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[Continued on next page]

(54) **Title**: DESIGNER CELLS FOR ENANTIOSELECTIVE REDUCTION OF KETONES AND USE THEREOF IN EFFICIENT PRODUCTION OF ENANTIOENRICHED ALCOHOLS

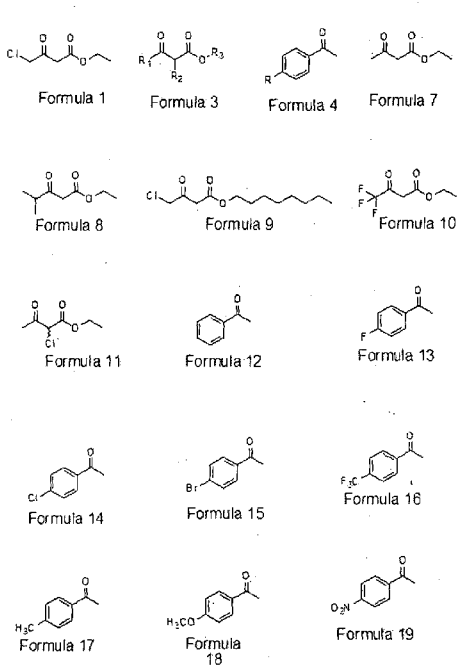

Figure 1: Examples of aliphatic and aromatic compounds serving as substrates

(57) **Abstract**: The present invention is to provide a preparation of variant recombinant whole cell biocatalysts, referred herein as "designer cells" having significantly enhanced carbonyl reductase activity for use in the efficient production of variant industrially important enantiomerically enriched alcohols. More specifically, the alcohol is optically pure ethyl (S)-4-chloro-3-hydroxybutyrate, which is useful as chiral building block and an intermediate for the production of hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors.

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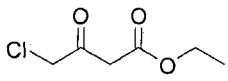
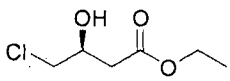
DESIGNER CELLS FOR ENANTIOSELECTIVE REDUCTION OF KETONES AND USE THEREOF IN EFFICIENT PRODUCTION OF ENANTIOENRICHED ALCOHOLS

FIELD OF THE INVENTION

The present invention relates to whole cell biocatalysts and use thereof in efficient production of enantioenriched alcohols. More specifically, the present invention relates to the development of whole cell-biocatalysts referred herein as "designer cells" having significantly enhanced conversion rate for asymmetric reduction of variant ketones to their alcohols in high enantiomeric excess. In particular, the present invention relates to the development of a designer cell having significantly enhanced conversion rate for efficient conversion of ethyl 4-chloro-3-oxobutyrate represented by formula 1 to produce ethyl (*S*)-4-chloro-3-hydroxybutyrate represented by formula 2 in >99.9% enantiomeric excess, which is useful as chiral building block and an intermediate for the production of hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors.



Formula 1



Formula 2

BACKGROUND OF THE INVENTION

In the prior art, examples of methods for preparation of industrially important optically active alcohols including ethyl (*S*)-4-chloro-3-hydroxybutyrate with the use of wild type whole cell biocatalysts isolated from variant sources like *Geotrichum candidum* (Sundby, E. et al. *Journal of Molecular Catalysis B: Enzymatic* **2003,** *21*, 63–66), *Candida parapsilosis* (Kaliaperumal, T. et al. *Journal of Industrial Microbiology & Biotechnology* **2010,** *37*, 159-165), *Candida magnoliae* (Yasohara, Y. et al. *Applied Microbiology and Biotechnology* **1999,** *51*, 847-851), *Cylindrocarpon sclerotigenum* (Saratani, Y. et al. *Bioscience, Biotechnology, and Biochemistry* **2001,** *65*, 1676-1679), *Kluveromyces lactis* (Yamamoto, H., et al. *Bioscience, Biotechnology, and Biochemistry* **2002,** *66*, 1775-1778), *Kluyveromyces aestuarii* (Yamamoto, H. et al. *Bioscience, Biotechnology, and Biochemistry* **2004,** *68*, 638-649), *Aureobasidium pullulans* (He, J. Y. et al. *Process Biochemistry* **2006,** *41*, 244–249), *Pichia stipitis* (Ye, Q. et al. *Biotechnology Letters* **2009,** *31*, 537-542) and *Streptomyces coelicolor* (Wang, L. J. et al.

Bioresource Technology **2011,** *102*, 7023-7028) have been described. Further, methods for producing ethyl (*S*)-4-chloro-3-hydroxybutyrate with the use of wild type whole cell biocatalysts have been disclosed in US patent No. 99/5891685; US patent No. 97/5700670; US patent No. 96/5559030; US patent No. 95/5413921; US patent No. 90/4933282 and US patent No. 87/4710468. Although, the methods that describe use of wild type microorganism for reduction of carbonyl group to corresponding alcohol exist, these suffer from drawbacks, such as lower efficiency, lower substrate concentration, lower optical purity, etc. Therefore, it is impractical to synthesize industrially important optically active alcohols including ethyl (*S*)-4-chloro-3-hydroxybutyrate using wild type natural whole cell biocatalysts.

Further known methods to improve optical purity of industrially important optically active alcohols including ethyl (*S*)-4-chloro-3-hydroxybutyrate, include the use of an enzyme purified from the native source, like *Candida magnoliae* (Wada, M. et al. *Bioscience, Biotechnology, and Biochemistry* **1998,** *62*, 280-285), *Sporobolomyces salmonicolor* (Kita, K. et al. *Journal of Molecular Catalysis B: Enzymatic* **1999,** *6*, 305-313), *Kluyveromyces lactis* (Yamamoto, H. et al. *Bioscience, Biotechnology, and Biochemistry* **2002,** *66*, 1775-1778), *Kluyveromyces aestuarii* (Yamamoto, H. et al. *Bioscience, Biotechnology, and Biochemistry* **2004,** *68*, 638-649), *Pichia stipitis* (Ye, Q., et al. *Bioresource Technology* **2009,** *100*, 6022-6027) and *Streptomyces coelicolor* (Wang, L. J. et al. *Bioresource Technology* **2011,** *102*, 7023-7028).

When a purified enzyme or transformant having carbonyl reductase activity reduces carbonyl group of ketones including ethyl 4-chloro-3-oxobutyrate, it requires a coenzyme, nicotinamide adenine dinucleotide, reduced (NADH) or nicotinamide adenine dinucleotide phosphate, reduced (NADPH) for preparation of industrially important optically active alcohols including ethyl (*S*)-4-chloro-3-hydroxybutyrate. As the reaction proceeds, coenzyme is converted into nicotinamide adenine dinucleotide phosphate, reduced (NADPH) or nicotinamide adenine dinucleotide phosphate (NADP). In the absence of cofactor regenerating system, stoichiometric amount of expansive cofactor is required. However, when the reaction is done in presence of a cofactor regenerating system, the amount of an expansive coenzyme is greatly reduced. A cofactor regenerating system typically consists of an enzyme which in presence of its substrate converts nicotinamide adenine dinucleotide phosphate, reduced (NADPH) or nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide, reduced (NADH) or nicotinamide adenine dinucleotide phosphate, reduced (NADPH). Coenzyme regeneration ability can be fulfilled either by the use of purified enzyme (Yasohara, Y. et al. *Bioscience, Biotechnology, and Biochemistry* **2000,** *64*, 1430-1436; Kaluzna, I. A. et al. *Journal of the American Chemical Society* **2004,** *126*, 12827-12832; Zhu, D. et al. *The Journal of Organic*

Chemistry **2006,** *71*, 4202-4205; Ye, Q. et al. *Biotechnology Letters* **2009,** *31*, 537-542), or a transformant having coenzyme regeneration ability in the cytoplasm (Xu, Z. et al. *Applied Microbiology and Biotechnology* **2006,** *70*, 40-46; Zhang, J. et al. *Chemical Communications* **2006**, 398–400).

Methods such as *in vitro* enzyme evolution using gene shuffling technologies have been employed to improve the activity of a ketoreductase that asymmetrically reduces ethyl 4-chloro-3-oxobutyrate by about 13-fold and glucose dehydrogenase that recycles cofactor nicotinamide adenine dinucleotide phosphate (NADP) or nicotinamide adenine dinucleotide phosphate, reduced (NADPH) using glucose a substrate by about 7-fold compared to corresponding wild type enzyme (Steve K. Ma et al. Green Chemistry **2010**, *12*, 81-86; US patent No. 10/0028972; US patent No. 10/7816111).

However, the use of an isolated enzyme requires additional steps, such as isolation, purification and stabilization of enzyme, which adds to the cost and makes the overall process for production of optically active alcohols economically unattractive. In addition, inhibition of enzyme by substrate and/or product can sometimes occur. The use of two enzymes, one for reduction of carbonyl compound and other for cofactor recycling along with two substrates, one for each enzyme leads to complex overall kinetics of the reaction. Put together, these factors make the use of isolated enzymes less attractive compared to use of whole cells in the preparation of enantiomerically enriched alcohols including ethyl (*S*)-4-chloro-3-hydroxybutyrate.

To overcome the problem of low efficiency associated with wild type strains, the gene encoding the carbonyl reductase activity can be deduced and overexpressed in a host cell. Further improvement in efficiency of whole cell biocatalyst for production of enantiomerically enriched alcohols including ethyl (*S*)-4-chloro-3-hydroxybutyrate is achieved by coexpressing both carbonyl reductase and coenzyme regeneration ability in the cytoplasm of same host (Kizaki, N. et al. *Applied Microbiology and Biotechnology* **2001,** *55*, 590-595, Kataoka, M. et al. *Enzyme and Microbial Technology* **2006,** *38*, 944–951; Ye, Q. et al. *Bioresource Technology* **2010,** *101*, 6761-6767).

However, the method that use either a transformant wherein carbonyl reductase ability is present or a transformant wherein both carbonyl reductase and coenzyme regenerating ability is present in the cytoplasm of cells suffers from drawbacks such as low efficiency due to barrier imposed by plasma membrane on substrate uptake and product efflux, complex kinetics of the overall process, etc.

Consequently, the current methods for the production of optically pure ethyl (*S*)-4-chloro-3-hydroxybutyrate and other optically enriched alcohols suffer from drawback such as low efficiency, low productivity, etc., which therefore results in increased cost of production.

The art of expressing a protein including enzymes on surface of cells is well known (Lee, S. Y. et al. *Trends in Biotechnology* **2003,** *21*, 45-52) and has been used in a wide range of biotechnological and industrial applications like whole-cell biocatalyst for bioconversion (Shimazu, M. et al. Protein. *Biotechnology Progress* **2001,** *17*, 76-80; Shimazu, M. et al. *Biotechnology and Bioengineering* **2001,** *76*, 318-324), bioadsorbent for the removal of harmful chemicals and heavy metals (Bae, W. et al. *Biotechnology and Bioengineering* **2000,** *70*, 518-524; Bae, W. et al. *J Inorg Biochem* **2002,** *88*, 223-227; Sousa, C. et al. *J. Bacteriol.* **1998,** *180*, 2280-2284; Xu, Z. et al. *Appl Environ Microbiol* **1999,** *65*, 5142-5147), screening of human antibodies libraries (Chao, G. et al. *Nat. Protocols* **2006,** *1*, 755-768), mutation detection (Aoki, T. et al. *Analytical Biochemistry* **2002,** *300*, 103-106), biosensor development by anchoring enzymes, receptors or other signal-sensitive components (Dhillon, J. K. et al. *Letters in Applied Microbiology* **1999,** *28*, 350-354; Shibasaki, S. et al. *Applied Microbiology and Biotechnology* **2001,** *57*, 702-707; Shibasaki, S. S. et al. *Applied Microbiology and Biotechnology* **2001,** *57*, 528-533).

More recently, a method for simultaneous display of a target protein including dehydrogenase but not carbonyl reductase and glucose dehydrogenase has been disclosed in US patent No. 11/7897366. Usually the cell surface proteins or their truncated form (carrier proteins) fused with the target peptide or protein (passenger protein) are used to display the protein on the surface. Gram (-) ve (*Escherichia coli*), gram (+) ve (*Bacillus subtilis*, *Staphylococcus* strains) bacteria and yeast (*Saccharomyces cerevisiae*, *Pichia pastoris*) have been explored for display of heterologous protein expression on the surface. Rigid structure of the cell wall of gram (+) ve bacteria makes it a suitable host, however, the gram (-) ve *Escherichia coli* has been the most popular and much explored for the cell surface display. Integral proteins of *Escherichia coli*, such as LamB, FhuA, and the porins OmpA, OmpC and OmpX, which give structural rigidity to outer membrane has been extensively used for insertion of short amino acid sequence (up to 60 amino acid) in extracellular loop and display it on the cell surface. Outer membrane lipoproteins are anchored in the membrane by a small lipid modified amino terminal. The first lipoprotein based cell surface display was Lpp–OmpA chimera consisting of the 20 amino acid signal sequence and first nine N-terminal residues of the mature *Escherichia coli* lipoprotein, and the residues 46–159 of the *Escherichia coli* outer membrane protein A (OmpA) were fused to the N-terminal of the passenger protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Examples of compounds serving as substrates.

Figure 2: Examples of compounds serving as products.

Figure 3: Schematic representation of "CRS polypeptide" construct corresponding to SEQ ID No: 1, 3, 5 or 7. The sequence consists of (i) N-terminal 20-amino acid signal sequence linked to first nine N-terminal residues of mature *Escherichia coli* lipoprotein (Lpp), (ii) residues 46-159 of *Escherichia coli* outer membrane proteinA (OmpA), which is expected to transport the passenger protein fused at its C-terminal across the membrane and (iii) full sequence of CRS, wherein CRS is wild type carbonyl reductase of *Candida magnoliae* SEQ ID NO: 13 or modified carbonyl reductase SEQ ID No: 15, 17 or 19, which differs from SEQ ID NO: 13 by having several amino acid substitutions. The 1st 29 aa residue signal + Lpp peptide was linked to 114 aa OmpA residue through Gly-Ile linker, which in turn was attached to N-terminal of CRS through Gly-Ile-Pro-Gly. Figure 4: Schematic representation of "GDH polypeptide" construct corresponding to SEQ ID No: 9 or 11. The sequence consists of (i) N-terminal 20-amino acid signal sequence linked to first nine N-terminal residues of mature *Escherichia coli* lipoprotein (Lpp), (ii) residues 46-159 of *Escherichia coli* outer membrane protein A (OmpA), which is expected to transport the passenger protein fused at its C-terminal across the membrane and (iii) full sequence of GDH, wherein GDH is wild type glucose dehydrogenase of *Bacillus megaterium* SEQ ID NO: 21 or modified glucose dehydrogenase SEQ ID NO: 23, which differs from SEQ ID NO: 21 by having several amino acid substitutions. The 1st 29 aa residue signal + Lpp peptide was linked to 114 aa OmpA residue through Gly-Ile linker, which in turn was attached to N-terminal of GDH through Gly-Ile-Pro-Gly.

