(12)特許協力条約に基づいて公開された国際出願

(19) 世界知的所有権機関
国際事務局

(43) 国際公開日
2003年4月17日 (17.04.2003)

PCT

(10) 国際公開番号
WO 03/031626 A1

(51) 国際特許分類[7]: C12N 15/53, 9/04, 1/21, C12P 21/02 // (C12N 15/53, C12R 1:01) (C12N 9/04, C12R 1:19) (C12N 1/21, C12R 1:19) (C12P 21/02, C12R 1:19) (C12N 9/04, C12R 1:01)

(21) 国際出願番号: PCT/JP02/10460

(22) 国際出願日: 2002年10月9日 (09.10.2002)

(25) 国際出願の言語: 日本語

(26) 国際公開の言語: 日本語

(30) 優先権データ:
特願2001-312043 2001年10月9日 (09.10.2001) JP

(71) 出願人 (米国を除く全ての指定国について): 鐘淵化学工業株式会社 (KANEKA CORPORATION) [JP/JP]; 〒530-8288 大阪府 大阪市 北区中之島３丁目２番４号 Osaka (JP).

(72) 発明者; および
(75) 発明者/出願人 (米国についてのみ): 難波 弘憲 (NANBA,Hirokazu) [JP/JP]; 〒676-0025 兵庫県 高砂市 西畑１丁目１６−２０ Hyogo (JP). 高岡 康子 (TAKAOKA,Yasuko) [JP/JP]; 〒655-0872 兵庫県 神戸市 垂水区塩屋町６丁目３１−１７ 三青荘 Hyogo (JP).

(74) 代理人: 安富 康男, 外(YASUTOMI,Yasuo et al.); 〒532-0011 大阪府 大阪市 淀川区西中島５丁目４番２０号 中央ビル Osaka (JP).

(81) 指定国 (国内): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) 指定国 (広域): ARIPO 特許 (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), ユーラシア特許 (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), ヨーロッパ特許 (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI 特許 (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

添付公開書類:
— 国際調査報告書
— 明細書とは別に規則１３の２に基づいて提出された生物材料の寄託に関する表示。

２文字コード及び他の略語については、定期発行される各PCTガゼットの巻頭に掲載されている「コードと略語のガイダンスノート」を参照。

(54) Title: NOVEL FORMATE DEHYDROGENASE TOLERANT TO HALOGEN COMPOUNDS AND PROCESS FOR PRODUCING THE SAME

(54) 発明の名称: ハロゲン化合物耐性新規ギ酸脱水素酵素及びその製造方法

(57) Abstract: It is intended to provide a formate dehydrogenase which has a high specific activity, a small Km values to formic acid and NAD, a temperature-stability over a wide range, a pH-stability over a wide range, a wide function temperature range and a wide function pH range, is not inactivated even in case of coexisting in an enzymatic reduction system of a haloketone compound, and can efficiently regenerate a coenzyme. A formate dehydrogenase-producing strain is searched for from soil and an enzyme having the above-described properties and suitable for industrial use is provided. Moreover, a DNA containing the gene of this enzyme, a recombinant DNA with a vector and its transformant constructed by using a plasmid are provided. Furthermore, a process for producing the formate dehydrogenase using a strain belonging to the genus *Thiobacillus* or a transformant constructed by using a formate dehydrogenase gene originating in this strain, and a method of regenerating a coenzyme in an enzymatic reduction system with the use of this enzyme are provided.

[続葉有]

WO 03/031626 A1

WO 03/031626 A1

(57) 要約:

本発明は、高い比活性を有し、ギ酸及びＮＡＤに対するＫｍ値が小さく、温度安定性やｐＨ安定性の範囲が広く、作用温度、作用ｐＨの範囲が広く、かつ、ハロケトン化合物の酵素的還元反応系に共存させた場合でも失活することなく、補酵素を効率的に再生できるギ酸脱水素酵素を提供する。

土壌よりギ酸脱水素酵素生産菌を探索し、上記の工業的利用に適した性質を有する酵素を提供する。また、本酵素の遺伝子を含むＤＮＡ、ベクターとの組換えＤＮＡ及びこのプラスミドによる形質転換体を提供する。さらに、チオバシラス（Thiobacillus）属菌株あるいは本菌株由来のギ酸脱水素酵素遺伝子を用いた形質転換体による本ギ酸脱水素酵素の製造方法、及び、当該酵素を用いた酵素的還元系における補酵素再生方法を提供する。

NOVEL FORMATE DEHYDROGENASE TOLERANT TO HALOGEN COMPOUNDS AND PROCESS FOR PRODUCING THE SAME

TECHNICAL FIELD

The present invention relates to a polypeptide tolerant to haloketone compounds and having formate dehydrogenase activity, a gene coding for it, a process for producing a formate dehydrogenase with the aid of a strain of microorganism capable of producing said polypeptide or a transformant thereof, and a process for regenerating a coenzyme with good efficiency which comprises permitting said enzyme to be present in an enzymatic reduction reaction system.

BACKGROUND ART

The enzyme formate dehydrogenase (enzyme no.[EC1.2.1.2]) is an enzyme which catalyzes the reaction giving rise to carbon dioxide and reduced nicotine adenine dinucleotide (hereinafter referred to as NADH) from a formate and oxidized nicotine adenine dinucleotide (hereinafter referred to as NAD). Formate dehydrogenase is a useful enzyme having advantages that when it is used for coenzyme regeneration in a NADH-dependent enzymatic reaction, an inexpensive formate can be utilized and the byproduct is carbon dioxide which is not accumulated in the system. Furthermore, as an enzyme having small Km values for formate and NAD, this enzyme acts effectively at low substrate concentrations and can be utilized for specific microassay of formate, thus being an industrially useful enzyme.

Formate dehydrogenase is known to exist in higher plants, methanol-utilizing yeasts, bacteria, and the like. The enzyme which has heretofore been purified and characterized includes the enzyme derived from the higher plant pea (*Pisum sativum*: J. Biochem., vol.77, 845, 1975)

and the enzymes derived from methanol-utilizing yeast species, namely *Candida boidinii* (Eur. J. Biochem., vol.62, 151, 1976), *Candida methylica* (Eur. J. Biochem. vol.152, 657, 1985), *Candida methanolica* (FEMS Microbiol. Lett., vol.48, 139, 1987), *Kloeckera sp.* (Agric. Biol. Chem., vol.38, 111, 1974), *Pichia pastoris* (Agric. Biol. Chem., vol.47, 2547, 1983), and *Lipomyces methanosilviensis* (JP-A-60-241887), among others. However, these enzymes invariably have some or other shortcomings to be overcome for industrial application, namely low specific activity, large Km values for formate and NAD, and/or a narrow pH range for activity.

There also are several enzymes which have been purified from bacteria and characterized but each has its own drawback in industrial application. For example, the enzymes derived from *Pseudomonas* sp. 101 (Eur. J. Biochem., vol.99, 569, 1979) and *Pseudomonas oxalaticus* (Eur. J. Biochem., vol.83, 485, 1978) have comparatively high specific activity but are unstable in the absence of a stabilizer. The enzyme derived from *Moraxella* sp. (J. Bacteriol., vol.170, 3189, 1988; JP-A-63-313580) is low in specific activity and has a large Km value for formate. The enzyme derived from *Hyphomicrobium* sp. (JP-A-2000-78970, reported at 1999 Congress of Japan Society for Bioscience, Biotechnology, and Agrochemistry, Synopsis of Papers Read Before the same Congress, p.234) is low in specific activity and has a low pH range for activity. Moreover, the enzyme derived from *Paracoccus* sp. (JP-A-03-61481) has a large Km value for formate.

A further problem is that while the products (e.g. haloalcohols) obtainable by reducing haloketone compounds are very useful compounds as raw materials of such as pharmaceutical products, the formate dehydrogenases heretofore reported are invariably inactivated in the presence of a haloketone compound so that if added to an enzymatic reduction reaction system for a haloketone

compound, none are able to regenerate the coenzyme. Thus, there is not known a formate dehydrogenase capable of regenerating the coenzyme with good efficiency in the enzymatic reduction reaction system of a haloketone compound.

Referring to formate dehydrogenases of the bacterial origin, enzymes which do not require NAD as the electron acceptor [EC1.2.2.1]), such as those derived from *Escherichia coli* (J. Biol. Chem., vol.250, 6693, 1975), *Clostridium pasteurianum* (J. Bacteriol., vol.159, 375, 1984), *Clostridium thermoaceticum* (J. Biol. Chem., vol.259, 1826, 1983), etc. are also known but these cannot be utilized for the purpose of regenerating the coenzyme NAD.

Referring to the expression of bacterial NAD-dependent formate dehydrogenase genes in transformants, the genes derived from *Pseudomonas* sp. 101 (Biotechnol. Appl. Biochem., vol.18, 201, 1993), *Mycobacterium vaccae* (Appl. Microbiol. Biotechnol., vol.44, 479, 1995, JP-A-10-23896), *Pyrococcus* KOD1 (JP-A-2000-69971), *Hyphomicrobium* sp. (JP-A-2000-78970, reported at the 1999 Congress of Japan Society for Bioscience, Biotechnology, and Agrochemistry, Synopsis of Papers Read Before the same Congress, p.234) but there has been no knowing of a formate dehydrogenase gene derived from any microorganism of the genus *Thiobacillus*.

It is known that bacteria of the genus *Thiobacillus* have formate dehydrogenase activity in the form of crude enzyme solutions (J. Bacteriol., 131, 389 (1977)) but there is no report on the actual purification and isolation of an enzyme or on the characterization thereof, nor has it been reported that a relevant gene was ever isolated.

SUMMARY OF THE INVENTION

There can be provided a formate dehydrogenase which is free from the above-mentioned

disadvantages of the hitherto-known formate dehydrogenases, namely low enzyme productivity, low specific activity, large Km values for formate and NAD, narrow temperature and pH ranges of stability, and narrow pH range for action, but also has industrially useful characteristics such as high tolerance to haloketone compounds and a process for producing the same. There can further be provided a process for regenerating the coenzyme with good efficiency even in enzymatic reduction reaction systems, particularly in the enzymatic reduction of haloketone compounds which would inactivate the conventional formate dehydrogenases.

