzyme activity was measured by means of the following photometric test (340 nm, 30 °C measurement temperature): 0.1 mM NADH; 100 mM potassium phosphate buffer pH 7.5; 1 mM DTT (= dithiothreitol); 10 µl enzyme solution per 1 ml  
20 (diluted if necessary). 1 unit NADH oxidase activity oxidises 1 µMole NADH / min.

#### b) Chromatographic purification of the enzyme

After the preparation of the crude extract in buffer with a  
25 pH of 4.5, the crude extract was immediately rebuffered and transferred to a Macro Q column (Biorad) equilibrated with 50 mM TEA pH 7.5 + 3 mM DTT + 200 mM NaCl (= buffer A) (100 ml column volume, flow rate 4 ml/min, 10 ml fractions). The chromatography was carried out on an FPLC  
30 apparatus (Pharmacia) (pressure 0.1 MPa, room temperature). The proteins were eluted by means of an NaCl step gradient to 1 M NaCl (buffer B: buffer A + 1 M NaCl), the following

steps being used : 0-2 column volumes: 0 % B (=200 mM NaCl), 2-5 column volumes: 40 % B, 5-5.5 column volumes: 100 % B. The enzyme activity and the protein content in the fractions were determined by means of the photometric test. The active fractions were combined and concentrated by evaporation in an Amicon stirred cell (YM10 membrane, 50 ml) while simultaneously rebuffering with hydroxylapatite equilibration buffer, see below.

10 This fraction pool was added to an hydroxylapatite ceramic type 20  $\mu$ m column (Biorad) equilibrated with 10 mM KPi buffer pH 6.7 + 3 mM DTT + 200 mM NaCl (=buffer A) (15 ml column volume, flow rate 2 ml/min, 5 ml fractions; FPLC apparatus, pressure 0.1 MPa, room temperature). The proteins were eluted with a 3-step gradient to 500 mM KPi (buffer B: buffer A + 500 mM KPi) with the following stages: 0-3 column volumes 0% B, 3-7 column volumes 30 % B, 7-9 column volumes 50 % B, then rising to 100 % B in 3 column volumes. The active fractions were combined and concentrated as described above, and rebuffered with the buffer required for the phenylsepharose.

This preparation was added, after the addition of ammonium sulfate (1.2 M final concentration in the sample), to a phenylsepharose High Sub column (Pharmacia) equilibrated with 50 mM TEA pH 7.5 + 3 mM DTT + 1.2 M  $(\text{NH}_4)_2\text{SO}_4$  (=buffer A) (25 ml column volume, flow rate 1 ml/min, 3 ml fractions; FPLC apparatus, pressure 0.1 MPa, room temperature). The proteins were eluted using a 3-step gradient to 0 M ammonium sulfate (buffer B: buffer A without ammonium sulfate) with the following stages: 1 column volume 0 % B, 1 column volume 20 % B, 1 column

volume 60 % B, 2 column volumes 100 % B). The active fractions were combined, concentrated by evaporation in an Amicon ultrafiltration cell, followed by the addition of further ammonium sulfate.

5

The last purification stage, which was carried out with the concentrated fraction from the phenylsepharose High Sub column, is a fine purification by chromatography on phenylsepharose 650C material (Tosohaas), equilibrated with  
 10 50 mM TEA pH 7.5 + 3 mM DTT + 1.2 M  $(\text{NH}_4)_2\text{SO}_4$  (=buffer A). Chromatography conditions: column volume 2 ml, flow rate 1 ml/min; FPLC apparatus, pressure 0.1 MPa, room temperature, 2 ml fractions). The proteins are eluted with a 4-stage gradient to 0 M ammonium sulfate (buffer B: buffer A  
 15 without ammonium sulfate) using the following stages: 10 column volumes 0% B, 5 column volumes 20 % B, 5 column volumes 50 % B, 5 column volumes 70 % B, 10 column volumes 100 % B).

20 The enzyme activity and protein yields of the various chromatography steps are summarised in Table 1.

Table 1: Purification of the NADH oxidase from  
*Lactobacillus brevis* DSM 20054 (OH-apatite =  
 25 hydroxyapatite; PS = phenylsepharose)

| Sample           | Activity<br>[U/ml] | Specific<br>Activity<br>[U/mg] | Total<br>Activity<br>[U] | Yield<br>[%] | Enrich-<br>ment<br>Factor |
|------------------|--------------------|--------------------------------|--------------------------|--------------|---------------------------|
| Crude<br>extract | 35.1               | 17.8                           | 702                      | 100          | 1                         |
| Macro Q          | 77.3               | 10.4                           | 270                      | 38           | 0.6                       |
| OH-apatite       | 32.2               | 12.2                           | 103                      | 15           | 0.7                       |
| PS               | 44                 | 58.6                           | 87                       | 12           | 3.3                       |
| PS 650C          | 98                 | 116                            | 98                       | 14           | 6.5                       |

An SDS-PAGE of the purified fractions according to Table 1 shows that the enzyme fraction after the last step still contains only 2 proteins, whose subunits have molecular weights of 38 and 50 kDa. These two protein bands can be individually N-terminally sequenced after blotting, and a comparison with sequences accessible in databanks shows that the 38 kDa band has very high similarities with known lactate dehydrogenases, while the 50 kDa band has very high similarities with NADH oxidases.