Figure 5: Method and structure of recombinant vector pET23(a)-omp-CRS.

Figure 6: Method and structure of recombinant vector pETDuet1-omp-CRS, omp-GDH.

Figure 7: Transmission electron micrographs (TEM) of 'Designer whole-cell biocatalyst' expressing CRS on the surface of *Escherichia coli* cells. The cells were probed with rabbit anti-*CRS* polyclonal antibody followed by nanogold labeled goat *anti-rabbit IgG* (whole molecule) secondary antibody. Arrowheads denote gold particles. Figure 7a is TEM of *Escherichia coli* BL21(DE3) + *pET 23(a)* i.e. negative control, which does not show any gold labeling. Figure 7b

shows TEM of *Escherichia coli* BL21(DE3) + *pET 23(a)-omp-CRS* that expresses CRS on surface, which shows intense gold labeling.

SUMMARY OF THE INVENTION

The present invention provides an *Escherichia coli* strain that expresses a CRS polypeptide on the surface of cell that has 250-fold to 300-fold enhanced rate of conversion per unit mass of CRS polypeptide compared to corresponding prior art *Escherichia coli* strain that expresses CRS in cytoplasm of cell for reduction of ethyl 4-chloro-3-oxobutyrate to ethyl 4-chloro-3-hydroxybutyrate.

In an embodiment, the recombinant *Escherichia coli* strain that expresses CRS polypeptide on the surface of cell has 50-fold to 275-fold enhanced rate of conversion per unit mass of CRS polypeptide compared to corresponding prior art *Escherichia coli* strain that expresses CRS in cytoplasm of cell for asymmetric reduction of variant keto compounds.

In an embodiment, recombinant *Escherichia coli* strain that expresses a CRS polypeptide on the surface of cell that has about 3-fold to 26-fold enhanced rate of conversion per unit cell mass compared to corresponding prior art *Escherichia coli* strain that expresses CRS in cytoplasm of cell for reduction of variant keto compounds

In another aspect the invention is directed to provide a recombinant *Escherichia coli* strain that simultaneously expresses a CRS polypeptide and a GDH polypeptide on the surface of cell that has 250-fold to 300-fold enhanced rate of conversion for reduction of ethyl 4-chloro-3-oxobutyrate to ethyl 4-chloro-3-hydroxybutyrate per unit mass of CRS polypeptide and 200-fold to 250-fold enhanced activity for oxidation of glucose to gluconate per unit mass GDH polypeptide compared to *Escherichia coli* strain that simultaneously expresses a CRS and a GDH in cytoplasm of *Escherichia coli* cell.

In an embodiment, the recombinant *Escherichia coli* strain that simultaneously expresses a CRS polypeptide and a GDH polypeptide on the surface of cell has about 50-fold to 270-fold enhanced rate of conversion per unit mass of CRS protein compared to corresponding prior art *Escherichia coli* strain that simultaneously expresses a CRS polypeptide and a GDH polypeptide in cytoplasm of cell for reduction of variant ketones.

In an embodiment, recombinant *Escherichia coli* strain that simultaneously expresses a CRS polypeptide and a GDH polypeptide on the surface of cell that has about 3-fold to 24-fold enhanced rate of conversion per unit cell mass compared to corresponding prior art *Escherichia*

coli strain that simultaneously expresses a CRS polypeptide and a GDH polypeptide in cytoplasm of cell for reduction of variant keto compounds

In one embodiment, the variant ketone is an aliphatic compound represented by formula 3; wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$ and R_2 = H, X or $CH_3(CH_2)_n$ and R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$ and X = Cl or Br and n = 1-4 and m = 1-8.

In a more preferred embodiments, R_1 is CH_2Cl, R_2 is H and R_3 is CH_2CH_3 or R_1 is CH_3, R_2 is H and R_3 is CH_2CH_3 or R_1 is CH_2Cl, R_2 is H and R_3 is $(CH_2)_7CH_3$ or R_1 is CH_3, R_2 is Cl and R_3 is CH_2CH_3 or R_1 is CF_3, R_2 is H and R_3 is CH_2CH_3 or R_1 is $(CH_3)_2CH$, R_2 is H and R_3 is CH_2CH_3.

In another embodiment, the variant ketone is an aromatic compound represented by general formula 4 wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3 and R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$ and n= 1 to 5.

In an embodiment, the *Escherichia coli* strain is selected from *Escherichia coli* BL21(DE3), *Escherichia coli* C41(DE3) or *Escherichia coli* C43(DE3).

In a preferred embodiment, the rate of conversion of ethyl 4-chloro-3-oxobutyrate to ethyl (*S*)-4-chloro-3-hydroxybutyrate with nicotinamide adenine dinucleotide phosphate, reduced (NADPH) as cofactor is 1.3-fold higher when *Escherichia coli* strain BL21(DE3) that simultaneously expresses a CRS polypeptide and a GDH polypeptide on surface of cell is replaced with *Escherichia coli C41(DE3)* that simultaneously expresses a CRS polypeptide and a GDH polypeptide on surface of cell.

In another preferred embodiment, the rate of conversion of ethyl 4-chloro-3-oxobutyrate to ethyl (*S*)-4-chloro-3-hydroxybutyrate with nicotinamide adenine dinucleotide phosphate, reduced (NADPH) as cofactor is about 1.6-fold higher with *Escherichia coli* strain C41(DE3) that simultaneously expresses a CRS polypeptide and a GDH polypeptide on surface of cell compared to *Escherichia coli* strain BL21(DE3) that expresses only CRS polypeptide on the surface of cell, requiring addition of external GDH for cofactor recycling.

In a specific embodiment, the present invention provide a production method for producing industrially important ethyl (*S*)-4-chloro-3-hydroxybutyrate in about 100% enantiomeric excess with high productivity and product accumulation of at least 150 g l^{-1}.

DETAILED DESCRIPTION OF THE INVENTION

While the invention is susceptible to various modifications and/or alternative processes and/or compositions, specific embodiment thereof has been shown by way of example in the drawings/figures and tables and will be described in detail below. It should be understood, however that it is not intended to limit the invention to the particular processes and/or

compositions disclosed, but on the contrary, the invention is to cover all modifications, equivalents, and alternative falling within the spirit and the scope of the invention as defined by the appended claims.

The graphs, figures, tables, formulas and protocols have been represented where appropriate by conventional representations in the drawings, showing only those specific details that are pertinent to understanding the embodiments of the present invention so as not to obscure the disclosure with details that will be readily apparent to those of ordinary skill in the art having benefit of the description herein.

The following description is of exemplary embodiments only and is not intended to limit the scope, applicability or configuration of the invention in any way. Rather, the following description provides a convenient illustration for implementing exemplary embodiments of the invention. Various changes to the described embodiments may be made in the function and arrangement of the elements described without departing from the scope of the invention.

The terms "comprises", "comprising", or any other variations thereof, are intended to cover a non-exclusive inclusion, such that one or more processes or composition/s or systems or methods proceeded by "comprises... a" does not, without more constraints, preclude the existence of other processes, sub-processes, composition, sub-compositions, minor or major compositions or other elements or other structures or additional processes or compositions or additional elements or additional features or additional characteristics or additional attributes.

Definitions:

For the purposes of this invention, the following terms will have the meaning as specified therein:

"CRS polypeptide'' refers to an amino acid sequence which is schematically shown in Figure 3.

"GDH polypeptide" refers to a sequence which is schematically shown in Figure 4.

"Carbonyl reductase" and "CRS" are used interchangeably and refer to polypeptide that converts a ketone and nicotinamide adenine dinucleotide phosphate, reduced (NADPH) to corresponding alcohol and nicotinamide adenine dinucleotide phosphate (NADP).

"Glucose dehydrogenase" and "GDH" are used interchangeably and refers to a polypeptide that converts glucose and nicotinamide adenine dinucleotide phosphate (NADP) to gluconate and nicotinamide adenine dinucleotide phosphate, reduced (NADPH).

In the description, the term 'designer cell' refers herein to a recombinant strain of *Escherichia coli* that catalyze the asymmetric reduction of variant ketones to produce enantioenriched alcohols using catalytic amount of reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a reducing agent with or without addition of external co-factor recycling system for oxidation of nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide phosphate, reduced (NADPH).

Further, the term "cofactor recycling system" refers herein to a set of reagents which are added to the reaction for conversion of spent cofactor back to its pre-reaction state. For example, in conversion of ketone to alcohol by CRS, cofactor nicotinamide adenine dinucleotide phosphate, reduced (NADPH) gets converted to nicotinamide adenine dinucleotide phosphate (NADP) (spent cofactor). Addition of reagents, GDH and glucose to the reaction will result in regeneration of nicotinamide adenine dinucleotide phosphate, reduced (NADPH) from nicotinamide adenine dinucleotide phosphate (NADP).

Further, the term "CRS polypeptide'' refers to an amino acid sequence which is schematically shown in Figure 3 in which CRS is wild type carbonyl reductase of *Candida magnoliae* SEQ ID NO: 13 or modified carbonyl reductase that differs from SEQ ID NO: 13 by having several amino acid substitutions, such as SEQ ID NO: 15 (P14A, S42N, A194V, I275V) or SEQ ID NO: 17 (P14A, S42N, V147A, A194V, E234G) or SEQ ID NO: 19 (E9G, P14A, N20S, S42N, T190A, A194V, E234G).

Further GDH polypeptide refers to a sequence which is schematically shown in Figure 4 in which GDH is wild type glucose dehydrogenase of *Bacillus megaterium* SEQ ID NO: 21 or modified glucose dehydrogenase that differs from SEQ ID NO: 21 by having several amino acid substitution such as SEQ ID NO: 23 (S16T, E170K, P194T, A197K, E222D, S237C).

As used herein, the terms "carbonyl reductase" and "CRS" are used interchangeably and refer to polypeptide that converts a ketone and nicotinamide adenine dinucleotide phosphate, reduced (NADPH) to corresponding alcohol and nicotinamide adenine dinucleotide phosphate (NADP). Further, the terms "glucose dehydrogenase" and "GDH" are used interchangeably and refers to a polypeptide that converts glucose and nicotinamide adenine dinucleotide phosphate (NADP) to gluconate and nicotinamide adenine dinucleotide phosphate, reduced (NADPH).

The present invention provides whole cell-biocatalyst referred herein as "designer cell" having significantly enhanced conversion rate for enantioselective reduction of variant ketones to their alcohols in high enantiomeric excess In particular, the present invention relates to the development of a designer cell having significantly enhanced conversion rate for efficient

conversion of ethyl 4-chloro-3-oxobutyrate represented by formula 1 to produce ethyl (*S*)-4-chloro-3-hydroxybutyrate represented by formula 2 in >99.9% enantiomeric excess, which is useful as chiral building block and an intermediate for the production of hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors.

The present invention provides for expression of carbonyl reductase polypeptide (CRS) on the cell surface. Further the present invention also provides for co-expression carbonyl reductase polypeptide (CRS) and gluconase dehryogenase (GDH) on the surface surface. Thus the present invention overcomes the existing drawback of whole-cell system expressing carbonyl reductase in cytoplasm, wherein the carbonyl reductase polypeptide (CRS) is anchored to the surface of cell and has shown unexpected and surpising enhanced conversion rate per unit mass of CRS polypeptide compared to carbonyl reductase expressed in cytoplasm of same host for the conversion of ethyl 4-chloro-3-oxobutyrate to industrially important ethyl (*S*)-4-chloro-3-hydroxybutyrate. Likewise the present invention also overcome the existing drawback of of whole-cell system co-expression CRS and GDH enzymes in cytoplasm, wherein the CRS and GDH are anchored to the surface of cell and coexpressed, wherein the coexpresion has shown unexpected and surpising enhanced expression. Moreover, an enzymes expressed in such a manner is expected to behave like a pure, immobilized enzymes, thereby obviating the need for cost-intensive isolation, purification and stabilization of the enzyme. Moreover, kinetics is expected to be much simpler because of the fact that substrate uptake and product efflux across plasma membrane is not required in this case.

Suprisingly the present invention works with the hypothesis that the efficiency of designer cell can be further improved if both CRS and glucose dehydrogenase (GDH) activities are coexpressed in the same host, possibly because with such a biocatalyst, the cofactor nicotinamide adenine dinucleotide phosphate, reduced (NADPH)/nicotinamide adenine dinucleotide phosphate (NADP) will not become completely free in solution; instead, it will get channelized between CRS and GDH which are localized in close proximity on the surface of cell.

Accordingly, the present invention provides for a designed transformant, wherein enzyme carbonyl reductase and coenzyme regenerating enzyme (e.g., a glucose dehydrogenase) are coexpressed together on the surface of cell, which has several fold improved efficiency compared to the transformant that expresses only CRS on the surface of a cell and requires external addition of GDH for cofactor recycling in conversion of ethyl (*S*)-4-chloro-3-oxobutanoate to ethyl (*S*)-4-chloro-3-hydroxybutyrate.

The present invention provides a method for production of industrially important ethyl (*S*)-4-chloro-3-hydroxybutyrate in about 100% enantiomeric excess with high productivity and product accumulation of at least 150 g l^{-1}.

The present invention also provides a 'designer cell' which is a recombinant strain of *Escherichia coli* that catalyze the asymmetric reduction of variant ketones to produce enantioenriched alcohols using catalytic amount of reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a reducing agent with or without addition of external co-factor recycling system for oxidation of nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide phosphate, reduced (NADPH). The strain of *Candia magnoliae* is AKU4643 and *Bacillus megaterium* is DSM 2894.