In view of the above state of the art, the inventors of the present invention did an extensive screening for soil microorganisms having formate dehydrogenase activity and, as a result, firstly isolated a strain of microorganism of the genus *Thiobacillus* which is capable of elaborating a formate dehydrogenase having laudable properties on a high production scale. From this strain of microorganism, the inventors isolated and purified the formate dehydrogenase and further succeeded in the isolation of a formate dehydrogenase gene and the expression of the gene in host microorganisms. Furthermore, it was found that the formate dehydrogenase of the invention is tolerant to haloketone compounds and capable of regenerating the coenzyme with good efficiency even in the enzymatic reduction system of a haloketone compound.

The present invention is concerned with a polypeptide of the following (a) or (b):

(a) a polypeptide comprising an amino acid sequence shown under SEQ ID NO:1 of the sequence listing

(b) a polypeptide comprising an amino acid sequence derived from the amino acid sequence shown under SEQ ID NO:1 of the sequence listing by substitution, insertion, deletion and/or addition of one or several amino acids and having formate dehydrogenase activity.

The present invention is further concerned with a DNA coding for the above polypeptide.

The present invention is further concerned with a DNA of the following (c) or (d):

(c) a DNA comprising a base sequence shown under SEQ ID NO:2 of the sequence listing

(d) a DNA having a base sequence derived from a base sequence shown under SEQ ID NO:2 of the sequence listing by substitution, insertion, deletion and/or addition of one or several bases and coding for the polypeptide having formate dehydrogenase activity.

The present invention is further concerned with a DNA

of the following (e) or (f):

(e) a DNA comprising a base sequence shown under SEQ ID NO:3 of the sequence listing

(f) a DNA having a base sequence derived from a base sequence shown under SEQ ID NO:3 of the sequence listing by substitution, insertion, deletion and/or addition of one or several bases and coding for the polypeptide having formate dehydrogenase activity.

The present invention is further concerned with a recombinant plasmid containing said DNA.

The present invention is further concerned with a transformant obtained by transforming a host microorganism with said recombinant plasmid.

The present invention is further concerned with a process for producing a formate dehydrogenase,

which comprises cultivating a strain of microorganism belongs to the genus *Thiobacillus* sp. KNK65MA (FERM BP-7671) to let it elaborate and accumulate said polypeptide in the resulting culture medium and harvesting the same.

The present invention is also concerned with a process for producing a formate dehydroganase, which comprises cultivating said transformant to let it elaborate and accumulate the formate dehydrogenase in the resulting culture medium and harvesting the same.

In a further aspect, the present invention is concerned with a process for regenerating a coenzyme,

which comprises permitting either said polypeptide or a strain of microorganism capable of producing said polypeptide or a processed matter thereof to be present in an enzymatic reduction reaction system.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is now described in detail.

The polypeptide of the invention is described in the first place.

In the present invention, the formate dehydrogenase activity of the polypeptide is assayed by quantitating the increase in absorbance at 340 nm resulting from the production of NADH at 30°C or 40°C in a 0.1 M phosphate buffer (pH 7) containing 500 mM sodium formate and 5 mM NAD.

The molecular weight determination is by gel permeation chromatography. The Km value for formate is determined by measuring the activity under the above conditions of activity assay (30°C, NAD 5 mM) while the concentration of sodium formate is varied, and the Km value for NAD is determiend under the above conditions of activity assay (30°C, sodium formate 500 mM) while the concentration of NAD is varied.

The temperature for action and the pH for action are determined by measuring the activity while the temperature or pH is varied under the above conditions of activity assay. The temperature stability is evaluated by treating the polypeptide at each temperature for 10 minutes and measuring the residual activity. The pH stability is evaluated by treating the polypeptide at 30°C for 22 hours at each pH level and measuring the residual activity.

The polypeptide of the invention is tolerant to haloketone compounds. The term "tolerant to haloketone

compounds" as used in this specification means that the residual activity after 5 minutes' incubation of a crude enzyme solution at 30°C in the presence of 10 mM ethyl 4-chloroacetoacetate is not less than 25%.

The polypeptide of the invention can be acquired from microorganisms having formate dehydrogenase activity. Therefore, as microorganisms for use as sources of the polypeptide of the invention, methanol-utilizing bacteria and formate-utilizing bacteria can be used with advantage. Thus, although this is not exclusive choices, microorganisms belonging to the genus *Thiobacillus*, for instance, can be used. In particular, *Thiobacillus* sp. are preferred and *Thiobacillus* sp. KNK65MA is especially preferred.

The *Thiobacillus* sp. KNK65MA mentioned above is the strain of microorganism which was isolated and obtained by the inventors of the present invention and has been deposited with the National Institute of Advanced Industrial Science and Technology International Patent Organism Depositary, Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan as of July 19, 2001 under the accession number of FERM BP-7671. The bacteriological characteristics of *Thiobacillus* sp. KNK65MA are shown below.

1. Morphology
   1) Bacilli ca 0.6 μm dia. x 1.5 to 2.0 μm
   2) Gram stain: negative
   3) Motility: non-motile
   4) Sporogenesis: non-sporulating
   5) Colonies on broth agar plate: round, smooth entire margin, low convex, lustrous, light yellow (translucent)
2. Cultural characteristics
   1) Broth liquid culture: suspended
   2) Litmuth milk: unchanged
3. Physiological characteristics
   1) Catalase: +

2) Oxidase: +
3) O/F test: -
4) Denitrification: +
5) MR test: +
6) VP test: -
7) Hydrogen sulfide production: -
8) Utilization of citric acid
Koser: -
Christensen: -
9) Utilization of inorganic nitrogen sources
$NaNO_3$: -
$(NH_4)_2SO_4$: -
10) Starch hydrolysis: -
11) Nitrate reduction: -
12) Indole production: -
13) Glucose acidification: -
14) Arginine dihydrolase: -
15) Urease: -
16) β-galactosidase: +
17) Esculin hydrolysis: +
18) Gelatin hydrolysis: -
19) Range for growth
pH
3.0: -
5.0: -
8.0: +
Temperature (°C)
30: +
50: -
20) Biotin requirement: +
21) Anaerobic growth: +
22) Utilization of sulfur compounds
Elemental sulfur: -
Sodium thiosulfate: +
23) Ethanol oxidation: $+_w$

24) Acid and gas production from carbohydrates in O-F medium (acid/gas)

L-arabinose: -/-
D-glucose: -/-
D-fructose: -/-
Maltose: -/-
Lactosse: -/-
D-sorbitol: -/-
Inositol: -/-
Starch: -/-
D-xylose: -/-
D-mannose: -/-
D-galactose: -/-
Sucrose: -/-
Trehalose: -/-
D-mannitol: +/-
Glycerol: -/-

25) Substrate utilization

Glucose: +
L-arabinose: -
D-mannose: -
D-mannitol: +
N-acetyl-D-glucosamine: -
Maltose: -
Potassium gluconate: +
n-Capric acid: -
Adipic acid: -
dl-Malic acid: -
Sodium citrate: -
Phenyl acetae: -

As the medium for cultivating a microorganism producing the polypeptide of the invention, an aqueous medium containing methanol as a major carbon source together with nitrogen sources and nutrients such as inorganic salts is used. Satisfactory results are obtained

in many cases when organic trace nutrients such as vitamins and amino acids are added. As the nitrogen sources, ammonium salts, aqueous ammonia, ammonia gas, urea, yeast extract, peptones, corn steep liquor, etc. can be used. As the inorganic salts, phosphates, magnesium salts, potassium salts, sodium salts, calcium salts, iron salts, sulfates, and chlorides can be used.

Culture can be made generally within the temperature range of 20°C to 40°C, preferably at 20°C to 30°C. The cultivation pH may range from 6.0 to 9.0, and is preferably from 7.0 to 9.0. The cultural process may be continuous or batch-wise.

Regarding the separation and purification from the culture medium, the cells are harvested, for example by centrifuging the culture medium, at completion of culture and disrupted, for example by sonication, to recover a crude enzyme solution. The polypeptide of the invention can be obtained by purifying the crude enzyme solution by salting-out or column chromatography, for instance.

The polypeptide of the invention may be a native enzyme acquired from a microorganism as above or a recombinant enzyme as produced by utilizing the recombinant gene technology.

As the native enzyme, the polypeptide shown under SEQ ID NO:1 of the sequence listing can be mentioned.

The polypeptide of the invention may also be a polypeptide comprising an amino acid sequence derived from the amino acid sequence shown under SEQ ID NO:1 by substitution, insertion, deletion and/or addition of one or several amino acids and having formate dehydrogenase activity.

The "amino acid sequence derived by substitution, insertion, deletion and/or addition of one or several amino acids" can be acquired by the technology well known to those skilled in the art, such as the method of site-

specific mutagenesis, through said substitution, insertion, deletion and/or addition of one or several amino acids. Specific relevant protocols are described in Nucleic Acid Res. 10, 6487 (1982), Methods in Enzymology 100, 448 (1983), among other literature.

The term "polypeptide having formate dehydrogenase activity" means any polypeptide showing at least 10%, preferably not less than 40%, more preferably not less than 60%, still more preferably not less than 80%, of the activity of the polypeptide comprising the amino acid sequence shown under SEQ ID NO:1 when determined under the above conditions of activity assay.

Now, the DNA according to the invention is described. The DNA of the invention may be any DNA that codes for a polypeptide such as the one described above. It may for example be a DNA comprising the base sequence shown under SEQ ID NO:2 or SEQ ID NO:3 of the sequence listing. Furthermore, it may be any DNA having a base sequence derived from the base sequence shown under SEQ ID NO:2 or SEQ ID NO:3 by substitution, insertion, deletion and/or addition of one or several bases and coding for a polypeptide having formate dehydrogenase activity.