3) Biochemical characteristic data of the NADH oxidase from *L. brevis*

The biochemical data were determined with an enzyme preparation purified by 4 chromatographic steps.

a) pH dependence of the oxidation

The pH optimum was measured in the range pH 4.0 to 10.0.

Different buffers were used depending on the pH value. As

a check on the influence of the buffer, when changing the buffer this pH value was measured twice in the different buffers (pH 7.0 and pH 8.5). 0.1 M citric acid /Na<sub>2</sub>PO<sub>4</sub> was used for the range pH 4.0-7.0, 0.1 M TEA was used for the  
5 range 7.0-8.5, and 0.1 M glycine/NaOH was used for the range pH 8.5-10.0. Fig. 1 shows that the optimum of the NADH oxidation is at pH 8.0 to 8.5.

b) pH stability of the NADH oxidase

10

In order to determine the pH stability the NADH oxidase was incubated for 8 hours in the buffers listed under 3a), then the residual activity was measured in the standard test. Fig. 2 shows that the enzyme has a first sharp stability  
15 optimum at pH 5.0, is moderately stable in the range 5.5 - 7.5, and exhibits a second optimum at pH 8.5. At pH 8.5 the enzyme is still up to 100% stable after 8 hours in TEA/NaOH buffer as well as in glycine/NaOH buffer. The figure illustrates that the enzyme stable in the slightly  
20 alkaline range (pH 8 - 9) is also stable in a wider range. This range is useful for application purposes if the NADH oxidase for the oxidation is to be coupled with an NAD-dependent dehydrogenase, since the pH optimum of many dehydrogenases for the oxidation lies in a slightly  
25 alkaline range.

c) Temperature optimum of the NADH oxidase

Activity tests at various temperatures show that the NADH oxidase exhibits maximum activity at 40 °C (Fig. 3).

30

d) Temperature stability of the NADH oxidase

In order to determine the temperature stability enzyme samples were stored at 30 °C and 42 °C, samples were taken at various times, and the residual activity was measured.

- 5 The evaluation shows that the enzyme at 30 °C is in practice stable for ca. 400 minutes, following which it appears to deactivate according to a slow reaction kinetics (half-value time ca. 18 hours). At 42 °C the enzyme deactivates initially (up to ca. 120 minutes) relatively  
10 quickly, following which it appears to be stable at ca. 50% residual activity over a relatively long time (at least 16 hours).

e) Determination of the  $K_M$  value for NADH for the NADH  
15 oxidase

- For the substrate NADH the concentration was varied in the range from 0.0013 to 0.52 mM and the activity was measured photometrically. The evaluation shows that maximum activity is reached at 0.1 mM. Higher NADH concentrations  
20 (up to 0.5 mM) exhibit only a weak excess inhibition. The  $K_M$  value is calculated to be 24  $\mu$ M. This high affinity of the enzyme for NADH is very advantageous for applications in which  $NAD^+$  is to be regenerated by oxidation of NADH, since a prerequisite for this is as complete an oxidation  
25 of NADH as possible.

4) Protein chemistry characteristic data of the NADH oxidase from *Lactobacillus brevis*

a) N-terminal amino acid sequence

- 30 The results of several enrichments always revealed a protein whose subunits have a molecular weight according to

SDS-PAGE of 50 kDa. The first 27 amino acids of the N-terminus were determined for this protein (automated Edman decomposition with an automated sequencer 4774 (Applied Biosystems) with online HPLC 120 A); the sequence is as follows: MKVTVVGCTHAGTFAIKQILAEPPDADXXVY (Seq 5). In the sequence a conserved motif is underlined that acts as FAD-binding motif in NADH oxidases, and is a slight modification of the known ADP-binding site GXGXXG. Databank searches (BLAST) already show for this protein fragment agreement with known NADH oxidases.

#### b) Determination of the protein and gene sequences

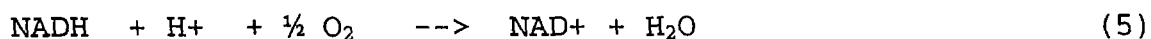
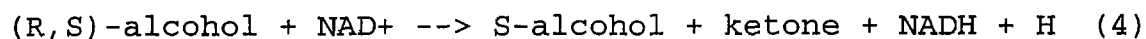
The results of the databank searches show in some partial regions sequences that are present in virtually all NADH oxidases or in related NADH peroxidases. The amino acid sequence DIFAAGDSA (Seq. 3) is regarded as such a conserved region. An antisense codon is used as "C-terminal" primer for this sequence, and together with a primer that codes for the N-terminus MKVTVVGCT (Seq. 4), is used in a PCR. The PCR yields a 777 bp long fragment that is 59% identical to the NADH oxidase (NOX) from *Enterococcus*. A gene library is established by digesting genome DNA with 4 different restriction endonucleases (EcoRV, Pvu II, Sca I and Stu I). Using specific primers derived from the cleavage sites of the restriction enzymes and sequences from the 777 bp fragment, further sequence fragments are then obtained by means of PCR, whereby the total sequence can be defined with the aid of overlapping DNA sequences and the stop codon.