The present invention provides for a designer cell which comprises of "CRS" polypeptide construct corresponding to SEQ ID No.1, 3, 5 or 7. The designer cell as described in the present invention has (a) N-terminal 20-amino acid signal sequence linked to first nine N-terminal residues of mature *Escherichia coli* lipoprotein (Lpp); (b) residues 46-159 of *Escherichia coli* outer membrane proteinA (OmpA), which is expected to transport the passenger protein fused at its C-terminal across the membrane; and (c) full sequence of CRS, wherein CRS is wild type carbonyl reductase of *Candida magnoliae* SEQ ID NO: 13 or modified carbonyl reductase SEQ ID No: 15, 17 or 19, which differs from SEQ ID NO: 13 by having several amino acid substitutions. The 1st 29 aa residue signal + Lpp peptide was linked to 114 aa OmpA residue through Gly-Ile linker, which in turn was attached to N-terminal of CRS through Gly-Ile-Pro-Gly (Figure 3). The modified carbonyl reductase that differs from SEQ ID NO: 13 by having several amino acid substitutions, such as SEQ ID NO: 15 (P14A, S42N, A194V, I275V) or SEQ ID NO: 17 (P14A, S42N, V147A, A194V, E234G) or SEQ ID NO: 19 (E9G, P14A, N20S, S42N, T190A, A194V, E234G).

The present invention also provides for a designer cell which comprises of "GDH" polypeptide construct corresponding to SEQ ID No.9 or 11. The designer cell as described in the present invention has (a) N-terminal 20-amino acid signal sequence linked to first nine N-terminal residues of mature *Escherichia coli* lipoprotein (Lpp); (b) residues 46-159 of *Escherichia coli* outer membrane proteinA (OmpA), which is expected to transport the passenger protein fused at its C-terminal across the membrane; and (c) full sequence of GDH, wherein GDH is wild type glucose dehydrogenase (GDH) of *Bacillus megaterium* SEQ ID NO: 21 or modified carbonyl reductase SEQ ID No: 23, which differs from SEQ ID NO: 21 by having several amino acid substitutions. The 1st 29 aa residue signal + Lpp peptide was linked to 114 aa OmpA residue through Gly-Ile linker, which in turn was attached to N-terminal of CRS

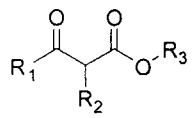
through Gly-Ile-Pro-Gly (Figure 3). The modified gucose dehydrogenase that differs from SEQ ID NO: 21 by having several amino acid substitution such as, SEQ ID NO: 23 (S16T, E170K, P194T, A197K, E222D, S237C).

The present invention also provides for a cofactor recycling system which refers herein to a set of reagents which are added to the reaction for conversion of spent cofactor back to its pre-reaction state. For example, in conversion of ketone to alcohol by designer cell, cofactor nicotinamide adenine dinucleotide phosphate, reduced (NADPH) gets converted to nicotinamide adenine dinucleotide phosphate (NADP) (spent cofactor). Addition of reagents, GDH and glucose to the reaction will result in regeneration of nicotinamide adenine dinucleotide phosphate, reduced (NADPH) from nicotinamide adenine dinucleotide phosphate (NADP).

The invention presented herein has multiple aspects. In one aspect the invention is directed to construction of a recombinant *Escherichia coli* strain that expresses a CRS polypeptide on the surface of cell that has 250-fold to 300-fold enhanced rate of conversion per unit mass of CRS polypeptide compared to corresponding prior art *Escherichia coli* strain that expresses CRS in cytoplasm of cell for reduction of ethyl 4-chloro-3-oxobutyrate to ethyl 4-chloro-3-hydroxybutyrate as measured by decrease in absorbance of nicotinamide adenine dinucleotide phosphate, reduced (NADPH). The relative amount of CRS polypeptide was determined by immunoblotting (Example 7).

In an embodiment, the recombinant *Escherichia coli* strain that expresses CRS polypeptide on the surface of cell has about 50-fold to 275-fold enhanced rate of conversion per unit mass of CRS protein compared to corresponding prior art *Escherichia coli* strain that expresses CRS in cytoplasm of cell for reduction of variant keto compounds as measured by decrease in absorbance of nicotinamide adenine dinucleotide phosphate, reduced (NADPH). The relative CRS protein content was determined by immunoblotting (Example 7).

In one embodiment, the ketone is an aliphatic compound represented by formula 3.



Formula 3

wherein $R_1 = CH_3$, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$

R_2 = H, X or $CH_3(CH_2)_n$;

R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

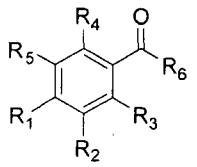
X = Cl or Br;

n = 1-4 and

m = 1-8.

In a more preferred embodiments, R_1 is CH_2Cl, R_2 is H and R_3 is CH_2CH_3 or R_1 is CH_3, R_2 is H and R_3 is CH_2CH_3 or R_1 is CH_2Cl, R_2 is H and R_3 is $(CH_2)_7CH_3$ or R_1 is CH_3, R_2 is Cl and R_3 is CH_2CH_3 or R_1 is CF_3, R_2 is H and R_3 is CH_2CH_3 or R_1 is $(CH_3)_2CH$, R_2 is H and R_3 is CH_2CH_3

In another embodiment, the ketone is an aromatic compound represented by formula 4



Formula 4

Wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$ and

n= 1 to 5.

As stated herein, the prior art *Escherichia coli* used for comparison of CRS activity refers to *Escherichia coli* strain that expresses wild type carbonyl reductase of *Candida magnoliae* SEQ ID NO: 13 or modified carbonyl reductase polypeptide SEQ ID NO: 15, SEQ ID NO: 17 or SEQ ID NO: 19 in the cytoplasm. These *Escherichia coli* strains were constructed by using the art disclosed in US Patent 2010/0028972.

In another aspect the invention is directed to construction of a recombinant *Escherichia coli* strain that expresses a CRS polypeptide on the surface of cell that has about 15-fold to 26-fold enhanced rate of conversion per unit cell mass compared to corresponding prior art *Escherichia coli* strain that expresses CRS in cytoplasm of cell for reduction of ethyl 4-chloro-3-oxobutyrate to ethyl 4-chloro-3-hydroxybutyrate as measured by decrease in absorbance of nicotinamide adenine dinucleotide phosphate, reduced (NADPH).

In an embodiment, recombinant *Escherichia coli* strain that expresses a CRS polypeptide on the surface of cell that has about 3-fold to 26-fold enhanced rate of conversion per unit cell mass compared to corresponding prior art *Escherichia coli* strain that expresses CRS in cytoplasm of cell for reduction of variant keto compounds as measured by decrease in absorbance of nicotinamide adenine dinucleotide phosphate, reduced (NADPH).

In one embodiment, the ketone is an aliphatic compound represented by formula 3 wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$ and R_2 = H, X or $CH_3(CH_2)_n$ and R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$ and X = Cl or Br and n = 1-4 and m = 1-8.

In a more preferred embodiments, R_1 is CH_2Cl, R_2 is H and R_3 is CH_2CH_3 or R_1 is CH_3, R_2 is H and R_3 is CH_2CH_3 or R_1 is CH_2Cl, R_2 is H and R_3 is $(CH_2)_7CH_3$ or R_1 is CH_3, R_2 is Cl and R_3 is CH_2CH_3 or R_1 is CF_3, R_2 is H and R_3 is CH_2CH_3 or R_1 is $(CH_3)_2CH$, R_2 is H and R_3 is CH_2CH_3

In another embodiment, the ketone is an aromatic compound represented by general formula 4 wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3 and R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$ and n= 1 to 5.

In yet another aspect, the invention is directed to construction of a recombinant *Escherichia coli* strain that simultaneously expresses a CRS polypeptide and a GDH polypeptide on the surface of cell that has 250-fold to 300-fold enhanced rate of conversion for reduction of ethyl 4-chloro-3-oxobutyrate to ethyl 4-chloro-3-hydroxybutyrate per unit mass of CRS polypeptide and 200-fold to 250-fold enhanced activity for oxidation of glucose to gluconate per unit mass GDH polypeptide compared to *Escherichia coli* strain that simultaneously expresses a CRS and a GDH in cytoplasm of *Escherichia coli* cell. CRS activity was measured by decrease in absorbance of nicotinamide adenine dinucleotide phosphate, reduced (NADPH). The relative amount of CRS polypeptide was determined by immunoblotting (Example 7). GDH activity and the relative amount GDH polypeptide was measured as described in Example 11.

In an embodiment, the recombinant *Escherichia coli* strain that simultaneously expresses a CRS polypeptide and a GDH polypeptide on the surface of cell has about 50-fold to 270-fold enhanced rate of conversion per unit mass of CRS protein compared to corresponding prior art *Escherichia coli* strain that simultaneously expresses a CRS polypeptide and a GDH polypeptide in cytoplasm of cell for reduction of variant keto compounds as measured by decrease in absorbance of nicotinamide adenine dinucleotide phosphate, reduced (NADPH). The relative CRS protein content was determined by immunoblotting (Example 7).

In one embodiment, the ketone is an aliphatic compound represented by formula 3 wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$ and R_2 = H, X or $CH_3(CH_2)_n$ and R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$ wherein n= 1 to 8 and X = Cl or Br and n = 1-4 and m = 1-8.

In a more preferred embodiments, R_1 is CH_2Cl, R_2 is H and R_3 is CH_2CH_3 or R_1 is CH_3, R_2 is H and R_3 is CH_2CH_3 or R_1 is CH_2Cl, R_2 is H and R_3 is $(CH_2)_7CH_3$ or R_1 is CH_3, R_2 is Cl and R_3 is CH_2CH_3 or R_1 is CF_3, R_2 is H and R_3 is CH_2CH_3 or R_1 is $(CH_3)_2CH$, R_2 is H and R_3 is CH_2CH_3

In another embodiment, the ketone is an aromatic compound represented by general formula 4 wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3 and R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$ and n= 1 to 5.

As stated herein, the prior art *Escherichia coli* used for comparison of CRS and GDH activity refers to *Escherichia coli* strain that co-expresses wild type carbonyl reductase of *Candida magnoliae* SEQ ID NO: 13 and wild type glucose dehydrogenase of *Bacillus megaterium* SEQ ID NO: 21 in the cytoplasm. These *Escherichia coli* strains were constructed by using the art disclosed by Kizaki, N. et al. *Applied Microbiology and Biotechnology* **2001,** *55,* 590-595. Further, it refers to *Escherichia coli* strains that co-express variant carbonyl reductase selected from SEQ ID NO: 15, SEQ ID NO: 17 or SEQ ID NO: 19 and variant glucose dehydrogenase selected from SEQ ID NO: 21 SEQ ID NO: 23 in the cytoplasm. These *Escherichia coli* strains were constructed based on the knowledge disclosed in Applied and Microbial Technology, Volume 55, pages 5590-595, year 2001, US Patent 2010/0028972 and US Patent 2010/7816111 with respect to peptide sequence, corresponding nucleotide sequences that code for these peptides and expression vectors.

In another aspect the invention is directed to construction of *Escherichia coli* strain that simultaneously expresses a CRS polypeptide and a GDH polypeptide on the surface of cell that has 11-fold to 24-fold enhanced activity for reduction of ethyl 4-chloro-3-oxobutyrate to ethyl 4-chloro-3-hydroxybutyrate per unit cell mass and 9-fold to 31-fold enhanced activity for oxidation of glucose to gluconate per unit per unit cell mass compared to *Escherichia coli* strain that simultaneously expresses CRS and GDH in cytoplasm of *Escherichia coli* cell. CRS activity was measured by decrease in absorbance of nicotinamide adenine dinucleotide phosphate, reduced (NADPH). GDH activity was measured by increase in absorbance of nicotinamide adenine dinucleotide phosphate (NADP) (Example 11).

In an embodiment, recombinant *Escherichia coli* strain that simultaneously expresses a CRS polypeptide and a GDH polypeptide on the surface of cell that has about 3-fold to 24-fold enhanced rate of conversion per unit cell mass compared to corresponding prior art *Escherichia coli* strain that simultaneously expresses a CRS polypeptide and a GDH polypeptide in cytoplasm of cell for reduction of variant keto compounds as measured by decrease in absorbance of nicotinamide adenine dinucleotide phosphate, reduced (NADPH).

In one embodiment, the ketone is an aliphatic compound represented by formula 3 wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$ and R_2 = H, X or $CH_3(CH_2)_n$ and R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$ wherein n= 1 to 8 and X = Cl or Br and n = 1-4 and m = 1-8.

In a more preferred embodiments, R_1 is CH_2Cl, R_2 is H and R_3 is CH_2CH_3 or R_1 is CH_3, R_2 is H and R_3 is CH_2CH_3 or R_1 is CH_2Cl, R_2 is H and R_3 is $(CH_2)_7CH_3$ or R_1 is CH_3, R_2 is Cl

and R_3 is CH_2CH_3 or R_1 is CF_3, R_2 is H and R_3 is CH_2CH_3 or R_1 is $(CH_3)_2CH$, R_2 is H and R_3 is CH_2CH_3

In another embodiment, the ketone is an aromatic compound represented by general formula 4 wherein $R_1=R_2=R_3=R_4=R_5$=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3 and R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$ and n= 1 to 5.

Accordingly, an object of the present invention is to provide a designer cell that has an amino acid sequence selected from SEQ ID NO:1, 3, 5 or 7 of the Sequence Listing with the one or several amino acids being deleted, substituted or added in the amino acid sequence of SEQ ID NO: 1, 3, 5 or 7 of the Sequence Listing, expressed on the surface of the transformant/s, having carbonyl reductase activity.

The DNA according to present invention codes for the above polypeptide has nucleotide sequence selected from SEQ ID NO: 2, 4, 6 or 8 of the Sequence Listing.

The plasmid according to present invention that codes for the above polypeptide is pET 23(a)-omp-CRS.

In the description, the amino acid sequence has been assigned odd number. The nucleotide sequence coding for a particular amino acid has been assigned immediately next even number. For example, for amino acid sequence SEQ ID NO: 1, the corresponding coding nucleotide sequence has been assigned sequence SEQ ID NO: 2.

The cell used according to present invention for creating a transformant is selected from *Escherichia coli* BL21(DE3), *Escherichia coli* C41(DE3) or *Escherichia coli* C43(DE3).

The transformed cell used according to present invention is transformant/s which is a cell transformed with the above plasmid pET 23(a)-omp-CRS.

In a preferred embodiment, the transformed cells is *Escherichia coli* BL21(DE3) + pET 23(a)-omp-CRS.

In more preferred embodiment the transformed cell is *Escherichia coli* C41(DE3) + pET 23(a)-omp-CRS.