The "base sequence derived by substitution, insertion, deletion, and/or addition of one or several bases" means a base sequence derived by substitution, insertion, deletion and/or addition of such a number of bases as can be substituted, inserted, deleted and/or added by the technology well known to those skilled in the art as described in, *inter alia*, Supplemental Issue, Tanpakushitsu, Kakusan, Koso (Protein, Nucleic Acid and Enzyme), PCR Method for Gene Amplification, TAKKAJ, 35 (17), 2951-3178 (1990) or Henry A. Erlich (ed.), translated into Japanese under the supervision of Ikunoshin Kato: PCR Technology (1990).

Furthermore, it may be a DNA which hybridizes with a

DNA comprising the base sequence shown under SEQ ID NO:2 or SEQ ID NO:3 under stringent conditions and coding for a polypeptide having formate dehydrogenase activity.

The "DNA which hybridizes with a DNA comprising the base sequence shown under SEQ ID NO:2 or SEQ ID NO:3 under stringent conditions" means a DNA which can be acquired by the colony hybridization, plaque hybridization, Southern hybridization, or the like technique using a DNA comprising the base sequence shown under SEQ ID NO:2 or 3 as a probe. Any one skilled in the art may easily carry out such hybridization procedures in accordance with the protocols described in Molecular Cloning 2nd Edt. (Cold Spring Harbor Laboratory Press, 1989) to acquire the objective DNA.

The DNA (formate dehydrogenase gene) according to the invention can be acquired from said microorganism having formate dehydrogenase activity. The objective DNA may be acquired by, *inter alia*, the following method.

In the first place, the amino acid sequence at the amino-terminus of a formate dehydrogenase purified from a strain of microorganism having formate dehydrogenase activity is determined by means of a gas-phase protein sequencer, for instance. The DNA primer designed on the basis of this amino acid sequence and the DNA primer designed on the basis of the sequence of high homology among the base sequences of known formate dehydrogenase genes are synthesized.

Then, from the source microorganism for formate dehydrogenase, the chromosomal DNA is isolated. The chromosomal DNA can be acquired by lysing and extracting cultured cells with a surfactant, CTAB, chloroform, phenol and the like, causing the extracted DNA to be precipitated from isopropyl alcohol, centrifuging the same, and washing the resulting DNA pellet with ethanol (e.g. Current Protocols in Molecular Biology (Greene Publishing Associates and Wiley-Interscience)).

Part of the objective gene can be obtained by carrying out PCR using this chromosomal DNA as a template and the above primers. Then, to acquire the full length of the objective gene, the chromosomal DNA obtained above is digested with suitable restriction enzymes such as HindIII and EcoRI and subjected to agarose electrophoresis. Using the DNA fragment which is part of the above formate dehydrogenase gene obtained by PCR as a probe, Southern hybridization is carried out and the DNA fragment containing the formate dehydrogenase gene and not cleaved by said restriction enzymes within the formate dehydrogenase gene is detected on the gel.

This DNA fragment is recovered from the gel and cyclized with e.g. T4 DNA ligase and using the resulting DNA as a template and synthetic DNA primers based on the base sequences corresponding to enzyme N-terminal and C-terminal regions of the partial formate dehydrogenase gene obtained by PCR as above and directed outwardly of said enzyme gene, PCR is carried out to construct DNA fragments coding for the further N-terminal and C-terminal sides of the partial gene already acquired with above primers. After sequencing of the DNA fragments, DNA primers are constructed based on the base sequences of the DNAs deduced to be present upstream of the DNA coding for the N-terminus and downstream of the DNA coding for the C-terminus of the enzyme and the DNA between these sequences is amplified to acquire a DNA fragment containing the full-length formate dehydrogenase gene. The acquired DNA fragment can be confirmed to contain the full length of the objective formate dehydrogenase gene by determination of molecular weight and partial base sequence analysis.

Then, the DNA fragment containing the formate dehydrogenase gene as obtained above can be integrated into a vector DNA with e.g. T4 DNA ligase to construct a recombinant plasmid. Using this plasmid, the DNA fragment

containing the formate dehydrogenase gene inserted into the vector is sequenced to confirm the presence of bases coding for the N-terminal amino acid sequence of the formate dehydrogenase. In addition, the site of translation initiation is determined and using the sequence down to the termination codon as an open reading frame, it is confirmed that this base sequence has relatively high homology with the known formate dehydrogenase gene, the encoded protein corresponds to the molecular weight determined by electrophoresis, etc., whereby the product is identified to be the objective gene.

By transforming a host strain of microorganism with the DNA thus acquired or a recombinant plasmid constructed by integrating this DNA into a vector, a transformant can be obtained.

As the host and vector, the host-vector systems described in "Recombinant DNA Experimentation Guidelines" (edited by Life Science Section, Research and Development Bureau of Science and Technology Agency, Japan, as amended March 22, 1996) can be utilized. For example, the host which can be used includes microorganisms belonging to any of the following genera: *Escherichia*, *Pseudomonas*, *Flavobacterium*, *Bacillus*, *Serratia*, *Corynebacterium*, *Brevibacterium*, *Agrobacterium*, *Acetobacter*, *Gluconobacter*, *Lactobacillus*, *Streptococcus*, and *Streptomyces*, among others. As the vector, plasmids and phages of the microbial origin, inclusive of derivatives thereof, which are capable of autonomous replication within these hosts can be employed. The particularly preferred host microorganism is *Escherichia coli* and the suitable vector is one capable of autonomous replication in this microorganism. Among such vectors are pUC18, pUC19, pBR322, pACYC184, pSC101, and pUCNT.

Referring to said transformant, the recombinant plasmid pFT001 constructed by integrating the above-

acquired DNA into the pUC19 vector or the recombinant plasmid pFT002 constructed by integrating the same into pUCNT (WO 94/03613), can be used to transform *Escherichia coli* HB101 to give the trnasformant *Escherichia coli* HB101 (pFT001) or *Escherichia coli* HB101 (pFT002). The recombinant plasmid pFT001 is shown in Fig.1 and the recombinant plasmid pFT002 is shown in Fig.2.

The above transformants *Escherichia coli* HB101 (pFT001) and *Escherichia coli* HB101 (pFT002) according to the invention have been deposited with the National Institute of Advanced Industrial Science and Technology International Patent Organism Depositary, Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan as of July 19, 2001 under the accession number of FERM BP-7672 and as of July 19, 2001 under the accession number of FERM BP-7673, respectively.

For increasing the enzyme output, a vector modified to have a potent structural promoter can be employed.

The recombinant DNA technology used in the present invention is well-known in the art and has been described, *inter alia*, in Molecular Cloning 2nd Edition (Cold Spring Harbor Laboratory Press, 1989), Current Protocols in Molecular Biology (Greene Publishing Associates and Wiley-Interscience).

The process for producing a formate dehydrogenase according to the invention is characterized in that a strain of microorganism belongs to the genus *Thiobacillus* sp. KNK65MA (FERM BP-7671) is cultivated to let it elaborate and accumulate said polypeptide in the resulting culture medium and the polypeptide so accumulated is harvested.

The process for producing a formate dehydrogenase according to the invention is characterized in that said transformant is cultivated to let it elaborate and accumulate a formate dehydrogenase in the resulting culture medium and the formate dehydrogenase so accumulated is

harvested.

The production of a formate dehydrogenase using the transformant according to the invention can be carried out by cultivating the transformant in a common medium. The medium for use to cultivate may be a common medium containing source of carbon, source of nitrogen and nutrients such as inorganic salts. Superior results are frequently obtained when organic trace nutrients such as vitamins and amino acids are further formulated. As the carbon source, carbohydrates such as glucose and sucrose, organic acids such as acetic and other acids, and alcohols can be judiciously used. As the nitrogen source, ammonium salts, aqueous ammonia, ammonia gas, urea, yeast extract, peptones, corn steep liquor, etc. can be utilized. As said inorganic salts, various phosphates, magnesium salts, potassium salts, sodium salts, calcium salts, iron salts, sulfates, and chlorides can be used.

Culture can be made within the temperature range of 25°C to 40°C, although the range of 25°C to 37°C is particularly preferred. The cultivation pH may range from 4 to 8 and is preferably from 5 to 7.5. The cultural process may be continuous or batch-wise.

Where necessary, an enzyme induction treatment such as addition of methanol, formic acid, isopropyl-1-thio-β-D-galactoside (IPTG), lactose, or the like may be carried out.

Regeneration of the coenzyme can be achieved by permitting either said polypeptide or a strain of microorganism capable of producing the polypeptide or a processed matter thereof to be present in the enzymatic reduction reaction system.

Thus, by permitting the formate dehydrogenase of the invention to be present in an enzymatic reduction reaction system, the coenzyme can be regenerated with good efficiency. Particularly, the coenzyme can be efficiently regenerated in the presence of this enzyme even in the

enzymatic reduction system of a haloketone compound or the like which would inactivate the conventional formate dehydrogenase.

For example, the enzymatic reduction reaction of a haloketone such as ethyl 4-chloroacetoacetate using the formate dehydrogenase of the invention for coenzyme regeneration can be carried out as follows.

This reaction can be carried out in water or a suitable organic solvent which is hardly soluble in water, for example ethyl acetate, butyl acetate, toluene, chloroform or n-hexane, or a biphasic system consisting of such an organic solvent and an aqueous medium. The reaction can be achieved by adding to such a solvent a substrate haloketone, the coenzyme NAD, and either a haloketone reductase or a microorganism having such reductive activity or a processed matter thereof, and either the polypeptide (i.e. haloketone-tolerant formate dehydrogenase) of the invention or a microorganism capable of producing this polypeptide or a processed matter thereof and stirring the mixture under judicious pH control with formic acid.

The haloketone compound is not particularly restricted provided that it can be a substrate for the reductase and microorganism to be used, thus including ethyl 4-chloroacetoacetate, ethyl 4-bromoacetoacetate, ethyl 4-iodoacetoacetate, methyl 4-chloroacetoacetate, ethyl 2-chloroacetoacetate, 2-chloro-1-(3-pyridinyl)ethanone, 1,1-dimethylethyl [(1S)-3-chloro-2-oxo-1-(phenylmethyl)propyl]carbamate, 2-chloroacetophenone, 2,3'-dichloroacetophenone, chloroacetone, 1-(benzoyloxy)-3-chloro-2-propanone, and 1-chloro-3-hydroxy-2-propanone, among others. The preferred substrates are α-haloketones such as ethyl 4-chloroacetoacetate, methyl 4-chloroacetoacetate, ethyl 2-chloroacetoacetate, 1,1-dimethylethyl [(1S)-3-chloro-2-oxo-1-

(phenylmethyl)propyl]carbamate, 2,3'-dichloroacetophenone, and 1-(benzoyloxy)-3-chloro-2-propanone.