30

The sequence for the NADH oxidase (NOX) from *L. brevis* is reproduced in the sequence protocol (Seq. 1) given above.

5) Use of the NADH oxidase from *Lactobacillus brevis* for the coenzyme regeneration

5 A typical use of the NADH oxidase is the regeneration of NAD<sup>+</sup>. This may be necessary for example if NAD-dependent dehydrogenases are used for the oxidation, as has been shown in a general way hereinbefore (reaction scheme 1). This is demonstrated in the following example for the  
10 production of an enantiomer-pure alcohol corresponding to the following equations:



15

with an R-specific alcohol dehydrogenase as catalyst for reaction (4), and the NADH oxidase for (5).

In this example an NAD mutant of the R alcohol  
20 dehydrogenase from *Lactobacillus brevis* (recADHG37D (NAD-mutant) DE10037101.9), coupled with the purified NADH oxidase from *Lactobacillus brevis*, was used as R-specific alcohol dehydrogenase. (R,S)-phenylethanol was used as alcohol, and the reaction was carried out as a batch  
25 reaction. The following reactants were used: 50 mM (R,S)-phenylethanol, 2 mM NADH, 50 mM TEA buffer pH 7.0 with 1mM MgCl<sub>2</sub> and with 5 mM DTT, 1 U recADHG37D and 2 U NADH oxidase.

30 Samples were taken after 0, 1, 2, 3, 4, 6, 9 and 24 hours and separated by gas chromatography (column: CP-Chirasil-

DEX CB, length: 25 m, diameter: 25  $\mu$ m (Chrompack).  
Temperature programme: 5 mins at 60 °C, then 5 °C/min up to 190 °C; column flow rate 1.3 ml/min; gas: helium. The acetophenone peak (product; retention time = 16.9 min) as well as the enantiomers of phenylethanol (educts; retention times R-phenylethanol = 20.8 min and S-phenylethanol = 21.1 min) were detected. Fig. 4 shows the GC spectrum of a sample in which the R-alcohol had been almost completely oxidised, which is why a corresponding amount of the oxidation product acetophenone can be seen. As a control the same experiment was carried out, but without the addition of NADH oxidase. Gas chromatography analysis does not show any significant change in the two alcohols, and simply a small amount of acetophenone can be detected, which may possibly be attributed to a spontaneous oxidation of NADH by atmospheric oxygen.

## Patent Claims:

1. NADH oxidase from *Lactobacillus*.
- 5 2. Oxidase according to claim 1 from *Lactobacillus brevis*, preferably DSM 20054.
3. Nucleic acids coding for an oxidase according to claim 1 or 2.
- 10 4. Plasmids, vectors, microorganisms containing one or more nucleic acids according to claim 3.
5. Nucleic acids hybridising with the single-strand  
15 nucleic acids or complementary single-strand nucleic acids according to claim 3 under stringent conditions.
6. Primers for the production of the nucleic acids according to claim 3 and/or 5 by means of PCR.
- 20 7. Process for the production of improved rec-NADH oxidases (NOX) starting from nucleic acids coding for an oxidase according to claim 1, characterised in that
  - a) the nucleic acids are subjected to a mutagenesis,
  - 25 b) the nucleic acids obtainable from a) are cloned in a suitable vector and this is transferred to a suitable expression system, and
  - c) the proteins formed having improved activity and/or selectivity are detected and isolated.

8. rec-NADH oxidases (NOX) or nucleic acids coding for the latter, obtainable according to claim 7.
- 5 9. Use of the NADH oxidases (NOX) according to claim 1 or 8 for the production of chiral enantiomer-enriched organic compounds such as for example alcohols or amino acids.
- 10 10. Use of the nucleic acids according to claim 3 or 8 for the production of whole-cell catalysts.
11. Whole-cell catalysts containing a cloned gene for a dehydrogenase and a cloned gene for an NADH oxidase.
- 15 12. Whole-cell catalyst according to claim 11, characterised in that it is an NADH oxidase (NOX) from *Lactobacillus*.
- 20 13. Whole-cell catalyst according to claim 12, characterised in that it is an NADH oxidase (NOX) from *Lactobacillus brevis*, preferably DSM 20054.
- 25 14. Process for the production of NADH oxidases from *Lactobacillus*, characterised in that the microorganisms are cultivated under aerobic conditions.