In a preferred embodiment designer cell has an amino acid sequence SEQ ID NO:1of the Sequence Listing with the one or several amino acids being deleted, substituted or added in the amino acid sequence of SEQ ID NO: 1 of the Sequence Listing, expressed on the surface of the transformant/s, having carbonyl reductase activity.

The DNA for the above polypeptide has nucleotide sequence SEQ ID NO: 2

The plasmid according to present invention that codes for the above polypeptide is pET 23(a)-omp-CRS.

The cell used according to present invention for creating a transformant is selected from *Escherichia coli* BL21(DE3), *Escherichia coli* C41(DE3) or *Escherichia coli* C43(DE3).

The transformed cell used according to present invention is transformant/s which is a cell transformed with the above plasmid pET 23(a)-omp-CRS.

In a preferred embodiment, the transformed cell is *Escherichia coli* BL21(DE3) + pET 23(a)-omp-CRS.

In more preferred embodiment the transformed cell is *Escherichia coli* C41(DE3) + pET 23(a)-omp-CRS.

The transformant according to the present invention that asymmetrically reduces carbonyl group of the substrate including ethyl 4-chloro-3-oxobutyrate for producing industrially important optically pure alcohols including ethyl (*S*)-4-chloro-3-hydroxybutyrate require coenzyme nicotinamide adenine dinucleotide phosphate, reduced (NADPH). As the reaction proceeds, coenzyme is converted to oxidation type nicotinamide adenine dinucleotide phosphate (NADP). Conversion of the oxidation-type into a reduction type needs another enzyme having coenzyme regeneration ability (e.g. glucose dehydrogenase).

In one embodiment, the glucose dehydrogenase is derived from *Bacillus megaterium*.

Another object of the present invention is to provide designer cell coexpressing on the surface of the transformant, of the two polypeptides, one having CRS activity has an amino acid sequence selected from SEQ ID NO: 1, 3, 5 or 7 of the Sequence Listing with the one or several amino acids being deleted, substituted or added in the amino acid sequence of SEQ ID NO: 1, 3, 5 or 7 of the Sequence Listing, which effectively reduces ethyl (*S*)-4-chloro-3-hydroxybutyrate and the other having GDH activity has an amino acid sequence selected from SEQ ID NO: 9 or of the Sequence Listing with the one or several amino acids being deleted, substituted or added in the amino acid sequence of SEQ ID NO: 9 or 11 of the Sequence Listing, having coenzyme regeneration activity for producing an optically pure ethyl (*S*)-4-chloro-3-hydroxybutyrate.

The DNA according to present invention codes for the above polypeptide having carbonyl reductase activity has nucleotide sequence selected from SEQ ID NO: 2, 4, 6 or 8 of the Sequence Listing and for coenzyme regenerating enzyme has nucleotide sequence selected from SEQ ID NO:10 or 12 of the Sequence Listing.

The plasmid according to present invention coding both polypeptides having carbonyl reductase activity and coenzyme regenerating ability is pETDuet1-omp-CRS,omp-GDH.

The transformed cell used according to present invention is transformant which is a cell transformed with the above plasmid pETDuet1-omp-CRS,omp-GDH.

The cell used according to present invention for creating transformant having both carbonyl reductase activity and coenzyme regenerating ability is selected from *Escherichia coli* BL21(DE3), *Escherichia coli* C41(DE3) or *Escherichia coli* C43(DE3).

In a preferred embodiment, the transformed cell is *Escherichia coli* BL21(DE3) + pET pETDuet1-omp-CRS,omp-GDH.

In more preferred embodiment, the transformed cell is *Escherichia coli* C41(DE3) + pETDuet1-omp-CRS, omp-GDH.

In a preferred embodiment, transformant coexpressing on the surface of the transformant, of the two polypeptides, one having CRS activity has an amino acid sequence SEQ ID NO: 1 of the Sequence Listing with the one or several amino acids being deleted, substituted or added in the amino acid sequence of SEQ ID NO: 1 of the Sequence Listing, which effectively reduces ethyl (*S*)-4-chloro-3-hydroxybutyrate and the other having GDH activity has an amino acid sequence SEQ ID NO: 9 of the Sequence Listing with the one or several amino acids being deleted, substituted or added in the amino acid sequence of SEQ ID NO: 9 of the Sequence Listing, having coenzyme regeneration activity for producing an optically pure ethyl (*S*)-4-chloro-3-hydroxybutyrate.

The DNA according to present invention codes for the above polypeptide having carbonyl reductase activity has nucleotide sequence SEQ ID NO: 2 of the Sequence Listing and for coenzyme regenerating enzyme has nucleotide sequence SEQ ID NO:10 of the Sequence Listing.

The plasmid according to present invention coding both polypeptides having carbonyl reductase activity and coenzyme regenerating ability is pETDuet1-omp-CRS; omp-GDH.

The transformed cell used according to present invention is transformant which is a cell transformed with the above plasmid pETDuet1-omp-CRS; omp-GDH.

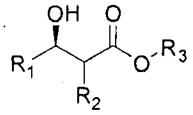
In a preferred embodiment, the transformed cell is *Escherichia coli* BL21(DE3) + pET pETDuet1-omp-CRS,omp-GDH.

The cell used according to present invention for creating transformant having both carbonyl reductase activity and coenzyme regenerating ability is selected from *Escherichia coli* BL21(DE3), *Escherichia coli* C41(DE3) or *Escherichia coli* C43(DE3).

In more preferred embodiment, the transformed cell is *Escherichia coli* C41(DE3) + pETDuet1-omp-CRS; omp-GDH.

Another object of the present invention is to provide for a production method for producing industrially important optically active alcohols includes culture condition for the transformant/s, harvesting the culture, reaction condition for substrates having carbonyl group and harvesting the produced optically active alcohols.

In one embodiment, the ketone is an aliphatic compound represented by formula 3 and the resultant optically active alcohol is represented by formula 5.



Formula 5

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$

R_2 = H, X or $CH_3(CH_2)_n$;

R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

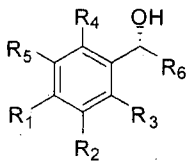
X = Cl or Br;

n = 1-4 and

m = 1-8.

In a more preferred embodiment, R_1 is CH_2Cl, R_2 is H and R_3 is CH_2CH_3 or R_1 is CH_3, R_2 is H and R_3 is CH_2CH_3 or R_1 is CH_2Cl, R_2 is H and R_3 is $(CH_2)_7CH_3$ or R_1 is CH_3, R_2 is Cl and R_3 is CH_2CH_3 or R_1 is CF_3, R_2 is H and R_3 is CH_2CH_3 or R_1 is $(CH_3)_2CH$, R_2 is H and R_3 is CH_2CH_3

In another embodiment, the ketone is an aromatic compound represented by formula 4 and the resultant optically active alcohol is represented formulae 6



Formula 6

Wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$ and

n= 1 to 5.

The designer whole cell biocatalyst can be isolated from fermentation broth by centrifugation or filtration. The isolated biocatalyst may be used as such or as lyophilized powder. For biotransformation of carbonyl compounds to hydroxyl compounds, the biocatalyst may be suspended in a suitable buffer pH 5.0-9.0, but optimally 6.5 at temperature of 10-45 °C, but optimally at 30 °C. Optionally, a cosolvent such as diethyl ether, di-n-butyl ether, methyl n-butyl ether, ethyl acetate, butyl acetate etc. may be added to the reaction mixture. The contents may be energetically mixed or shaken on an orbital shaker.

In a preferred embodiment, the reaction is done by contacting the carbonyl compound with whole cell biocatalyst in phosphate buffer pH 6.5 containing di-n-butyl ether at 30 °C.

In a specific embodiment, the present invention provides for a production method for producing industrially important ethyl (*S*)-4-chloro-3-hydroxybutyrate in about 100% enantiomeric excess, the method comprising

(i) providing ethyl 4-chloro-3-oxobutyrate and

(ii) contacting the ethyl 4-chloro-3-oxobutyrate with *Escherichia coli* strain BL21(DE3) that expresses CRS polypeptide on the surface of the cell, nicotinamide adenine dinucleotide phosphate, reduced (NADPH), glucose dehydrogenase and buffer solution to form the reaction mixture for converting ethyl 4-chloro-3-oxobutyrate to ethyl (*S*)-4-chloro-3-hydroxybutyrate. Optionally an organic solvent such as ethyl acetate, butyl acetate, diethylether, methyl n-butyl ether or di-n-butyl ether may be added to the reaction mixture.

In yet another specific embodiment the present invention provides for a production method for producing industrially important ethyl (*S*)-4-chloro-3-hydroxybutyrate in 100% enantiomeric excess and high yield via designer whole cell biocatalyzed reduction of ethyl 4-chloro-3-oxobutyrate, the method comprising

(i) providing ethyl 4-chloro-3-oxobutyrate and

(ii) contacting the ethyl 4-chloro-3-oxobutyrate with *Escherichia coli* strain BL21(DE3) that simultaneously expresses a CRS polypeptide and a GDH polypeptide on surface of cell, nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and buffer solution to form the reaction mixture for converting ethyl 4-chloro-3-oxobutyrate to ethyl (*S*)-4-chloro-3-hydroxybutyrate. Optionally an organic solvent such as ethyl acetate, butyl acetate, diethylether, methyl n-butyl ether or di-n-butyl ether may be added to the reaction mixture.

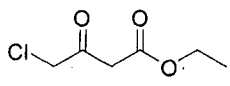
In a preferred embodiment, the rate of conversion of ethyl 4-chloro-3-oxobutyrate to ethyl (*S*)-4-chloro-3-hydroxybutyrate with nicotinamide adenine dinucleotide phosphate, reduced (NADPH) as cofactor is 1.3-fold higher when *Escherichia coli* strain BL21(DE3) that simultaneously expresses a CRS polypeptide and a GDH polypeptide on surface of cell is replaced with *Escherichia coli C41(DE3)* that simultaneously expresses a CRS polypeptide and a GDH polypeptide on surface of cell.

In a preferred embodiment, the rate of conversion of ethyl 4-chloro-3-oxobutyrate to ethyl (*S*)-4-chloro-3-hydroxybutyrate with nicotinamide adenine dinucleotide phosphate, reduced (NADPH) as cofactor is about 1.6-fold higher with *Escherichia coli* strain C41(DE3) that simultaneously expresses a CRS polypeptide and a GDH polypeptide on surface of cell

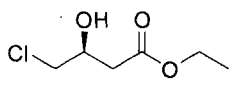
compared to *Escherichia coli* strain BL21(DE3) that expresses only CRS polypeptide on the surface of cell, requiring addition of external GDH for cofactor recycling.

In a specific embodiment, the present invention provides for a production method for producing industrially important ethyl (*S*)-4-chloro-3-hydroxybutyrate in about 100% enantiomeric excess with high productivity and product accumulation of at least 150 g l^{-1}.

Accordingly, the main embodiment of the present invention provides a designer cell that expresses a non-naturally occurring carbonyl reductase polypeptide of sequence selected from SEQ ID NO: 1, 3, 5 or 7 of the Sequence Listing on the surface of cell having 250-fold to 300-fold higher activity per unit mass of CRS polypeptide compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 or 15 or 17 or 19 of the sequence listing in cytoplasm of cell for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2.



Formula 1



Formula 2

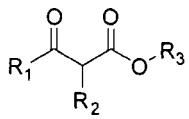
Another embodiment of the present invention provides a designer cell as described in the present invention that expresses a non-naturally occurring carbonyl reductase polypeptide SEQ ID NO: 1 of the Sequence Listing on the surface of cell having about 275-fold higher activity per unit mass of CRS polypeptide compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 of the sequence listing in cytoplasm of cell for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2.

Another embodiment of the present invention provides a designer cell as described in the present invention that expresses a non-naturally occurring carbonyl reductase polypeptide of sequence selected from SEQ ID NO: 1, 3, 5 or 7 of the Sequence Listing on the surface of cell having 15-fold to 26-fold higher activity per unit cell mass compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 or 15 or 17 or 19 of the Sequence Listing in cytoplasm of cell for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2.

Another embodiment of the present invention provides a designer cell as described in the present invention that expresses a non-naturally occurring carbonyl reductase polypeptide of

SEQ ID NO: 1 of the Sequence Listing on the surface of cell having at least 15-fold higher activity per unit cell mass compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 of the sequence listing in cytoplasm of cell for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2.

Another embodiment of the present invention provides a designer cell as described in the present invention that expresses a non-naturally occurring carbonyl reductase polypeptide of SEQ ID NO: 1 of the Sequence Listing on the surface of cell having 50-fold to 275-fold higher activity per unit mass of CRS polypeptide compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 of the Sequence Listing in cytoplasm of cell for reduction of compound of formula 3



Formula 3

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$

R_2 = H, X or $CH_3(CH_2)_n$;

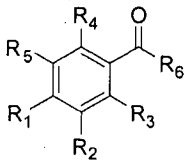
R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8;

Another embodiment of the present invention provides a designer cell as described in the present invention that expresses a non-naturally occurring carbonyl reductase polypeptide of SEQ ID NO: 1 of the Sequence Listing on the surface of cell having 50-fold to 180-fold higher activity per unit mass of CRS polypeptide compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 of the Sequence Listing in cytoplasm of cell for reduction of compound of formula 4



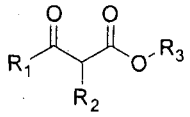
Formula 4

wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

Another embodiment of the present invention provides a designer cell as described in the present invention that expresses a non-naturally occurring carbonyl reductase polypeptide of SEQ ID NO: 1 of the Sequence Listing on the surface of cell having about 3-fold to 15-fold higher activity per unit cell mass compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 in cytoplasm of cell for reduction of compound of formula 3 or compound of formula 4



Formula 3

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$

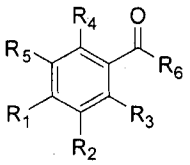
R_2 = H, X or $CH_3(CH_2)_n$;

R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8;



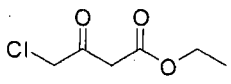
Formula 4

wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

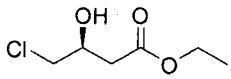
R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

Another embodiment of the present invention provides a designer cell as described in the present invention that simultaneously expresses a non-naturally occurring CRS polypeptide of sequence selected from SEQ ID NO: 1, 3, 5 or 7 and a non-naturally occurring GDH polypeptide of sequence selected from SEQ ID NO: 9 or 11 of the Sequence Listing on the surface of cell that has 250-fold to 300-fold higher activity for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 per unit mass of CRS polypeptide and 200-fold to 250-fold enhanced activity for oxidation of glucose to gluconate per unit mass of GDH polypeptide compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 or 15 or 17 or 19 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 or 23 of the Sequence Listing in cytoplasm of cell.