As said reductase or microorganism capable of reducing a haloketone, the recombinant *Escherichia coli* HB101 (pNTS1M1) (FERM BP-8059) capable of reducing ethyl 4-chloroacetoacetate to a corresponding alcohol, for instance, can be employed. This recombinant *E. coli* HB101 (pNTS1M1) strain was originally deposited with the National Institute of Advanced Industrial Science and Technology, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan as of June 22, 2001 and transferred to the Budapest Treaty depositry as of May 27, 2002 with the accession number of FERM BP-8059 being assigned. It is to be understood that when an aldehyde dehydrogenase or an amino acid dehydrogenase, or a microorganism having the corresponding activity or a processed matter thereof is used as the reductase, a corresponding aldehyde or amine can be obtained as the product.

As the microorganism capable of producing the polypeptide of the invention, the above-mentioned transformant *Escherichia coli* HB101 (pFT001) or *Escherichia coli* HB101 (pFT002), for instance, can be employed.

The term "processed matter" as used herein with reference to said microorganism means any of the crude extract, lyophilized preparation and acetone-dried preparation of cultured cells, or a ground matter of such cells. Furthermore, these may be used in the form of the enzyme or cells immobilized in the conventional manner. The immobilization can be effected by various techniques known to those skilled in the art (e.g. crosslinking, physical adsorption, entrapment, etc.).

The reaction is carried out at 10°C to 70°C, preferably 20°C to 60°C, and at pH 4 to 10, preferably pH 5.5 to 9.5. The substrate concentration of the charge is 0.1% to 90% (w/v) but the substrate may be supplied

continuously. The reaction can be carried out batch-wise or continuously. The reaction of the invention can be conducted by utilizing the immobilized enzyme, a membrane reactor, and/or other contrivances.

As described above, the method of the invention is such that in the reduction reaction for enzymatic production of ethyl 4-chloro-3-hydroxybutyrate and other alcohols, amines, aldehydes, or the like, it provides for efficient coenzyme regeneration contributing to a drastic reduction in the level of use of an expensive coenzyme.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1 is a restriction enzyme map of the recombinant plasmid pFT001 containing the formate dehydrogenase gene of the invention.

Fig.2 is a restriction enzyme map of the recombinant plasmid pFT002 containing the formate dehydrogenase gene of the invention.

Fig.3 is a graph for determining the Km value for formate of the formate dehydrogenase of the invention.

Fig.4 is a graph for determining the Km value for NAD of the formate dehydrogenase of the invention.

Fig.5 is a graph for determining the temperature range for action and optimum temperature of the formate dehydrogenase of the invention.

Fig.6 is a graph for determining the pH range for action and optimum pH of the formate dehydrogenase of the invention.

Fig.7 is a graph for determining the temperature stability of the formate dehydrogenase of the invention.

Fig.8 is a graph for determining the pH stability of the formate dehydrogenase of the invention.

BEST MODES FOR CARRYING OUT THE INVENTION

The following examples illustrate the present

invention in further detail. It should, however, be understood that the invention is by no means restricted by these examples.

Example 1 Acquisition of the *Thiobacillus* sp. KNK65MR strain

The formate dehydrogenase high-producer strain of the invention, namely *Thiobacillus* sp. KNK65MA, was isolated as follows. Soil samples collected from various geographic locations were respectively suspended in 0.9% saline. Each of the supernatants obtained in size of 1% was inoculated into 7 ml of the liquid medium of the composition shown in Table 1, which contained methanol as a sole carbon source, followed by shake culture at 30°C under aerobic conditions.

Table 1

Methanol	8 g
$(NH_4)_2SO_4$	5 g
KH_2PO_4	0.15 g
K_2HPO_4	4.85 g
$MgSO_4$	0.5 g
NaCl	0.1 g
$CaCl_2$	0.1 g
Meat extract	0.1 g
Yeast extract	0.1 g
Polypeptone	0.1 g
Biotin	20 µg
Calcium pantothenate	2 mg
Folic acid	2 µg
Inositol	10 mg
Niacin	400 µg
p-aminobenzoic acid	200 µg
Pyridoxine hydrochloride	400 µg
Riboflavin	200 µg
Thiamin hydrochloride	400 µg

Boric acid	500 µg
$CuSO_4$	40 µg
KI	100 µg
$FeCl_3$	200 µg
$MnSO_4$	400 µg
Na_2MoO_4	200 µg
$ZnSO_4$	400 µg

The above composition was made up to 1 L with water, adjusted to pH 9, sterilized by autoclaving, and used. However, methanol was added after sterilization.

Each culture medium showing bacterial growth was analyzed for cell concentration in terms of absorbance at 600 nm. The cells obtained by centrifuging 1.5 ml of the above culture medium was suspended in 0.5 ml of a substrate solution (0.1 M phosphate buffer, 0.5 M sodium formate, 1 mM NAD, 1% Triton X-100, pH 7) and shake-cultured at 30°C for 20 hours for cellular reaction. The reaction mixture was centrifuged and the amount of NADH in the supernatant was determined at 340 nm. The strain giving a high quotient when the NADH output was divided by cell concentration was selected as a strain having high formate dehydrogenase activity. Then, 5 ml of the culture medium of the selected strain was centrifuged and the resulting cells was suspended in 0.5 ml of 0.1 M phosphate buffer (pH 7), sonicated, and centrifuged to obtain a crude enzyme solution as supernatant.

The formate dehydrogenase activity of the crude enzyme solution was assayed by quantitating the increase in absorbance at 340 nm resulting from production of NADH in 0.1 M phosphate buffer (pH 7) containing 500 mM sodium formate and 5 mM NAD at 30°C.

Protein assays were carried out by the method of Bradford (Anal. Biochem., vol.72, 248, 1976) using BSA as

standard protein.

The α-haloketone tolerance of the formate dehydrogenase was evaluated by mixing 150 µl of the crude enzyme solution with 150 µl of 20 mM ethyl 4-chloroacetoacetate, incubating the mixture at 30°C for 5 minutes, assaying formate dehydrogenase activity in the same manner as above, and comparing the result with the pre-treatment activity.

Then, in cases where the specific activity of the crude enzyme solution was high and the tolerance to ethyl 4-chloroacetoacetate was high, the strain having formate dehydrogenase activity was isolated from the culture medium by the monocolony method and the isolated strain of cells thus obtained were cultivated to prepare a crude enzyme solution in the same manner as above and the solution was analyzed for formate dehydrogenase activity, protein concentration, and haloketone tolerance. Then, a comparison of specific activity and haloketone tolerance of the crude enzyme solution was carried out for each strain of cells and accordingly *Thiobacillus* sp. KNK65MA (FERM BP-7671) was elected as a high producer of formate dehydrogenase with high specific activity and high haloketone tolerance.

Example 2 Isolation and purification of the formate dehydrogenase

A colony of *Thiobacillus* sp. KNK65MA was inoculated into 7 ml of a medium of the composition shown in Table 2 and shake-cultured aerobically at 28°C for 2 days. The resulting culture medium each in size of 1% based on the medium volume was inoculated into 100 ml per flask of a production medium of the composition shown in Table 3 and shake-cultured aerobically at 28°C for 7 days.

Table 2

Methanol	20 g
Glycerol	10 g
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

The composition was made up to 1 L with water, adjusted to pH 7, sterilized by autoclaving, and used. However, methanol was added after sterilization.

Table 3

Methanol	20 g
$(NH_4)_2SO_4$	5 g
KH_2PO_4	0.15 g
K_2HPO_4	4.85 g
$MgSO_4$	0.5 g
NaCl	0.1 g
$CaCl_2$	0.1 g
Meat extract	0.1 g
Yeast extract	0.1 g
Polypeptone	0.1 g
Biotin	20 µg
Calcium pantothenate	2 mg
Folic acid	2 µg
Inositol	10 mg
Niacin	400 µg
p-Aminobenzoic acid	200 µg
Pyridoxine hydrochloride	400 µg
Riboflavine	200 µg
Thiamin hydrochloride	400 µg
Boric acid	500 µg
$CuSO_4$	40 µg
KI	100 µg
$FeCl_3$	200 µg
$MnSO_4$	400 µg

Na_2MoO_4	200 µg
$ZnSO_4$	400 µg

The composition was made up to 1 L with water, adjusted to pH 9, sterilized by autoclaving, and used. However, methanol was added after sterilization.

After completion of cultivation, the cells were harvested by centrifugation and suspended in 0.1 M phosphate buffer (pH 7.0) containing 1 mM dithiothreitol (DTT) and EDTA. The suspension was sonicated to disrupt the cells and centrifuged. The supernatant was salted out with 25 to 60% saturation of ammonium sulfate and the precipitate was recovered by centrifugation. This fraction was dissolved in 0.01 M phosphate buffer (pH 6.5) containing 1 mM DTT and EDTA and the solution was dialyzed against the same buffer. Then, DEAE-Sepharose (product of Pharmacia) column chromatography was carried out and after the column was washed with the same buffer, elution was carried out with 0.1 M phosphate buffer (pH 6.5) containing 1 mM DTT and EDTA. Active fractions were pooled and, after addition of ammonium sulfate to 24% saturation, applied to a chromatographic column of TSKgel Phenyl Toyopearl 650 M (product of Tosoh Corporation) and elution was carried out on an ammonium sulfate gradient of 24 to 0% saturation in 0.1 M phosphate buffer (pH 6.5) containing 1 mM DTT and EDTA. After addition of ammonium sulfate at a final concentration of 70% saturation to the active fractions, the pellet obtained by centrifugation was dissolved in 0.01 M phosphate buffer (pH 6.5) containing 1 mM DTT and EDTA, followed by dialysis against the same buffer. After this dialysis, the resulting material was applied to a chromatographic column of Blue Sepharose 6 Fast Flow (product of Pharmacia) and after the column was washed with the same buffer, elution was carried out on a NaCl gradient

of 0.5 M to 0 M in the same buffer. The active fractions were pooled, concentrated with an ultrafiltration membrane (cut-off molecular weight 10,000), and dialyzed against 0.01 M phosphate buffer (pH 6.5) containing 1 mM DTT and EDTA. The resulting material was applied to a column of Gigapite (product of Seikagaku Corporation) for chromatography. After the column was washed with 2 mM phosphate buffer (pH 6.5) containing 1 mM DTT and EDTA, elution was carried out with 50 mM phosphate buffer (pH 6.5) containing 1 mM DTT and EDTA. The active fractions were pooled and analyzed by SDS-polyacrylamide electrophoresis. As a result, the formate dehydrogenase was detected as a single band, attesting to the high purity of the thus-purified enzyme.