Formula 1



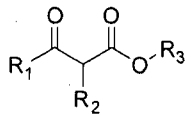
Formula 2

Another embodiment of the present invention provides a designer cell as described in the present invention that simultaneously expresses a non-naturally occurring CRS polypeptide SEQ ID NO: 1 and a non-naturally occurring GDH polypeptide SEQ ID NO: 9 of the Sequence Listing on the surface of cell that has about 270-fold higher activity for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 per unit mass of CRS polypeptide and about 225-fold enhanced activity for oxidation of glucose to gluconate per unit mass of GDH polypeptide compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 of the Sequence Listing in cytoplasm of cell.

Another embodiment of the present invention provides a designer cell as described in the present invention that simultaneously expresses a non-naturally occurring CRS polypeptide of sequence selected from SEQ ID NO: 1, 3, 5 or 7 and a non-naturally occurring GDH polypeptide of sequence selected from SEQ ID NO: 9, 11, 13 or 15 of the Sequence Listing on the surface of cell that has about 11-fold to 24-fold higher activity for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 per unit cell mass and 9-fold to 31-fold enhanced activity for oxidation of glucose to gluconate per unit cell mass compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 or 15 or 17 or 19 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 or 23 of the Sequence Listing in cytoplasm of cell.

Another embodiment of the present invention provides a designer cell as described in the present invention that simultaneously expresses a non-naturally occurring CRS polypeptide of SEQ ID NO: 1 and a non-naturally occurring GDH polypeptide of SEQ ID NO: 9 of the Sequence Listing on the surface of cell that has about 24-fold higher activity for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl 4-chloro-3-hydroxybutyrate of formula 2 per unit cell mass and about 31-fold enhanced activity for oxidation of glucose to gluconate per unit cell mass compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 of the Sequence Listing in cytoplasm of cell.

Another embodiment of the present invention provides a designer cell as described in the present invention that simultaneously expresses a non-naturally occurring CRS polypeptide of SEQ ID NO: 1 and a non-naturally occurring GDH polypeptide of SEQ ID NO: 9 of the Sequence Listing on the surface of cell having about 55-fold to 270-fold higher activity per unit mass of CRS polypeptide compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 of the Sequence Listing in cytoplasm of cell for reduction of compound of formula 3



Formula 3

wherein $R_1 = CH_3$, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$

R_2 = H, X or $CH_3(CH_2)_n$;

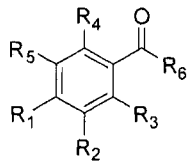
R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8

Another embodiment of the present invention provides a designer cell as described in the present invention that simultaneously expresses a non-naturally occurring CRS polypeptide of SEQ ID NO: 1 and a non-naturally occurring GDH polypeptide of SEQ ID NO: 9 of the Sequence Listing on the surface of cell having about 40-fold to 156-fold higher activity per unit mass of CRS polypeptide compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 of the Sequence Listing in cytoplasm of cell for reduction of compound of formula 4



Formula 4

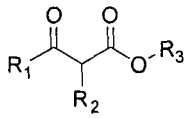
wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

Another embodiment of the present invention provides a designer cell as described in the present invention that simultaneously expresses a non-naturally occurring CRS polypeptide of

SEQ ID NO: 1 and a non-naturally occurring GDH polypeptide of SEQ ID NO: 9 of the Sequence Listing on the surface of cell having about 3-fold to 24-fold higher activity per unit cell mass compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 of the Sequence Listing in cytoplasm of cell for reduction of compound of compound of formula 3 or compound of formula 4



Formula 3

wherein $R_1 = CH_3$, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$;

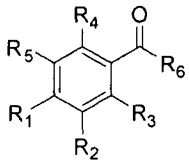
R_2 = H, X or $CH_3(CH_2)_n$;

R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8



Formula 4

wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

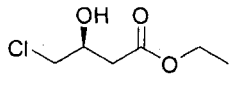
n= 1 to 5.

Another embodiment of the present invention provides a designer cell as described in the present invention, wherein designer cell is recombinant *Escherichia coli* BL21(DE3) or recombinant *Escherichia coli* C41(DE3) or recombinant *Escherichia coli* C43(DE3).

Yet another embodiment of the present invention provides a recombinant expression vector comprising polynucleotide encoding the polypeptide having the amino acid sequence of SEQ ID NO 1 or 3 or 5 or 7 showing carbonyl reductase activity.

Yet another embodiment of the present invention provides a recombinant expression vector comprising polynucleotides encoding the polypeptide having the amino acid sequence of SEQ ID NO 1 or 3 or 5 or 7 showing carbonyl reductase activity and the polypeptide having the amino acid sequence of SEQ ID NO 9 or 11 showing GDH activity.

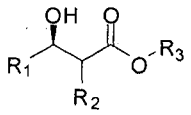
Yet another embodiment of the present invention provides a use of the designer cell as described in the present invention for the production of ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 in about 100% enantiomeric excess



Formula 2

Yet another embodiment of the present invention provides a use of the designer cell as claimed in claim 8 for the production of ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 in about 100% enantiomeric excess

Yet another embodiment of the present invention provides a use of the designer cell as described in the present invention for the production of compound of formula 5



Formula 5

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$;

R_2 = H, X or $CH_3(CH_2)_n$;

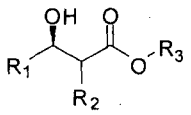
R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8

Yet another embodiment of the present invention provides use of the designer cell as as described in the present invention for the production of compound of formula 5



Formula 5

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$;

R_2 = H, X or $CH_3(CH_2)_n$;

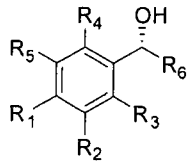
R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8

Yet another embodiment of the present invention provides use of the designer cell as described in the present invention for the production of compound of formula 6



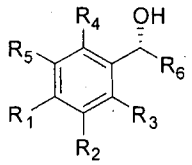
Formula 6

wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

Yet another embodiment of the present invention provides use of designer cell as described in the present invention for the production of compound of formula 6



Formula 6

wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

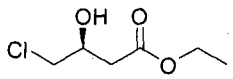
n= 1 to 5.

Another embodiment of the present invention provides for designer cells having Accession No. MTCC No.5806, MTCC No. 5807, MTCC No. 5808 and MTCC No. 5809, wherein the designer cells comprise of nucleotide sequences having SEQ ID Nos. 2, 4, 6 and 8 and wherein the nucleotide sequeces having SEQ ID Nos. 2, 4, 6 and 8 are capable of expressing amino acid sequences having SEQ ID Nos. 1, 3, 5 amd 7.

Another embodiment of the present invention provides for for designer cells having Accession No. MTCC No.5810, MTCC No. 5811, MTCC No. 5812, MTCC No. 5813; MTCC No. 5814. MTCC No. 5815, MTCC No. 5816 and MTCC No. 5817, wherein MTCC No.5810 comprise of nucleotide sequences having SEQ ID NO. 2 and SEQ ID NO.10; wherein designer cells having accession no. having MTCC No.5811 comprise of nucleotide sequences having SEQ ID NO. 2 and SEQ ID NO.12; wherein designer cells having accession no. having MTCC No.5812 comprise of nucleotide sequences having SEQ ID NO. 4 and SEQ ID NO.10 ; wherein designer cells having accession no. having MTCC No.5813 comprise of nucleotide sequences

having SEQ ID NO. 4 and SEQ ID NO.12; wherein designer cells having accession no. having MTCC No.5814 comprise of nucleotide having SEQ ID NO. 6 and SEQ ID NO.10; wherein designer cells having accession no. having MTCC No.5815 comprise of nucleotide sequences having SEQ ID NO. 6 and SEQ ID NO.12; wherein designer cells having accession no. having MTCC No.5816 comprise of nucleotide sequences having SEQ ID NO. 8 and SEQ ID NO.10; wherein designer cells having accession no. having MTCC No.5817 comprise of nucleotide sequences having SEQ ID NO. 8 and SEQ ID NO.12 and that wherein designer cells having accession no. having MTCC No.5810 is capable of expressig amino acid sequences having SEQ ID NO. 1 and SEQ ID NO. 9; wherein designer cells having accession no. having MTCC No.5811 is capable of expressing amino acid sequences having SEQ ID NO. 1 and SEQ ID NO.11; wherein designer cells having accession no. having MTCC No.5812 is capable of expressing amino acid sequences having SEQ ID NO. 3 and SEQ ID NO. 9; wherein designer cells having accession no. having MTCC No.5813 is capable of expressing amino acid sequences having SEQ ID NO. 3 and SEQ ID NO.11; wherein designer cells having accession no. having MTCC No.5814 is capable of expressing amino acid sequences having SEQ ID NO. 5 and SEQ ID NO.9; wherein designer cells having accession no. having MTCC No.5815 is capable of expressing amino acid sequences having SEQ ID NO. 5 and SEQ ID NO.11; wherein designer cells having accession no. having MTCC No.5816 is capable of expressing amino acid sequences having SEQ ID NO. 7 and SEQ ID NO.9; wherein designer cells having accession no. having MTCC No.5817 is capable of expressing amino acid sequences having SEQ ID NO. 7 and SEQ ID NO.11.

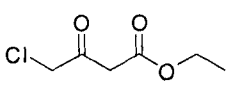
Another embodiment of the present invention provides a process for production of ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 in about 100% enantiomeric excess,



Formula 2

and the said process comprising the steps of;

a. providing ethyl 4-chloro-3-oxobutyrate of formula 1;



Formula 1

b. contacting the ethyl 4-chloro-3-oxobutyrate with designer cell as described in the present invention and 0.005 to 0.02 mol % nicotinamide adenine dinucleotide

phosphate, reduced (NADPH) and 100 to 500 units glucose dehydrogenase and a buffer solution of pH 5.0 to 9.0;

c. adding to the reaction mixture obtained in step (b) an organic solvent such as ethyl acetate, butyl acetate, diethylether, methyl n-butyl ether or di-n-butyl ether in the ratio ranging between 10:1 to 1:1;

d. energetically mixing the reaction mixture on magnetic stirrer at constant temperature of 20 to 40 °C;

e. extracting the product obtained in step (d) in ethyl acetate followed by isolating the product ethyl (*S*)-4-chloro-3-hydroxybutyrate.

Another embodiment of the present invention provides for a process as described in the present invention wherein pH is preferably 6.5.

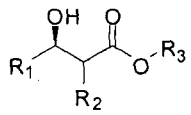
Another embodiment of the present invention provides for a process as described in the present invention wherein temperature is preferably 30 °C.

Another embodiment of the present invention provides for a process as described in the present invention wherein organic solvent is preferably di-n-butyl ether.

Another embodiment of the present invention provides for a process as described in the present invention wherein designer cell is a strain of recombinant *Escherichia coli* selected from recombinant *Escherichia coli* BL21(DE3), recombinant *Escherichia coli* C41(DE3) and recombinant *Escherichia coli* C43(DE3).

Another embodiment of the present invention provides for a process as described in the present invention wherein designer cell is preferably recombinant *Escherichia coli* C41(DE3).

Another embodiment of the present invention provides for a process for the production of optically enriched aliphatic alcohols of formula 5 as described in the present invention:



Formula 5

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$;

R_2 = H, X or $CH_3(CH_2)_n$;

R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

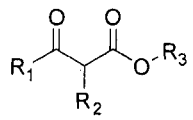
X = Cl or Br;

n = 1-4 and

m = 1-8

and the said process comprising the steps of;

a. providing a ketone of formula 3



Formula 3

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$;

R_2 = H, X or $CH_3(CH_2)_n$;

R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8

b. contacting the ketone of formula 3 as provided in step (a) with designer cell as described in the present invention and 0.005 to 0.02 mol % nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and 100 to 500 units glucose dehydrogenase and buffer solution of pH 5.0 to 9.0 to form the reaction mixture;

c. adding to the reaction mixture obtained in step (b) an organic solvent such as ethyl acetate, butyl acetate, diethylether, methyl n-butyl ether or di-n-butyl ether in the ratio ranging between 10:1 to 1:1;

d. energetically mixing the reaction mixture on magnetic stirrer at constant temperature of 20 to 40 °C;

e. extracting the product obtained in step (d) in ethyl acetate followed by isolating the compound of formula 5.

Another embodiment of the present invention provides for a process as described in the present invention wherein wherein pH is preferably 6.5.

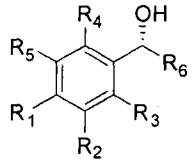
Another embodiment of the present invention provides for a process as described in the present invention wherein temperature is preferably 30 °C.

Another embodiment of the present invention provides for a process as described in the present invention wherein wherein organic solvent is preferably di-n-butyl ether.

Another embodiment of the present invention provides for a process as described in the present invention wherein designer cell is a strain of recombinant *Escherichia coli* selected from recombinant *Escherichia coli* BL21(DE3), recombinant *Escherichia coli* C41(DE3) and recombinant *Escherichia coli* C43(DE3).

Another embodiment of the present invention provides for a process as described in the present invention wherein designer cell is preferably recombinant *Escherichia coli* C41(DE3).

Another embodiment of the present invention provides for a process for production of optically enriched aryl alcohols of formula 6 as described in the present invention:



Formula 6

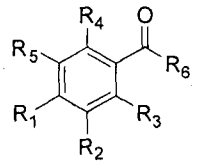
wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

and the said process comprising the steps of;

a. providing a ketone of formula 4



Formula 4

Wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

b. contacting the ketone of formula 3 as provided in step (a) with designer cell as described in the present invention and 0.005 to 0.02 mol % nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and 100 to 500 units glucose dehydrogenase and buffer solution of pH 5.0 to 9.0 to form the reaction mixture;

c. adding to the reaction mixture obtained in step (b) an organic solvent such as ethyl acetate, butyl acetate, diethylether, methyl n-butyl ether or di-n-butyl ether in the ratio ranging between 10:1 to 1:1;

d. energetically mixing the reaction mixture on magnetic stirrer at constant temperature of 20 to 40 °C;

e. extracting the product obtained in step (d) in ethyl acetate followed by isolating the compound of formula 6.

Another embodiment of the present invention provides for a process as described in the present invention wherein wherein pH is preferably 6.5.

Another embodiment of the present invention provides for a process as described in the present invention wherein wherein temperature is preferably 30 °C.

Another embodiment of the present invention provides for a process as described in the present invention wherein wherein organic solvent is preferably di-n-butyl ether.

Another embodiment of the present invention provides for a process as described in the present invention wherein wherein designer cell is a strain of recombinant *Escherichia coli* selected from recombinant *Escherichia coli* BL21(DE3), recombinant *Escherichia coli* C41(DE3) and recombinant *Escherichia coli* C43(DE3).

Another embodiment of the present invention provides for a process as described in the present invention wherein wherein designer cell is preferably recombinant *Escherichia coli* C41(DE3).

Another embodiment of the present invention provides for designer cells having Accession No. MTCC No.5806, MTCC No. 5807, MTCC No. 5808 and MTCC No. 5809.

Yet another embodiment of the present invention provides for designer cells wherein the designer cell having Accession Nos. MTCC 5806-5809 expressig amino acid sequences having SEQ ID No. 1, 3, 5 and 7.

Yet another embodiment of the present invention provides for designer cells as described in the present invention wherein the amino acid sequences having SEQ ID Nos. 1, 3, 5, 7 correspond to nucleotide sequences having SEQ ID No. 2, 4, 6 and 8.

Yet another embodiment of the present invention provides for designer cells, wherein the SEQ ID Nos. 1-8, are non-naturally occurring sequences of carbony reductase enzyme.

Yet another embodiment of the present invention provides for designer cells capable of expressing high activity of carbonyl reductase on the cell surface.

Yet another embodiment of the present invention provides for a recombinant vector construct comprising and capable of expressing SEQ ID Nos. 1-8.

Yet another embodiment of the present invention provides for a recombinant vector construct comprising non-naturally occurring sequences of carbony reductase enzyme.

Yet another embodiment of the present invention provides for a recombinant vector construct as described in the present invention capable of expressing high activity of carbonyl reductase on the cell surface.

Yet another embodiment of the present invention provides for designer cells having Accession No. MTCC No.5810, MTCC No. 5811, MTCC No. 5812, MTCC No. 5813; MTCC No. 5814. MTCC No. 5815, MTCC No. 5816 and MTCC No. 5817.

Yet another embodiment of the present invention provides for designer cells as, wherein designer cells having accession no. having MTCC No.5810 expressig amino acid sequences having SEQ ID NO. 1 and SEQ ID NO.9; wherein designer cells having accession no. having MTCC No.5811 expressig amino acid sequences having SEQ ID NO. 1 and SEQ ID NO.11;

wherein designer cells having accession no. having MTCC No.5812 expressig amino acid sequences having SEQ ID NO. 3 and SEQ ID NO. 9; wherein designer cells having accession no. having MTCC No.5813 expressig amino acid sequences having SEQ ID NO. 3 and SEQ ID NO.11; wherein designer cells having accession no. having MTCC No.5814 expressig amino acid sequences having SEQ ID NO. 5 and SEQ ID NO.9; wherein designer cells having accession no. having MTCC No.5815 expressig amino acid sequences having SEQ ID NO. 5 and SEQ ID NO.11; wherein designer cells having accession no. having MTCC No.5816 expressig amino acid sequences having SEQ ID NO. 7 and SEQ ID NO.9; wherein designer cells having accession no. having MTCC No.5817 expressig amino acid sequences having SEQ ID NO. 7 and SEQ ID NO.11.

Yet another embodiment of the present invention provides for designer cells wherein the amino acid sequences having SEQ ID Nos. 1, 3, 5, 7, 9 and 11 correspond to nucleotide sequences having SEQ ID Nos. 2, 4, 6, 8, 10 and 12.

Yet another embodiment of the present invention provides for designer cells, capable of co-expressing enzyme carbonyl reductase and gluconase dehydrogenase together on cell surface.

Yet another embodiment of the present invention provides for a recombinant vector comprising and capable of expressing amino acid sequences having SEQ ID Nos. 1, 3, 5, 7, 9 and 11 and nucleotide sequences having SEQ ID Nos. 2, 4, 6, 8, 10 and 12.

Aother embodiment of the present invention provides for a recombinant vector as described in the present invention capable of co-expressing enzyme carbonyl reductase and gluconase dehydrogenase together on cell surface.

Another embodiment of the present invention provides for a method of producing compound of Formula 5 or Formula 6 or Formula 2 such as herein described in the present invention said method comprising (a) providing as ketone having a formula 3 such as herein described in the present invention; (b) contacting the ketone of formula 3 of step (a) with a designer cell such as herein described in the present invention comprising SEQ ID Nos. 1-12 alone or in combination such as herein described in the present invention and 0.005 to 0.02 mol % nicotinamide adenine dinucleotide phosphate, reduced (NADPH) optionally, if required, adding 100 to 500 units glucose dehydrogenase and buffer solution of pH 5.0 to 9.0 to form the reaction mixture; (c) adding to the reaction mixture obtained in step (b) an organic solvent such as ethyl acetate, butyl acetate, diethylether, methyl n-butyl ether or di-n-butyl ether in the ratio ranging between 10:1 to 1:1; (d) energetically mixing the reaction mixture on magnetic stirrer at constant temperature of 20 to 40 ^{o}C and extracting the product obtained in step (d) in ethyl acetate followed by isolating the compound of Formula 5 or Formula 6 or Formula 2

Advantages of the invention

1. The invention provides a whole cell biocatalyst that has at least 250-fold improved conversion rate per unit mass of CRS compared to prior art biocatalyst for conversion of ethyl 4-chloro-3-oxobutyrate to industrially important ethyl (*S*)-4-chloro-3-hydroxybutyrate.

2. The invention provides a whole cell biocatalyst that has higher efficiency with respect to cofactor loading due to presence of both reductase and coenzyme regenerating activities on the surface of same cell. In such a system the cofactor nicotinamide adenine dinucleotide phosphate, reduced (NADPH)/nicotinamide adenine dinucleotide phosphate (NADP) gets channelized between CRS and GDH enzymes which are localized in close proximity on the surface of cells.

3. Enantiomerically enriched alcohols are produced at significantly higher conversion rate. More specifically, ethyl (*S*)-4-chloro-3-hydroxybutyrate is produced at significantly higher conversion rate in about 100% enantiomeric excess with product accumulation of at least 150 g l^{-1}, which is useful as chiral building block and an intermediate for the production of hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors.

Plasmid/s and Transformant/s

Expression plasmid of the present invention includes pET23(a)-omp-CRS having the DNA sequence represented by SEQ ID NO: 2 or 4 or 6 or 8 of Sequence listing encoding the polypeptide having the amino acid sequence represented by SEQ ID NO 1 or 3 or 5 or 7 showing carbonyl reductase activity.

Expression plasmid of the present invention includes pETDuet1-omp-CRS; omp-GDH having the DNA sequences represented by SEQ ID NO 2 or 4 or 6 or 8 encoding the CRS polypeptide having the amino acid sequence represented by SEQ ID NO 1 or 3 or 5 or 7 showing carbonyl reductase activity and SEQ ID NO 10 or 12 encoding the GDH polypeptide having the amino acid sequence represented by SEQ ID NO 9 or 11 showing GDH activity.

The plasmid containing the DNA of the present invention can be introduced into a chemically competent host cell by a known method. *Escherichia coli* DH5α were used as a cloning host and *Escherichia coli* BL21(DE3); *Escherichia coli* C41(DE3) and *Escherichia coli* C43(DE3) as expression host.

As an example of a transformant according to the present invention, mention may be made of *Escherichia coli* BL21(DE3) + pET23(a)-omp-CRS.

More significantly, as an example of a transformant according to the present invention, mention may be made of *Escherichia coli* C41(DE3) + pET23(a)-omp-CRS.

Transformant/s having carbonyl reductase activity

When a transformant having carbonyl reductase activity reacted with the carbonyl group of the substrate including ethyl 4-chloro-3-oxobutyrate and a coenzyme nicotinamide adenine dinucleotide phosphate, reduced (NADPH), it asymmetrically reduces the compound having the carbonyl group including ethyl 4-chloro-3-oxobutyrate to produce an optically pure corresponding alcohols including ethyl (*S*)-4-chloro-3-hydroxybutyrate. Transformant/s of the present invention mentioned above having expression plasmid pET23(a)-omp-CRS and pETDuet1-omp-CRS; omp-GDH exhibit carbonyl reductase activity. More precisely, transformant/s of the present invention mentioned above having expression plasmid pET23(a)-omp-CRS and pETDuet1-omp-CRS; omp-GDH expressed polypeptide having carbonyl reductase activity on the surface of the cell.

Transformant having both carbonyl reductase and coenzyme regeneration activity

Transformant of the present invention mentioned above having both CRS and GDH expression plasmid together i.e. pETDuet1-omp-CRS; omp-GDH exhibits both carbonyl reductase and coenzyme regeneration activity. More precisely, transformant/s of the present invention mentioned above having expression plasmid pETDuet1-omp-CRS; omp-GDH expressed polypeptides having both carbonyl reductase and coenzyme regeneration activity on the surface of the cell.

Strain designation and deposit details

The synthesized recombinant *Escherichia coli* strains expressing "CRS polypeptide'' and "GDH polypeptide" have been deposited with The International Depository Authority, Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Sector 39A, Chandigarh 160036, India. The strain designation and assigned accession number are given below. The strains MTCC 5806, 5807, 5808 and 5809 express "CRS polypeptide'' SEQ ID No. 1, 3, 5 and 7, respectively. The strains MTCC 5810 to 5817 co-express "CRS polypeptide'' SEQ ID No. 1, 3, 5 and 7 and "GDH polypeptide" SEQ ID No. 9 and 11. In the

description, the amino acid sequence has been assigned odd number. The nucleotide sequence coding for a particular amino acid has been assigned immediately next even number. For example, for amino acid sequence SEQ ID NO: 1, the corresponding coding nucleotide sequence has been assigned sequence SEQ ID NO: 2.

S. No.	Strain	MTCC No.
1	E. coli BL21(DE3) + pET 23(a)-omp-CRS (SEQ ID 2)	MTCC 5806
2	E. coli BL21(DE3) + pET 23(a)-omp-CRS (SEQ ID 4)	MTCC 5807
3	E. coli BL21(DE3) + pET 23(a)-omp-CRS (SEQ ID 6)	MTCC 5808
4	E. coli BL21(DE3) + pET 23(a)-omp-CRS (SEQ ID 8)	MTCC 5809
5	E. coli BL21(DE3) + pET Duet1-omp-CRS, omp-GDH (SEQ ID 2 + 10)	MTCC 5810
6	E. coli BL21(DE3) + pET Duet1-omp-CRS, omp-GDH (SEQ ID 2 + 12)	MTCC 5811
7	E. coli BL21(DE3) + pET Duet1-omp-CRS, omp-GDH (SEQ ID 4 + 10)	MTCC 5812
8	E. coli BL21(DE3) + pET Duet1-omp-CRS, omp-GDH (SEQ ID 4 + 12)	MTCC 5813
9	E. coli BL21(DE3) + pET Duet1-omp-CRS, omp-GDH (SEQ ID 6 + 10)	MTCC 5814
10	E. coli BL21(DE3) + pET Duet1-omp-CRS, omp-GDH (SEQ ID 6 + 12)	MTCC 5815
11	E. coli BL21(DE3) + pET Duet1-omp-CRS, omp-GDH (SEQ ID 8 + 10)	MTCC 5816
12	E. coli BL21(DE3) + pET Duet1-omp-CRS, omp-GDH (SEQ ID 8 + 12)	MTCC 5817

Demonstration of the expression of CRS on the surface of *Escherichia coli*

The recombinant cells expressing CRS were lysed, cell debris was removed by centrifugation and the supernatant was then subjected to ultra-centrifugation at 1,00,000*g* for 2 hr at 4 °C for separation of membrane fraction and soluble fraction. The sediment containing the membrane fraction was washed with buffer and re-suspended in membrane solublization buffer (25 mM Tris HCl, 20% Glycerol and 2% Triton X100, pH 7.5). All the three fractions, cell-free extract, membrane fraction and soluble protein fraction were assayed for activity using ethyl 4-

chloro-3-hydroxybutyrate as substrate. Most of the activity was recovered from membrane fraction.

The presence of CRS on the surface of *Escherichia coli* cells was further confirmed by EM immunogold labeling studies carried out with ultrathin sections of *Escherichia coli.* Anti-CRS polyclonal antibody was raised against the purified CRS in rabbit and was assayed for their specificity by Western blotting. The purified CRS was run on SDS-PAGE under the reducing conditions and after electro-blotting on to nitrocellulose membrane was probed with rabbit anti-CRS polyclonal antibody which was further probed with alkaline phosphatase conjugated goat *anti-rabbit IgG* (whole molecule) secondary antibody. The blot was then developed by dipping it in the substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 0.15 mg/ml), nitro blue tetrazolium (NBT, 0.30 mg/ml), Tris HCl (100 mM) and $MgCl_2$ (5 mM), pH 9.5 for 10 minute. The polyclonal antibody that specifically labeled pure CRS was also able to specifically label omp-CRS.

After several dehydration steps, *Escherichia coli* cells were embedded in LR white resin, which was then dehydrated in several steps using 0.2% glutaraldehyde as fixative. Thin sections cut using an ultramicrotome were incubated with rabbit anti-*CRS* polyclonal antibody followed by nanogold-labeled goat *anti-rabbit IgG* (whole molecule) secondary antibody and visualized under the transmission electron microscope. Different fields were observed and the gold particles were found to be exclusively present on the surface of the cells (Figure 7). No labeling occurred with cells as the negative control.

Use of transformant/s

When a transformant, of the present invention, containing DNA encoding the polypeptide having the carbonyl reductase activity is used, an optically pure alcohol can be produced. Furthermore, if the transformant, having the expression plasmid pET23(a)-omp-CRS expressing the polypeptide having carbonyl reductase activity is used, an optically pure alcohol can be produced. When a transformant having the expression vector pETDuet1-omp-CRS; omp-GDH expressing the polypeptides having carbonyl reductase activity and coenzyme regeneration activity are used, an optically pure alcohol can be produced. In particular, when a transformant having the expression vector pETDuet1-omp-CRS; omp-GDH expressing polypeptides having carbonyl reductase activity and coenzyme regeneration activity is used, an optically active alcohol can be produced more effectively.