Example 3 Properties of the enzyme

The properties of the purified formate dehydrogenase obtained in Example 2 were studied as follows.

[Specific activity]

The activity of the formate dehydrogenase obtained was assayed by quantitating the increase in absorbance at 340 nm resulting from formation of NADH in 0.1 M phosphate buffer (pH 7) containing 500 mM sodium formate and 5 mM NAD at 30°C or 40°C. In this connection, the amount of the enzyme yielding 1 μmol of NADH in 1 minute was defined as 1 unit. Protein assay was performed by the method of Lowry using BSA as standard protein. The specific activity of the purified formate dehydrogenase was 7.6 μ/mg protein (30°C) or 13.3 μ/mg protein (40°C).

[Determination of Km values]

The Km values for formate and NAD were determined as follows. Thus, the Km value for formate was determined by measuring the activity with the concentration of sodium

formate varied under the above specific activity assay conditions (30°C, NAD 5 mM) and the Km value for NAD was determined by measuring the activity with the concentration of NAD varied under the above specific activity assay conditions (30°C, sodium formate 500 mM). In Fig.3 and Fig.4, V represents the reaction velocity of the enzyme, S represents the concentration of the substrate, and the intercept of the abscissa represents -1/Km. The Km value for formate is found from Fig.3 to be 1.6 mM and that for NAD is found from Fig.4 to be 0.048 mM.

[Temperature range for action and optimum temperature]

The temperature range for action and optimum temperature were investigated. Fig.5 is a graph showing the relative activity at each temperature level, with the activity at 30°C being taken as 100%. It should be understood that the pH was kept constant at 7 for all temperatures. The enzyme of the invention acted well within the range of 20°C to 65°C, with the optimum temperature being 50°C to 60°C.

[pH range for action and optimum pH]

The pH range for action and optimum pH were investigated. Fig.6 is a graph showing the relative activity at each pH level, with the activity at pH 5.6 being taken as 100%. It should be understood that the temperature was kept constant at 30°C for all pH levels. The enzyme of the invention acted well within the range of pH 5.0 to 10.5, with the optimum pH being 5.5 to 9.5.

[Temperature stability]

The temperature stability was investigated. Fig.7 is a graph constructed by plotting residual activity after 10 minutes' treatment at each temperature in 0.1 M phosphate buffer (pH 7), with the activity at 0°C being taken as 100%.

The enzyme of the invention was stable at temperatures not over 50°C.

[pH stability]

The pH stability was investigated. Fig.8 is a graph constructed by plotting residual activity after 22 hours' treatment at 30°C with pH varied, with the activity at pH 7.5 being taken as 100%. The enzyme of the invention was comparatively stable within the range of pH 4.5 to 9.0.

[Molecular weight determination]

As determined by gel permeation chromatography, the molecular weight was approximately 9×10^4 ($9 \times 10^4 \pm 5 \times 10^3$).

Example 4 Isolation of the formate dehydrogenase gene

In the first place, the cells obtained by cultivating *Thiobacillus* sp. KNK65MA in the same manner as in Example 1 were lysed and extracted with a surfactant, CTAB, chloroform, phenol and the like, and the extracted DNA was precipitated from isopropyl alcohol and centrifuged. The DNA obtained as a pellet was washed with ethanol to give the chromosomal DNA (Current Protocols in Molecular Biology (Greene Publishing Associates and Wiley-Interscience)). To acquire the objective formate dehydrogenase gene, the amino-terminal amino acid sequence of the purified formate dehydrogenase was determined with a gas-phase protein sequencer or the like, and using the DNA mix primer (Primer-1) shown under SEQ ID NO:4 of the sequence listing as designed based on the above amino acid sequence and the DNA mix primer (Primer-2) shown under SEQ ID NO:5 of the sequence listing as designed based on the sequence of high homology among the base sequences of known formate dehydrogenase genes, a PCR using the above-obtained chromosomal DNA as a template was carried out. Referring

to SEQ ID NO:4 and NO:5 of the sequence listing, n represents adenine, guanine, cytosine or thymine, r represents adenine or guanine, and y represents cytosine or thymine. As the result of PCR, part of the objective formate dehydrogenase gene was obtained. Then, to acquire the full length of the objective gene, the previously obtained-chromosomal DNA fragments digested by the restriction enzymes SacI, XbaI, HindIII, ClaI, BamHI, PstI and EcoRI was electrophoresed on agarose gel and Southern hybridization was carried out using DNA fragments of the partial formate dehydrogenase gene obtained by PCR as probes. As a result, it was the band containing the formate dehydrogenase gene which was detected in the case of EcoRI or HindIII digestion that showed that the formate dehydrogenase gene was not cleaved by the restriction enzyme as used and the DNA fragment containing the formate dehydrogenase gene was small. After recovery of these DNA fragments from the gels, cyclization was performed with T4 DNA ligase and using the resulting DNA derived from the EcoRI or HindIII digest as a template, the DNA primers (Primer-3, Primer-4) shown under SEQ ID NO:6 and NO:7 of the sequence listing directed outwardly of the enzyme gene were synthesized based on the base sequences corresponding to segments on the N-terminal and C-terminal sides, respectively, of the enzyme of the partial formate dehydrogenase gene obtained by PCR as above, and inverse PCR was carried out. Thus acquired was a DNA fragment containing outward gene segments not possessed by the partial gene obtained previously. After determination of the base sequence of this DNA fragment, using a DNA primer (Primer-5) having the sequence corresponding to the base sequence deduced to be situated upstream of the DNA encoding the N-terminus of the enzyme shown under SEQ ID NO:8 of the sequence listing and a restriction enzyme PstI restriction site combined thereto and a DNA primer (Primer-

6) having the sequence corresponding to the base sequence deduced to be situated downstream of the DNA encoding the C-terminus shown under SEQ ID NO:9 of the sequence listing and a restriction enzyme EcoRI restriction site combined thereto, the DNA between these flanking regions was amplified by PCR to give a DNA fragment (SEQ ID NO:2 of the sequence listing) cotaining the full length of the formate dehydrogenase gene. Based on determination of molecular weight and analysis of partial base sequence of the DNA fragment thus obtained, the fragment was confirmed to contain the full length (SEQ ID NO:3 of the sequence listing) of the formate dehydrogenase gene.

Example 5 Construction of a recombinant plasmid containing the formate dehydrogenase gene and analysis of the gene

The DNA fragment containing the formate dehydrogenase gene as acquired in Example 4 was cleaved with restriction enzymes PstI and EcoRI and, using T4 DNA ligase, combined to the vector plasmid pUC19 cleaved with the same enzymes to construct a recombinant plasmid pFT001 containing the formate dehydrogenase gene as represented by the restriction enzyme map in Fig.1. In Fig.1, "FDH gene" represents the formate dehydrogenase gene of the invention.

Using the plasmid pFT001 thus acquired, the DNA fragment flanked by PstI and EcoRI sites as obtained in Example 4 was analyzed for base sequence. As a result, the presence of bases coding for the N-terminal amino acid sequence determined with the purified formate dehydrogenase was confirmed. In addition, the translation initiation site was accordingly determined and using the sequence down to the termination codon as an open reading frame, it was confirmed that this base sequence was homologous with the known formate dehydrogenase gene and that the protein encoded thereby corresponds to the molecular weight

determined by electrophoresis. The base sequence of the thus-obtained DNA fragment containing the full length of the formate dehydrogenase gene is shown under SEQ ID NO:2 of the sequence listing, the base sequence of the open reading frame is shown under SEQ ID NO:3 of the sequence listing, and the amino acid sequence deduced from the base sequence is shown under SEQ ID NO:1 of the sequence listing.

Example 6 Construction of a recombinant plasmid for high expression of the formate dehydrogenase gene

Using the primers (Primer-7, Primer-8) having the sequences obtained by combining NdeI and PstI restriction sites to the N-terminal and C-terminal regions, respectively, of the formate dehydrogenase gene obtained in Example 4, the sequences of which are shown under SEQ ID NO:10 and NO:11, the DNA intermediate therebetween was amplified by PCR to acquire the open reading frame DNA fragment shown under SEQ ID NO:3 of the sequence listing.

This DNA fragment was cleaved with restriction enzymes NdeI and PstI and, using a DNA ligase, combined to the vector plasmid pUCNT (WO 94/03613) cleaved with the same enzymes to construct a recombinant plasmid pFT002 designed so that the formate dehydrogenase gene might be expressed at a higher level as compared with pFT001 as represented by the restriction enzyme map in Fig.2. In Fig.2, "FDH gene" represents the formate dehydrogenase gene of the invention.

Example 7 Construction of a transformant using a recombinant DNA containing the formate dehydrogenase gene

The recombinant plasmids pFT001 and pFT002 obtained in Example 5 and Example 6 were respectively admixed with competent cells of *Escherichia coli* HB101 for transformation and then plated on the agar medium shown in Table 4 to obtain the transformant *Escherichia coli* HB101

(pFT001) or *Escherichia coli* HB101 (pFT002) having the recombinant DNA containing the formate dehydrogenase gene as a colony.

Table 4

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
Ampicillin	100 mg

The composition was made up to 1 L with deionized water, adjusted to pH 7, and sterilized by autoclaving. However, ampicillin was added after sterilization.

The transformant colony obtained was inoculated into 10 ml of the liquid medium shown in Table 5 and shake-cultured aerobically at 37°C for 20 hours.

Table 5

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g
Ampicillin	100 mg

The composition was made up to 1 L with deionized water, adjusted to pH 7, and sterilized by autoclaving. However, ampicillin was added after sterilization.

The cells were harvested by centrifuging the resulting culture medium, suspended in 0.1 M phosphate buffer (pH 7), disrupted by sonication, and centrifuged to remove cell-derived insolubles and give a transformant FDH enzyme solution. A 0.1 ml portion of this enzyme solution was mixed with 1.5 ml of 1 M sodium formate (pH 7 in 0.1 M

phosphate buffer), 0.15 ml of 0.1 M NAD, and 1.25 ml of 0.1 M phosphate buffer (pH 7) and the change in absorbance at 340 nm was investigated at 30°C. As a result, increases in absorbance due to production of NADH were observed, indicating that both transformants had formate dehydrogenase activity.

Example 8 Haloketone tolerance of the formate dehydrogenase

The tolerance of this enzyme and other FDHs to various haloketones was investigated as follows. The recombinant *Escherichia coli* HB101 (pFT002) having the KNK65MA FDH gene-integrated pFT002 as obtained in Example 7 was inoculated into 100 ml of the liquid medium shown in Table 5 sterilizated in a 500 ml Sakaguchi flask, and shake-cultured aerobically at 37°C for 20 hours. The resulting culture medium was centrifuged to harvest the cells, which were then suspended in 0.1 M phosphate buffer (pH 6.5), disrupted by sonication, and centrifuged to remove cell-derived insolubles and give a transformant FDH enzyme solution.

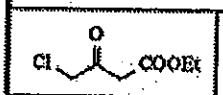
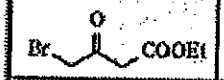
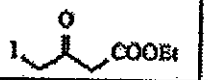
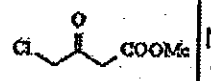
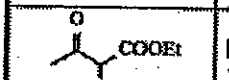
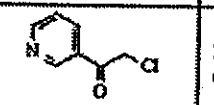
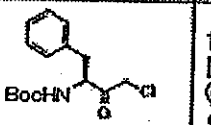
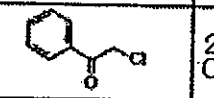
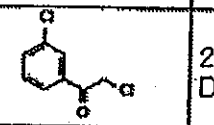
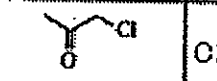
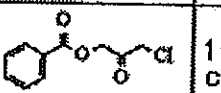
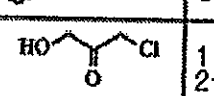
In the same manner, the recombinant *Escherichia coli* HB101 (pFA002) expressing the FDH derived from the KNK607 strain (WO 02/46427) was also cultivated to acquire a transformant FDH enzyme solution. Furthermore, *Candida boidinii* (ATCC 32195) was inoculated into 100 ml of the medium described in Example 1 (pH 6.0, however) and shake-cultured aerobically at 28°C for 72 hours to similarly give an FDH enzyme solution.

To each of the three cell-free extracts thus prepared was added each of the various haloketones shown in Table 6 at 20 mM concentration (however, 2 mM for 1,1-dimethylethyl [(1S)-3-chloro-2-oxo-1-(phenylmethyl)propyl]carbamate and 10 mM for 1-(benzoyloxy)-3-chloro-2-propanone) in a ratio of 1:1 and after the mixture was allowed to stand at 30°C

for 5 hours (however, 28.5 hours in the case of 2-chloroacetophenone), the residual FDH activity was determined. The FDH activity was assayed by the method described in Example 7.

As shown in Table 6, the enzyme of the invention was confirmed to be highly tolerant to various haloketone compounds as compared with other FDHs. The residual FDH activity was expressed in the percent residual activity after treatment, with the activity of each untreated cell-free extract being taken as 100%.

Table 6

Haloketone		Residual FDH activity (%)		
		KNK65MA	KNK607	Candida boidinii
	Ethyl 4-chloroacetoacetate	100.7	20.1	14.3
	Ethyl 4-bromoacetoacetate	24.1	0.0	0.0
	Ethyl 4-iodoacetoacetate	12.1	0.0	0.0
	Methyl 4-chloroacetoacetate	100.0	13.1	14.9
	Ethyl 2-chloroacetoacetate	100.0	2.5	5.4
	2-Chloro-1-(3-pyridinyl)ethanone	90.1	6.1	1.5
	1,1-Dimethylethyl [(1S)-3-chloro-2-oxo-1-(phenylmethyl)propyl] carbamate	100.0	81.6	31.3
	2-Chloroacetophenone	100.7	100.0	29.4
	2,3'-Dichloroacetophenone	97.9	27.0	7.4
	Chloroacetone	100.0	46.2	38.2
	1-(Benzoyloxy)-3-chloro-2-propanone	100.7	1.6	4.5
	1-Chloro-3-hydroxy-2-propanone	80.1	11.7	14.7

Example 9 Process for regenerating a coenzyme by permitting an FDH gene-integrated recombinant *Escherichia coli* to be present in the enzymatic reduction system of ethyl 4-chloroacetoacetate

Escherichia coli HB101 (pFT002) expressing the KNK65MA-derived FDH as acquired in Example 7 and *Escherichia coli* HB101 (pFA002) expressing KNK607-derived FDH (WO 02/46427) were respectively inoculated into 60 ml of the liquid medium shown in Table 5 sterilized in a 500 ml Sakaguchi flask and shake-cultured at 37°C for 9 hours. The resulting culture medium was inoculated into 100 ml of the liquid medium shown in Table 5 sterilized in a 500 ml Sakaguchi flask in size of 1% and shake-cultured at 37°C for 46 hours. The resulting culture medium was centrifugally concentrated 5-fold and sonicated to give an FDH enzyme solution. On the other hand, for use as the reductase enzyme for reducing a 4-haloacetoacetate, the recombinant *Escherichia coli* HB101 (pNTS1M1) (FERM BP-8059) was inoculated into 100 ml of the medium shown in Table 5 sterilized in a 500 ml Sakaguchi flask, shake-cultured at 37°C for 23 hours, and concentrated 10-fold and the cells were disrupted. To 6 ml of the above 5-fold concentrate of the FDH enzyme solution were added 9.25 ml of disrupted HB101 (pNTS1M1), 25 mg of NAD^+, 1 g of sodium formate, and 6 g of ethyl 4-chloroacetoacetate, followed by addition of 14.75 ml of ion-exchange water and 30 ml of butyl acetate to make a total of 60 ml. With the pH being controlled at 6.5 with 5 M aqueous formic acid solution, the reaction was carried out at 30°C for 3.5 hours with stirring. After completion of the reaction, the reaction mixture was extracted with butyl acetate and the extract was analyzed. In the system where the coenzyme was regenerated by utilizing the FDH derived from KNK65MA according to the invention, ethyl 4-chloro-3-hydroxybutyrate was formed at a conversion rate of 100%. On the other hand, in the system utilizing the FDH derived from KNK607, ethyl 4-chloro-3-hydroxybutyrate was formed at a conversion rate of 26%.

The quantitation of ethyl 4-chloro-3-hydroxybutyrate was carried out by gas chromatography. Thus, using a glass

column (ID 3 mm x 1 m) packed with PEG-20M Chromosorb WAW DMCS 10%, 80/100 mesh (product of GL Sciences Inc.), chromatography was carried out at 150°C. The detection was made by FID.

INDUSTRIAL APPLICABILITY

The present invention provides a formate dehydrogenase having high specific activity, small Km values for formate and NAD, broad temperature and pH ranges of stability, broad temperature and pH ranges for action, and high tolerance to haloketones, thus being suited for industrial use. The invention further provides a DNA containing the gene coding for the above enzyme, a recombinant DNA constructed using a vector, and a transformant obtained by using this plasmid. The invention further provides an efficient process for producing said enzyme which is meritorious and has novel properties, and a process for regenerating a coenzyme with good efficiency which comprises permitting said enzyme to be present in the enzymatic reduction system of a haloketone or other compound.

CLAIMS

1. A polypeptide of the following (a) or (b):

(a) a polypeptide comprising an amino acid sequence shown under SEQ ID NO:1 of the sequence listing

(b) a polypeptide comprising an amino acid sequence derived from the amino acid sequence shown under SEQ ID NO:1 of the sequence listing by substitution, insertion, deletion and/or addition of one or several amino acids and having formate dehydrogenase activity.

2. The polypeptide according to Claim 1, which is tolerant to haloketone compounds.

3. The polypeptide according to Claim 1 or 2, which is derived from a microorganism belonging to the genus *Thiobacillus*.

4. The polypeptide according to Claim 3,

wherein the microorganism is *Thiobacillus* sp. KNK65MA (FERM BP-7671).

5. A DNA coding for the polypeptide according to any one of claims 1 to 4.

6. A DNA of the following (c) or (d):

(c) a DNA comprising a base sequence shown under SEQ ID NO:2 of the sequence listing

(d) a DNA having a base sequence derived from a base sequence shown under SEQ ID NO:2 of the sequence listing by substitution, insertion, deletion and/or addition of one or several bases and coding for the polypeptide having formate dehydrogenase activity.

7. A DNA of the following (e) or (f):

(e) a DNA comprising a base sequence shown under SEQ ID NO:3 of the sequence listing

(f) a DNA having a base sequence derived from a base sequence shown under SEQ ID NO:3 of the sequence listing by substitution, insertion, deletion and/or addition of one or several bases and coding for the polypeptide having formate dehydrogenase activity.

8. A recombinant plasmid containing the DNA according to any one of Claims 5 to 7.

9. The recombinant plasmid according to Claim 8,

wherein a vector is pUC18, pUC19, pBR322, pACYC184, pSC101 or pUCNT.