The transformant containing both DNA encoding the polypeptide having carbonyl reductase activity of the present invention and DNA encoding the polypeptide having coenzyme

regeneration ability can be obtained by introducing both DNA encoding the polypeptide having carbonyl reductase activity, of the present invention and DNA encoding the polypeptide having the coenzyme regeneration ability into the single vector.

As an example of a vector into which both DNA encoding the polypeptide having the carbonyl reductase activity and DNA encoding a polypeptide having the coenzyme regeneration ability are introduced, mention may be made of pETDuet1-omp-CRS; omp-GDH.

Further, as an example of a transformant containing both DNA encoding the polypeptide having carbonyl reductase activity and the DNA encoding a polypeptide having a coenzyme regeneration ability, mention may be made *Escherichia coli* BL21(DE3) + pETDuet1-omp-CRS; omp-GDH obtained by transforming *Escherichia coli* BL21(DE3) with the vector.

Furthermore, as an example of a transformant containing both DNA encoding the polypeptide having carbonyl reductase activity and the DNA encoding a polypeptide having a coenzyme regeneration ability, mention may be made *Escherichia coli* C41(DE3) + pETDuet1-omp-CRS; omp-GDH obtained by transforming *Escherichia coli* C43(DE3) with the vector.

Culturing a transformant containing DNA encoding the polypeptide having carbonyl reductase activity and a transformant containing both DNA encoding the polypeptide having carbonyl reductase activity and the DNA encoding a polypeptide having a coenzyme regeneration ability can be performed in a liquid nutrition medium generally used and containing a carbon source, nitrogen source, inorganic salts and organic nutrients, etc., as long as they are proliferated.

Assay methods for enzyme activity

The activity of the transformant expressing polypeptide having a carbonyl reductase can be measured by a conventional method. For example, the activity of the carbonyl reductase can be obtained in which 1 ml of reaction mixture in 50 mM phosphate buffer, pH 6.5 contains 2 mM ECAA, 0.2 mM nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and 5-20 µl of the whole cell biocatalyst or 5-10 µ g of purified protein or $10^6 - 10^7$ whole cell biocatalyst and the reaction were monitored spectrophotometrically for 15 to 60 min at 340 nm (molar absorption coefficient of 6.22 $mM^{-1}cm^{-1}$) for the oxidation of nicotinamide adenine dinucleotide phosphate, reduced (NADPH).

The activity of the transformant expressing polypeptide having a coenzyme regeneration activity can be measured by a conventional method. For example, the activity of the glucose dehydrogenase can be obtained in which 1 ml of reaction mixture in 100 mM tris-HCl buffer, pH 8.0 contains 10 mM glucose , 0.5 mM nicotinamide adenine dinucleotide phosphate (NADP)

and 5-20 µl of the whole cell biocatalyst or 5-10 µ g of purified protein or $10^6 - 10^7$ whole cell biocatalyst and the reaction were monitored spectrophotometrically for 15 to 60 min at 340 nm (molar absorption coefficient of 6.22 $mM^{-1}cm^{-1}$) for the reduction of nicotinamide adenine dinucleotide phosphate (NADP).

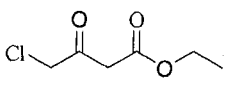
Production of optically active alcohols

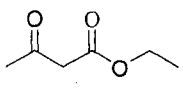
Production of an optically active alcohols using either transformant having the carbonyl reductase activity and commercially available glucose dehydrogenase or transformant having both the carbonyl reductase activity and coenzyme regeneration activity can be performed by adding a compound containing carbonyl group serving as a substrate including ethyl 4-chloro-3-oxobutyrate, a coenzyme nicotinamide adenine dinucleotide phosphate (NADP) and glucose to an appropriate solvent, and stirring the mixture while adjusting the pH.

The reaction may be performed in an aqueous solvent or in a mixture of aqueous solvent and organic solvent. Examples of the organic solvent include n-butyl acetate, ethyl acetate, diethyl ether, methyl n-butyl ether, di-n-butyl ether etc. Preferably, the reaction may be performed at 30 °C and the pH of the reaction solution is maintained at 6.5 with 5N NaOH.

Substrates and Products

Examples of the aliphatic compound having a carbonyl group serving as a substrate, includes ethyl 4-chloro-3-oxobutyrate represented by formula 1, ethyl-3-oxobutanoate represented by formula 7, ethyl 4-methyl-3-oxopentanoate represented by formula 8, octyl 4-chloro-3-oxobutanoate represented by formula 9, ethyl 4,4,4-trifluoro-3-oxobutanoate represented by formula 10 and ethyl 2-chloro-3-oxobutyrate represented by formula 11.


Formula1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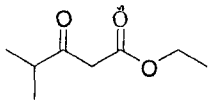
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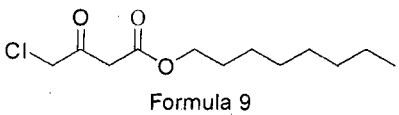
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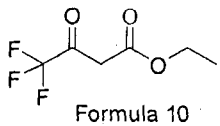
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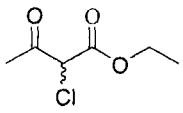
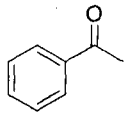

Formula 11

Examples of the aromatic compound having a carbonyl group serving as a substrate, includes acetophenone represented by formula 12, 1-(4-fluorophenyl)ethanone represented by formula 13, 1-(4-chlorophenyl)ethanone represented by formula 14, 1-(4-bromophenyl)ethanone represented by formula 15, 1-(4-(trifluoromethyl)phenyl)ethanone represented by formula 16, 1-p-

tolylethanone represented by formula 17, 1-(4-methoxyphenyl)ethanone represented by formula 18 and indoline-2,3-dione represented by formula 19 below: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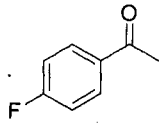
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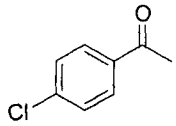
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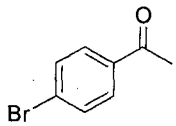
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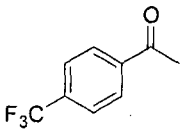
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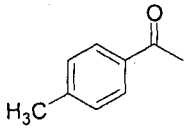
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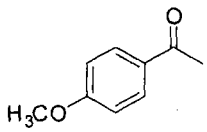
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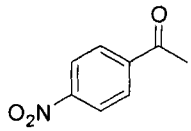

Formula 19

In the aforementioned reaction conditions, when ethyl 4-chloro-3-oxobutyrate represented by formula 1 above is used as a substrate, ethyl (*S*)-4-chloro-3-hydroxybutyrate represented by formula 2 below can be obtained.

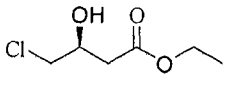
When ethyl-3-oxobutanoate represented by formula 7 above is used as a substrate, ethyl (*R*)-3-hydroxybutanoate represented by formula 20 below can be obtained

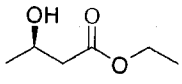
When ethyl 4-methyl-3-oxopentanoate represented by formula 8 above is used as a substrate, ethyl (*S*)-3-hydroxy-4-methylpentanoate represented by formula 21 below can be obtained.

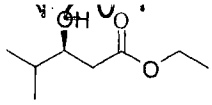
When octyl 4-chloro-3-oxobutanoate represented by formula 9 above is used as a substrate, octyl (*S*)-4-chloro-3-hydroxybutanoate represented by formula 22 below can be obtained.

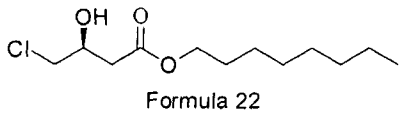
When ethyl 4,4,4-trifluoro-3-oxobutanoate represented by formula 10 above is used as a substrate, octyl (*S*)-4-chloro-3-hydroxybutanoate represented by formula 23 below can be obtained.

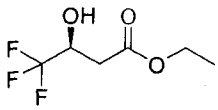
When ethyl 2-chloro-3-oxobutanoate represented by formula 11 above is used as a substrate, (2*R*,3*R*)-ethyl 2-chloro-3-hydroxybutanoate represented by formula 24 below can be obtained.

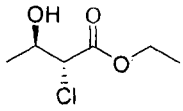

Formula 2


Formula 20


Formula 21


Formula 22


Formula 23


Formula 24

When acetophenone represented by formula 12 above is used as a substrate, (*R*)-1-phenylethanol represented by formula 25 below can be obtained.

When 1-(4-fluorophenyl)ethanone represented by formula13 above is used as a substrate, (*R*)-1-(4-fluorophenyl)ethanol represented by formula 26 below can be obtained.

When 1-(4-chlorophenyl)ethanone represented by formula 14 above is used as a substrate, (*R*)-1-(4-chlorophenyl)ethanol represented by formula 27 below can be obtained.

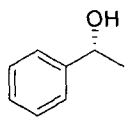
When 1-(4-bromophenyl)ethanone represented by formula 15 above is used as a substrate, (*R*)-1-(4-bromophenyl)ethanol represented by formula 28 below can be obtained.

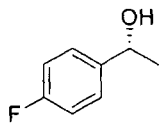
When 1-(4-(trifluoromethyl)phenyl)ethanone represented by formula 16 above is used as a substrate, (*R*)-1-(4-(trifluoromethyl)phenyl)ethanol represented by formula 29 below can be obtained.

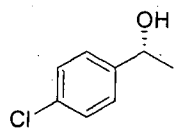
When 1-p-tolylethanone represented by formula 17 above is used as a substrate, (*R*)-1-p-tolylethanol represented by formula 30 below can be obtained.

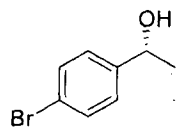
When 1-(4-methoxyphenyl)ethanone represented by formula 18 above is used as a substrate, (*R*)-1-(4-methoxyphenyl)ethanol represented by formula 31 below can be obtained.

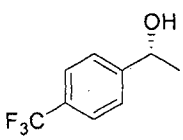
When 1-(4-nitrophenyl)ethanone represented by formula 19 above is used as a substrate, (*R*)-1-(4-nitrophenyl)ethanol represented by formula 32 below can be obtained.

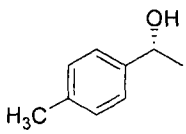

Formula 25

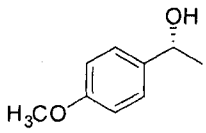

Formula 26

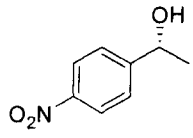

Formula 27


Formula 28


Formula 29


Formula 30


Formula 31


Formula 32

Purification and analysis

An optically pure alcohol produced by the reaction can be purified by a conventional method, for example, by extracting a reaction mixture containing an optically pure alcohol produced by the reaction with an organic solvent ethyl acetate, removing the organic solvent by distillation under reduced pressure, and subjecting the resultant mixture to distillation, recrystallization or chromatographic process.

The optical purity of the product was measured by high performance liquid chromatography by using Chiracel OB-H, Chiracel OD-H, Chiracel OJ product of Daicel Chemical Industries or by determining the optical rotation by polarimeter.

The structure of all compounds was confirmed by NMR. NMR was run using $CDCl_3$, CD_3OD or CD_3SOCD_3 as solvent. Chemical shifts are reported as downfield from TMS used as internal standard. Values of coupling constants *J* are reported in Hz.

Analytical data

The alcohols obtained from bioctalyzed reduction of ketones were purified by flash chromatography over silica gel.

Compound of Formula 2: ^{1}**H NMR** (300 MHz, $CDCl_3$): 1.28 (3H, t, J = 7.2 Hz); 2.63 (3H, m); 3.61 (2H, dd, J = 7.2, 5.4 Hz); 4.18 (2H, q, J = 7.2 Hz); 4.23 (1H, m). HPLC: Chiracel OB-H, λ_{217}, hexane:isopropanol 96:4, 1 ml/min. Retention times 13.2 min (*R*) and 14.3 min(*S*). $[\alpha]_D^{25}$ = -22.1 (c = 8.72, $CHCl_3$).

Compound of Formula 20: ^{1}**H NMR** (300 MHz, $CDCl_3$): 1.21 (3H, d, J = 6.5 Hz); 1.26 (3H, t, J = 6.8 Hz); 2.46 (2H, m); 3.00 (1H, bs); 4.16 (3H, m). HPLC: Chiracel OB-H, λ_{217}, hexane:isopropanol 96:4, 1 ml/min. Retention times 9.4 min (*S*) and 10.5 min (*R*). $[\alpha]_D^{25}$ = -44.2 (c = 2.03, $CHCl_3$).

Compound of Formula 21: ^{1}**H NMR** (300 MHz, $CDCl_3$): 0.90 and 0.93 (each 3H, each t, J = 6.8 Hz); 1.26 (3H, t, J = 7.2 Hz); 1.69 (1H, m); 2.38 (1H, dd, J = 9.6, 16.5 Hz); 2.52 (1H, dd, J = 2.8, 16.5 Hz); 2.94 (1H, bs); 3.75 (1H, m,); 4.14 (2H, q, J = 7.2 Hz). HPLC: Chiracel OD-H,

λ_{217}, hexane:isopropanol 95:5, 1 ml/min. Retention times 5.3 min (*S*) and 7.7 min (*R*). $[\alpha]_D^{25}$ = -40.8 (c = 2.56, $CHCl_3$).

Compound of Formula 22: ^{1}H NMR (300 MHz, $CDCl_3$): 0.86 (3H, t, *J* = 6.9 Hz, H_2 CH_3); 1.27 (10H, m, 5 x CH_2); 1.61 (2H, m); 2.62 (2H, m); 3.60 (2H, m); 4.11 (2H, t, *J* = 6.8 Hz); 4.14 (1H, m, H3). HPLC: Chiracel OB-H, λ_{217}, hexane:isopropanol 96:4, 1 ml/min. Retention times 5.7 min (*R*) and 7.6 min (*S*). $[\alpha]_D^{25}$ = -15.9 (c = 4.60, $CHCl_3$).

Compound of Formula 23: ^{1}H NMR (300 MHz, $CDCl_3$): 1.29 (3H, t, *J* = 7.2 Hz); 2.70 (2H, m); 4.21 (2H, q, J = 7.2 Hz); 4.43 (1H, m). $[\alpha]_D^{25}$ = -20.3 (c = 1.87, $CHCl_3$).