10. The recombinant plasmid according to Claim 8 or 9,

which is pFT001.

11. The recombinant plasmid according to Claim 8 or 9,

which is pFT002.

12. A transformant obtained by transforming a host microorganism with the recombinant plasmid according to any one of Claims 8 to 11.

13. The transformant according to Claim 12,

wherein the host microorganism is selected from among microorganisms belonging to the following genera: *Escherichia*, *Pseudomonas*, *Flavobacterium*, *Bacillus*, *Serratia*, *Corynebacterium*, *Brevibacterium*, *Agrobacterium*, *Acetobacter*, *Gluconobacter*, *Lactobacillus*, *Streptococcus* and *Streptomyces*.

14. The transformant according to Claim 12,

wherein the host microorganism is *Escherichia coli*.

15. The transformant according to Claim 12,

wherein the host microorganism is *Escherichia coli* HB101.

16. The transformant according to Claim 12,

which is *Escherichia coli* HB101(pFT001) (FERM BP-7672) or *Escherichia coli* HB101(pFT002) (FERM BP-7673).

17. A process for producing a formate dehydrogenase,

which comprises cultivating a strain of microorganism belongs to the genus *Thiobacillus* sp. KNK65MA (FERM BP-7671) to let it elaborate and accumulate the polypeptide according to any one of Claims 1 to 4 in the resulting culture medium and harvesting the same.

18. A process for producing a formate dehydroganase, which comprises cultivating the transformant according to any one of Claims 12 to 16 to let it elaborate and accumulate the formate dehydrogenase in the resulting culture medium and harvesting the same.

19. A process for regenerating a coenzyme, which comprises permitting either the polypeptide according to any one of Claims 1 to 4 or a strain of microorganism capable of producing said polypeptide or a processed matter thereof to be present in an enzymatic reduction reaction system.

20. The process according to Claim 19, wherein the enzymatic reduction reaction is the enzymatic reduction reaction of a haloketone compound.

21. The process according to Claim 20, wherein the haloketone compound is an α-haloketone compound.

ABSTRACT

The invention provides a formate dehydrogenase having high specific activity, small Km values for formate and NAD, broad temperature and pH ranges of stability, broad temperature and pH ranges for action, and capable of regenerating a coenzyme with good efficiency in case of being present in an enzymatic reduction reaction system without inactivated.

The invention provides the enzyme mentioned above which has characteristics suited for industrial application by screening for soil formate dehydrogenase-producing microorganisms. The invention further provides a DNA containing the gene coding for the enzyme of the invention, a recombinant DNA constructed using a vector, and a transformant obtained by using this plasmid. The invention further provides a process for producing the formate dehydrogenase of the invention by using any microorganism of the genus *Thiobacillus* or a transformant constructed by using a formate dehydrogenase gene derived from above strain of microorganism, and a process for regenerating a coenzyme by using said enzyme in an enzymatic reduction system.

SEQUENCE LISTING

<110> KANEKA CORPORATION

<120> NOVEL FORMATE DEHYDROGENASE TORELANT TO HALOGEN COMPOUNDS AND PR OCESS FOR PRODUCING THE SAME

<130> T696/FDH-2

<150> JP2001-312043

<151> 2001-10-9

<160> 11

<170> PatentIn Ver. 2.1

<210> 1

<211> 401

<212> PRT

<213> Thiobacillus sp.

<400> 1

Met Ala Lys Ile Leu Cys Val Leu Tyr Asp Asp Pro Val Asp Gly Tyr
1 5 10 15

Pro Lys Thr Tyr Ala Arg Asp Asp Leu Pro Lys Ile Asp His Tyr Pro
20 25 30

Gly Gly Gln Thr Leu Pro Thr Pro Lys Ala Ile Asp Phe Thr Pro Gly
35 40 45

Gln Leu Leu Gly Ser Val Ser Gly Glu Leu Gly Leu Arg Lys Tyr Leu

50 55 60

Glu Ala Asn Gly His Thr Phe Val Val Thr Ser Asp Lys Asp Gly Pro

65 70 75 80

Asp Ser Val Phe Glu Lys Glu Leu Val Asp Ala Asp Val Val Ile Ser

85 90 95

Gln Pro Phe Trp Pro Ala Tyr Leu Thr Pro Glu Arg Ile Ala Lys Ala

100 105 110

Lys Asn Leu Lys Leu Ala Leu Thr Ala Gly Ile Gly Ser Asp His Val

115 120 125

Asp Leu Gln Ser Ala Ile Asp Arg Gly Ile Thr Val Ala Glu Val Thr

130 135 140

Tyr Cys Asn Ser Ile Ser Val Ala Glu His Val Val Met Met Ile Leu

145 150 155 160

Gly Leu Val Arg Asn Tyr Ile Pro Ser His Asp Trp Ala Arg Lys Gly

165 170 175

Gly Trp Asn Ile Ala Asp Cys Val Glu His Ser Tyr Asp Leu Glu Gly

180 185 190

Met Thr Val Gly Ser Val Ala Ala Gly Arg Ile Gly Leu Ala Val Leu

195 200 205

Arg Arg Leu Ala Pro Phe Asp Val Lys Leu His Tyr Thr Asp Arg His

210 215 220

Arg Leu Pro Glu Ala Val Glu Lys Glu Leu Gly Leu Val Trp His Asp

225 230 235 240

Thr Arg Glu Asp Met Tyr Pro His Cys Asp Val Val Thr Leu Asn Val

245 250 255

Pro Leu His Pro Glu Thr Glu His Met Ile Asn Asp Glu Thr Leu Lys

260 265 270

Leu Phe Lys Arg Gly Ala Tyr Ile Val Asn Thr Ala Arg Gly Lys Leu

275 280 285

Ala Asp Arg Asp Ala Ile Val Arg Ala Ile Glu Ser Gly Gln Leu Ala
290 295 300

Gly Tyr Ala Gly Asp Val Trp Phe Pro Gln Pro Ala Pro Lys Asp His
305 310 315 320

Pro Trp Arg Thr Met Lys Trp Glu Gly Met Thr Pro His Ile Ser Gly
325 330 335

Thr Ser Leu Ser Ala Gln Ala Arg Tyr Ala Ala Gly Thr Arg Glu Ile
340 345 350

Leu Glu Cys Phe Phe Glu Gly Arg Pro Ile Arg Asp Glu Tyr Leu Ile
355 360 365

Val Gln Gly Gly Ala Leu Ala Gly Thr Gly Ala His Ser Tyr Ser Lys
370 375 380

Gly Asn Ala Thr Gly Gly Ser Glu Glu Ala Ala Lys Phe Lys Lys Ala
385 390 395 400

Gly

<210> 2

<211> 1264

<212> DNA

<213> Thiobacillus sp.

<400> 2

atacgcgcga gaggagatcc cggaa atg gcg aaa ata ctt tgc gtt ctc tat 52
Met Ala Lys Ile Leu Cys Val Leu Tyr
1 5

gat gat ccg gtc gac ggc tat ccg aag acc tat gcg cgc gac gat ctg 100
Asp Asp Pro Val Asp Gly Tyr Pro Lys Thr Tyr Ala Arg Asp Asp Leu

10 15 20 25

ccg aag atc gac cac tat ccg ggc ggg cag acc ctg ccg acg ccg aag 148
Pro Lys Ile Asp His Tyr Pro Gly Gly Gln Thr Leu Pro Thr Pro Lys
30 35 40

gcg atc gac ttc acc ccc ggc cag ctt ctc ggc tcg gtc tcc ggc gag 196
Ala Ile Asp Phe Thr Pro Gly Gln Leu Leu Gly Ser Val Ser Gly Glu
45 50 55

ctg ggc ctg cgc aaa tat ctc gaa gcc aac ggc cac acc ttc gtc gtc 244
Leu Gly Leu Arg Lys Tyr Leu Glu Ala Asn Gly His Thr Phe Val Val
60 65 70

acc tcc gac aag gac ggc ccg gat tcg gtg ttc gag aag gag ctc gtc 292
Thr Ser Asp Lys Asp Gly Pro Asp Ser Val Phe Glu Lys Glu Leu Val
75 80 85

gac gcc gac gtg gtg atc tcc cag ccc ttc tgg ccg gcc tat ctg aca 340
Asp Ala Asp Val Val Ile Ser Gln Pro Phe Trp Pro Ala Tyr Leu Thr
90 95 100 105

cct gag cgc atc gcc aag gcg aag aac ctg aag ctg gcg ctc acc gcc 388
Pro Glu Arg Ile Ala Lys Ala Lys Asn Leu Lys Leu Ala Leu Thr Ala
110 115 120

ggc atc ggc tcc gat cac gtc gat ctt cag tcg gcg atc gac cgc ggc 436
Gly Ile Gly Ser Asp His Val Asp Leu Gln Ser Ala Ile Asp Arg Gly
125 130 135

atc acc gtg gcc gaa gtc acc tac tgc aac tcg atc agc gtc gcc gag 484
Ile Thr Val Ala Glu Val Thr Tyr Cys Asn Ser Ile Ser Val Ala Glu
140 145 150

cat gtg gtg atg atg atc ctc ggc ctg gtg cgc aac tac att ccc tcg 532
His Val Val Met Met Ile Leu Gly Leu Val Arg Asn Tyr Ile Pro Ser
155 160 165

cat gac tgg gcg cgc aag ggc ggc tgg aac ata gcc gac tgc gtg gag 580
His Asp Trp Ala Arg Lys Gly Gly Trp Asn Ile Ala Asp Cys Val Glu
170 175 180 185

cac tcc tac gac ctc gag ggc atg acc gtc ggc tcc gtt gcc gcc ggc 628
His Ser Tyr Asp Leu Glu Gly Met Thr Val Gly Ser Val Ala Ala Gly
190 195 200

cgc atc ggc ctc gcc gtg ctg cgc cgc ctc gcg ccc ttc gac gtg aag 676
Arg Ile Gly Leu Ala Val Leu Arg Arg Leu Ala Pro Phe Asp Val Lys
205 210 215

ctg cac tac acc gac cgc cac cgc ctg ccg gaa gcg gtc gag aag gag 724
Leu His Tyr Thr Asp Arg His Arg Leu Pro Glu Ala Val Glu Lys Glu
220 225 230

ttg ggc ctg gtc tgg cac gac acc cgc gag gac atg tac cca cat tgc 772
Leu Gly Leu Val Trp His Asp Thr Arg Glu Asp Met Tyr Pro His Cys
235 240 245

gac gtg gtc acg ctc aac gtg ccg ctg cat ccc gag acg gag cac atg 820
Asp Val Val Thr Leu Asn Val Pro Leu His Pro Glu Thr Glu His Met
250 255 260 265

atc aat gac gag acg ctg aag ctg ttc aag cgc ggc gcc tat atc gtc 868
Ile Asn Asp Glu Thr Leu Lys Leu Phe Lys Arg Gly Ala Tyr Ile Val
270 275 280

aac acc gcc cgc ggc aag ctc gcc gac cgc gac gcc atc gtg cgt gcg 916
Asn Thr Ala Arg Gly Lys Leu Ala Asp Arg Asp Ala Ile Val Arg Ala
285 290 295

atc gag agc ggg cag ctc gcc ggc tat gct ggc gac gtg tgg ttc ccg 964
Ile Glu Ser Gly Gln Leu Ala Gly Tyr Ala Gly Asp Val Trp Phe Pro
300 305 310

cag ccg gcg ccg aag gat cac ccc tgg cgc acc atg aag tgg gaa ggc 1012
Gln Pro Ala Pro Lys Asp His Pro Trp Arg Thr Met Lys Trp Glu Gly
315 320 325

atg acg ccg cac att tcc ggc acc tcg ctc tcc gcc cag gcg cgc tat 1060
Met Thr Pro His Ile Ser Gly Thr Ser Leu Ser Ala Gln Ala Arg Tyr
330 335 340 345

gcg gcg ggc acg cgc gag atc ctc gaa tgc ttc ttc gaa ggt cgg ccg 1108
Ala Ala Gly Thr Arg Glu Ile Leu Glu Cys Phe Phe Glu Gly Arg Pro
350 355 360

atc cgc gac gag tac ctg atc gtg cag ggc ggc gca ctc gcc ggc acg 1156

Ile Arg Asp Glu Tyr Leu Ile Val Gln Gly Gly Ala Leu Ala Gly Thr
365 370 375

ggg gcg cat tcc tac tcc aag ggc aat gcg acc ggc ggc tcg gaa gag 1204
Gly Ala His Ser Tyr Ser Lys Gly Asn Ala Thr Gly Gly Ser Glu Glu
380 385 390

gcc gcg aag ttc aag aag gcc ggc tga tccctcccga gacacagacc 1251
Ala Ala Lys Phe Lys Lys Ala Gly
395 400

gaccaggcga acg 1264

<210> 3

<211> 1206

<212> DNA

<213> Thiobacillus sp.

<400> 3

atg gcg aaa ata ctt tgc gtt ctc tat gat gat ccg gtc gac ggc tat 48
Met Ala Lys Ile Leu Cys Val Leu Tyr Asp Asp Pro Val Asp Gly Tyr
1 5 10 15

ccg aag acc tat gcg cgc gac gat ctg ccg aag atc gac cac tat ccg 96
Pro Lys Thr Tyr Ala Arg Asp Asp Leu Pro Lys Ile Asp His Tyr Pro
20 25 30

ggc ggg cag acc ctg ccg acg ccg aag gcg atc gac ttc acc ccc ggc 144

Gly Gly Gln Thr Leu Pro Thr Pro Lys Ala Ile Asp Phe Thr Pro Gly
35 40 45

cag ctt ctc ggc tcg gtc tcc ggc gag ctg ggc ctg cgc aaa tat ctc 192
Gln Leu Leu Gly Ser Val Ser Gly Glu Leu Gly Leu Arg Lys Tyr Leu
50 55 60

gaa gcc aac ggc cac acc ttc gtc gtc acc tcc gac aag gac ggc ccg 240
Glu Ala Asn Gly His Thr Phe Val Val Thr Ser Asp Lys Asp Gly Pro
65 70 75 80

gat tcg gtg ttc gag aag gag ctc gtc gac gcc gac gtg gtg atc tcc 288
Asp Ser Val Phe Glu Lys Glu Leu Val Asp Ala Asp Val Val Ile Ser
85 90 95

cag ccc ttc tgg ccg gcc tat ctg aca cct gag cgc atc gcc aag gcg 336
Gln Pro Phe Trp Pro Ala Tyr Leu Thr Pro Glu Arg Ile Ala Lys Ala
100 105 110

aag aac ctg aag ctg gcg ctc acc gcc ggc atc ggc tcc gat cac gtc 384
Lys Asn Leu Lys Leu Ala Leu Thr Ala Gly Ile Gly Ser Asp His Val
115 120 125

gat ctt cag tcg gcg atc gac cgc ggc atc acc gtg gcc gaa gtc acc 432
Asp Leu Gln Ser Ala Ile Asp Arg Gly Ile Thr Val Ala Glu Val Thr
130 135 140

tac tgc aac tcg atc agc gtc gcc gag cat gtg gtg atg atg atc ctc 480
Tyr Cys Asn Ser Ile Ser Val Ala Glu His Val Val Met Met Ile Leu

145 150 155 160

ggc ctg gtg cgc aac tac att ccc tcg cat gac tgg gcg cgc aag ggc 528
Gly Leu Val Arg Asn Tyr Ile Pro Ser His Asp Trp Ala Arg Lys Gly
165 170 175

ggc tgg aac ata gcc gac tgc gtg gag cac tcc tac gac ctc gag ggc 576
Gly Trp Asn Ile Ala Asp Cys Val Glu His Ser Tyr Asp Leu Glu Gly
180 185 190

atg acc gtc ggc tcc gtt gcc gcc ggc cgc atc ggc ctc gcc gtg ctg 624
Met Thr Val Gly Ser Val Ala Ala Gly Arg Ile Gly Leu Ala Val Leu
195 200 205

cgc cgc ctc gcg ccc ttc gac gtg aag ctg cac tac acc gac cgc cac 672
Arg Arg Leu Ala Pro Phe Asp Val Lys Leu His Tyr Thr Asp Arg His
210 215 220

cgc ctg ccg gaa gcg gtc gag aag gag ttg ggc ctg gtc tgg cac gac 720
Arg Leu Pro Glu Ala Val Glu Lys Glu Leu Gly Leu Val Trp His Asp
225 230 235 240

acc cgc gag gac atg tac cca cat tgc gac gtg gtc acg ctc aac gtg 768
Thr Arg Glu Asp Met Tyr Pro His Cys Asp Val Val Thr Leu Asn Val
245 250 255

ccg ctg cat ccc gag acg gag cac atg atc aat gac gag acg ctg aag 816
Pro Leu His Pro Glu Thr Glu His Met Ile Asn Asp Glu Thr Leu Lys
260 265 270

ctg ttc aag cgc ggc gcc tat atc gtc aac acc gcc cgc ggc aag ctc 864
Leu Phe Lys Arg Gly Ala Tyr Ile Val Asn Thr Ala Arg Gly Lys Leu
275 280 285

gcc gac cgc gac gcc atc gtg cgt gcg atc gag agc ggg cag ctc gcc 912
Ala Asp Arg Asp Ala Ile Val Arg Ala Ile Glu Ser Gly Gln Leu Ala
290 295 300

ggc tat gct ggc gac gtg tgg ttc ccg cag ccg gcg ccg aag gat cac 960
Gly Tyr Ala Gly Asp Val Trp Phe Pro Gln Pro Ala Pro Lys Asp His
305 310 315 320

ccc tgg cgc acc atg aag tgg gaa ggc atg acg ccg cac att tcc ggc 1008
Pro Trp Arg Thr Met Lys Trp Glu Gly Met Thr Pro His Ile Ser Gly
325 330 335

acc tcg ctc tcc gcc cag gcg cgc tat gcg gcg ggc acg cgc gag atc 1056
Thr Ser Leu Ser Ala Gln Ala Arg Tyr Ala Ala Gly Thr Arg Glu Ile
340 345 350

ctc gaa tgc ttc ttc gaa ggt cgg ccg atc cgc gac gag tac ctg atc 1104
Leu Glu Cys Phe Phe Glu Gly Arg Pro Ile Arg Asp Glu Tyr Leu Ile
355 360 365

gtg cag ggc ggc gca ctc gcc ggc acg ggg gcg cat tcc tac tcc aag 1152
Val Gln Gly Gly Ala Leu Ala Gly Thr Gly Ala His Ser Tyr Ser Lys
370 375 380

ggc aat gcg acc ggc ggc tcg gaa gag gcc gcg aag ttc aag aag gcc 1200
Gly Asn Ala Thr Gly Gly Ser Glu Glu Ala Ala Lys Phe Lys Lys Ala
385 390 395 400

ggc tga 1206
Gly

<210> 4
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer-1

<220>
<221> misc_feature
<222> (3)..(3)
<223> n stands for any base

<220>
<221> misc_feature
<222> (9)..(9)
<223> n stands for any base

<220>
<221> misc_feature
<222> (12)..(12)

<223> n stands for any base

<400> 4

gcnaaratnc tntgygt 17

<210> 5

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer-2

<220>

<221> misc_feature

<222> (6)..(6)

<223> n stands for any base

<220>

<221> misc_feature

<222> (15)..(15)

<223> n stands for any base

<400> 5

ggytgnggra accanacrtc 20

<210> 6

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer-3

<400> 6

tagtggtcga tcttcggcag atcgt 25

<210> 7

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer-4

<400> 7

gagacgctga agctgttcaa 20

<210> 8

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer-5

<400> 8

acactgcaga tacgcgcgag aggagat 27

<210> 9

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer-6

<400> 9

tctgaattcg ttcgcctggt cggtctgt 28

<210> 10

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer-7

<400> 10

acgcatatgg cgaaaatact ttgc 24

<210> 11

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer-8

<400> 11

agtctgcagt tatcagccgg ccttcttgaa 30