Compound of Formula 24: ^{1}H NMR (300 MHz, $CDCl_3$): 1.34 (m, 6H); 4.25 (m, 4H). de = >96% by GC: Factorfour™ (Varian, 30m x 0.25mm, 140 °C, N_2 1 kg min^{-1}), retention time 9.98 (*syn*), 8.85 (*anti*); >99% *anti*. Ee. >98% GC betaDex™ (Supelco, 30m x 0.25mm, 140 °C, N_2 1 kg min^{-1}), tentatively assigned (*2R*, *3R*) configuration based on comparison of optical rotation with literature. $[\alpha]_D^{25}$ = -3.8 (c = 1.13, $CHCl_3$).

Compound of Formula 25: ^{1}H NMR (300 MHz, $CDCl_3$): 1.49 (3H, d, *J* = 6.5 Hz); 2.10 (1H, bs); 4.87 (1H, q, *J* = 6.5 Hz); 7.35 (5H, m). HPLC: Chiracel OB-H, λ_{217}, hexane:isopropanol 96:4, 1 ml/min. Retention times 6.2 min (*R*) and 9.2 min (*S*). $[\alpha]_D^{25}$ = +54.8 (c = 2.74, $CHCl_3$).

Compound of Formula 26: ^{1}H NMR (300 MHz, $CDCl_3$): 1.49 (3H, d, *J* = 6.5 Hz); 2.03 (1H, bs); 4.89 (1H, q, *J* = 6.5 Hz); 7.01 and 7.03 (each 2H, each d, *J* = 8.6 Hz). HPLC: Chiracel OB-H, λ_{217}, hexane:isopropanol 96:4, 1 ml/min. Retention times 7.8 min(*S*) and 8.8 min(*R*).$[\alpha]_D^{25}$ = +48.8 (c = 1.4, $CHCl_3$).

Compound of Formula 27: ^{1}H NMR (300 MHz, $CDCl_3$): 1.47 (3H, d, *J* = 6.5 Hz); 2.1 (1H, bs, OH); 4.87 (1H, q, *J* = 6.5 Hz); 7.31 (4H, s). HPLC: Chiracel OB-H, λ_{217}, hexane:isopropanol 96:4, 1 ml/min. Retention times 8.9 min (*S*) and 10.5 min (*R*). $[\alpha]_D^{25}$ = +49.2 (c = 1.83, ether).

Compound of Formula 28: ^{1}H NMR (300 MHz, $CDCl_3$): 1.47 (3H, d, *J* = 6.5 Hz); 2.08 (1H, bs); 4.87 (1H, q, *J* = 6.5 Hz); 7.25 and 7.47 (each 2H, each d, *J* = 8.7 Hz). HPLC:Chiracel OB-H, λ_{217}, hexane:isopropanol 96:4, 1 ml/min. Retention times 9.4 min (*S*) and 11.4 min (*R*). $[\alpha]_D^{25}$ = +38.3 (c = 1.55, $CHCl_3$).

Compound of Formula 29: ^{1}**H NMR** (300 MHz, $CDCl_3$): 1.5 (3H, d, J = 6.51 Hz); 2.33 (1H, bs); 4.98 (1H, q, J = 6.5 Hz); 7.48 and 7.60 (each 2H, each d, J = 8.2 Hz).$[\alpha]_D^{25}$ = +27.2 (c 2.08, MeOH).

Compound of Formula 30: ^{1}**H NMR** (300 MHz, $CDCl_3$): 1.48 (3H, d, J = 6.5 Hz); 2.01 (1H, bs); 2.38 (1H, s); 4.87 (1H, q, J = 6.5 Hz); 7.15 and 7.26 (each 2H, each d, J = 7.9 Hz). HPLC:Chiracel OB-H, λ_{217}, hexane:isopropanol 95:5, 1 ml/min. Retention times 10.1 min (*S*) and 12.9 min (*R*). $[\alpha]_D^{25}$ = +52.1 (c = 1.98, $CHCl_3$).

Compound of Formula 31: ^{1}**H NMR**(300 MHz, $CDCl_3$): 1.46 (3H, d, J = 6.5 Hz); 2.08 (1H, bs); 3.80 (3H, s); 4.84 (1H, q, J = 6.5 Hz); 6.86 and 7.28 (each 2H, each d, J = 8.2). $[\alpha]_D^{22}$= +51.4 (c 1.72, $CHCl_3$).

Compound of Formula 32: ^{1}**H NMR** (300 MHz, $CDCl_3$): 1.52 (3H, t, J = 6.5 Hz, CH_3); 2.50 (1H, bs, OH); 5.02 (1H, q, J = 6.5 Hz, CH); 7.54 and 8.12 (each 2H, each d, J = 8.9 Hz, aryl).$[\alpha]_D^{25}$ = +31.4 (c = 3.99, $CHCl_3$).

The following description is of exemplary embodiments only and is not intended to limit the scope, applicability or configuration to the invention in any way, Rather, the following description provides a convenient illustration for implementing exemplary embodiments of the invention various changes to the described embodiments may be made in the functions and arrangement of the elements described without departing from the scope of the invention.

EXAMPLES

The specific manipulation methods regarding recombinant DNA techniques used in the following examples are described in the following publication (Sambrook, J. et al. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.1989; Vol. 2.).

Example 1

Construction of expression plasmid pET23(a)-omp-CRS expressing carbonyl reductase on the surface of *Escherichia coli*

The artificial custom synthesized plasmid pUC 19-omp-CRS having DNA according to present invention has nucleotide sequence of SEQ ID NO:2 of the Sequence Listing codes for the polypeptide has an amino acid sequence of SEQ ID NO:1 of the Sequence Listing were double digested by Ndel and EcoR1 and product was separated by agarose gel electrophoresis, 1.3 Kb DNA fragment has nucleotide sequence of SEQ ID NO:2 of the Sequence Listing, was excised from the gel and purified using *Qiaquick kit* (Qiagen). Plasmid *pET 23(a)* was separately digested with Ndel & EcoR1, followed by the dephosphorylation with calf intestinal alkaline phosphatase to avoid self-ligation. Ndel & EcoR1 (NEB Ltd, UK) digested 1.3 Kb DNA fragment has nucleotide sequence of SEQ ID NO:2 of the Sequence Listing and *pET 23(a)* were ligated with T4 DNA Ligase (NEB Ltd, UK) and transformed in *Escherichia coli* DH5α. The resultant clones were grown in LB broth containing ampicillin (100 mg/ml) and plasmid was isolated, which was then double digested by Ndel and EcoR1. Plasmid giving the 3.6 Kb *pET 23(a)* backbone and 1.3 Kb DNA insert after double digestion, suggesting nucleotide sequence of SEQ ID NO:2 of the Sequence Listing was cloned downstream of the *lac* promoter of *pET 23(a)* giving 4.9 Kb plasmid *pET 23(a)-omp*-CRS.

A restriction map of plasmid *pET 23(a)-omp*-CRS is illustrated in Figure 5.

Escherichia coli BL21(DE3), *Escherichia coli* C41(DE3) or *Escherichia coli* C43(DE3) was transformed with plasmid *pET 23(a)-omp-CRS* which expresses carbonyl reductase on the surface of the transformants.

Example 2

Construction of expression vector pET23(a)-CRS expressing carbonyl reductase in the cytoplasm of transformants

It was constructed by using the knowledge disclosed in US Patent 2010/0028972. CRSF (SEQ ID NO:26) and CRSR (SEQ ID NO:28) primers for cloning were synthesized based on the nucleotide sequence of carbonyl reductase gene represented by SEQ ID NO:14 of the Sequence Listing, which code a polypeptide has an amino acid sequence of SEQ ID NO:13 of the Sequence Listing. The custom synthesized pUC 19-omp-CRS plasmid was subjected to polymerase chain reaction (PCR) for 30 cycles of a reaction. The PCR conditions were, initial denaturing at 95 °C for 5 min followed by 30 cycles of 95 °C for 60 s, 58 °C for 60 s and 72 °C for 60 s as well as a final extension step of 72 °C for 5 min. The purified PCR product was digested with Ndel & Xhol, and was run on the 1% agarose gel. The 0.87 Kb Ndel and Xhol digested fragment was excised from the gel and purified using *Qiaquick kit* (Qiagen). Plasmid

pET 23(a) was separately digested with Nde1 & Xho1, followed by the dephosphorylation with calf intestinal alkaline phosphatase to avoid self-ligation. Nde1 and Xho1 digested 0.87 Kb has nucleotide sequence of SEQ ID NO:14 of the Sequence Listing and *pET 23(a)* were ligated with T4 DNA Ligase and transformed in *Escherichia coli* DH5α. The resultant clones were grown in LB broth containing ampicillin and plasmid were isolated, which were then double digested with Nde1 and Xho1. Plasmid gave 3.6 Kb *pET 23(a)* backbone and 0.87 Kb insert after double digestion, suggesting 0.87 Kb gene was cloned downstream of the *lac* promoter of *pET 23(a)* giving 4.4 Kb plasmid *pET 23(a)-CRS*.

Escherichia coli BL21(DE3) was transformed with plasmid *pET 23(a)-CRS* which express carbonyl reductase in the cytoplasm.

Example 3

Construction of expression vector pETDuet1-omp-CRS; omp-GDH co expressing carbonyl reductase and glucose dehydrogenase on the surface of transformants

Construction of expression vector pETDuet1-omp-CRS;omp-GDH, which co expressing the two polypeptides, one having the carbonyl reductase activity has an amino acid sequence of SEQ ID NO: 1 of the Sequence Listing and the other polypeptide has an amino acid sequence of SEQ ID NO: 3 of the Sequence Listing having coenzyme regeneration activity on the surface of the transformant/s. To achieve this goal, inventors have chosen plasmid *pETDuet1* that contains two multiple cloning sites, each of which is preceded by a T7 promoter/lac operator and a ribosome binding site (*rbs*). *omp-CRS* and *omp-GDH* gene were cloned in *pETDuet1* plasmid, which is a two-step procedure.

In first step, DNA according to present invention has nucleotide sequence of SEQ ID NO:2 of the Sequence Listing codes for the polypeptide has an amino acid sequence of SEQ ID NO:1 of the Sequence Listing was amplified from the plasmid *pUC19-omp-CRS* that was used as a template for polymerase chain reaction (PCR) with the primers oc1F (SEQ ID NO:30) and oc1R (SEQ ID NO:32). The PCR conditions were, initial denaturing at 95 °C for 5 min followed by 30 cycles of 95 °C for 60 s, 55 °C for 60 s and 72 °C for 90 s as well as a final extension step of 72 °C for 5 min. The purified PCR product was digested by Nco1 and Hind3 and was run on the 1% agarose gel. The 1.3 Kb Nco1 and Hind3 fragment was excised from the gel and purified using *Qiaquick kit* (Qiagen). Plasmid *pETDuet* was separately digested with Nco1 and Hind3, followed by the dephosphorylation with calf intestinal alkaline phosphatase to avoid self-ligation. Nco1 and Hind3 digested *omp-CRS* and *pETDuet1* were ligated with T4 DNA Ligase

and transformed in *Escherichia coli* DH5α. The resultant clones were grown in LB broth containing ampicillin (100 μg/ml) from which plasmid was isolated and then double digested with Nco1 and Hind3. Plasmid gave ~5.4 Kb *pETDuet1* backbone and 1.3 Kb SEQ ID NO:2 insert after double digestion, suggesting SEQ ID NO:2 was cloned downstream of the *lac* promoter of *pETDuet1* in the 1st multiple cloning site giving 6.7 Kb plasmid *pETDuet1-omp-CRS*.

In second step, DNA according to present invention has nucleotide sequence of SEQ ID NO:4 of the Sequence Listing codes for the polypeptide has an amino acid sequence of SEQ ID NO:3 of the Sequence Listing was amplified from the artificial custom made plasmid *pUC19-omp-GDH* that was used as a template in polymerase chain reaction (PCR) with the primers ogF (SEQ ID NO:34) and ogR (SEQ ID NO:36). The PCR conditions were, initial denaturing at 95°C for 5 min followed by 30 cycles of 95 °C for 60 s, 52 °C for 60 s and 72 °C for 90 s as well as a final extension step of 72 °C for 5 min. The purified PCR product was digested by Nde1 and Xho1 and was run on the agarose gel. The 1.2 Kb Nde1 and Xho1 digested fragment was excised from the gel and purified using *Qiaquick kit* (Qiagen). 6.7 Kb plasmid *pETDuet1-omp-CRS* constructed as described above was separately digested with Nde1 and Xho1, followed by the dephosphorylation with calf intestinal alkaline phosphatase to avoid self-ligation. Nde1 and Xho1 digested SEQ ID NO:4 and *pETDuet1-omp-CRS* were ligated with T4 DNA Ligase and transformed in *Escherichia coli* DH5α. The resultant clones were grown in LB broth containing ampicillin and from which plasmid was isolated, which was then double digested by Nde1 and Xho1. Plasmid gave the ~6.7 Kb *pETDuet1-omp-CRS* backbone and 1.2 Kb *omp-GDH* insert after double digestion, suggesting *omp-GDH* gene was cloned downstream of the *lac* promoter of *pETDuet1-omp-CRS* in the 2nd multiple cloning site in 7.8 Kb plasmid *pETDuet1-omp-CRS,omp-GDH*.

A restriction map of plasmid *pETDuet1-omp-CRS,omp-GDH* is illustrated in Figure 6.

Escherichia coli BL21(DE3), *Escherichia coli* C41(DE3) and *Escherichia coli* C43(DE3) were transformed with plasmid *pETDuet1-omp-CRS,omp-GDH* which co express carbonyl reductase and glucose dehydrogenase on the surface of transformant/s.

Example 4

Preparation of Transformant

Using the recombinant plasmid pET23(a)-omp-CRS constructed in Example 1, the *Escherichia coli* BL21(DE3), *Escherichia coli* C41(DE3) and *Escherichia coli* C43(DE3) competent cells were transformed to obtain *Escherichia coli* BL21 (DE3) + pET23(a)-omp-CRS, *Escherichia*

coli C41 (DE3) + pET23(a)-omp-CRS and *Escherichia coli* C43(DE3) + pET23(a)-omp-CRS expressing carbonyl reductase on the surface.

Similarly, using the recombinant plasmid pETDuet1-omp-CRS; omp-GDH constructed in example 3, the *Escherichia coli* BL21(DE3), *Escherichia coli* C41(DE3), *Escherichia coli* C43(DE3) and *Escherichia coli* competent cells were transformed to obtain *Escherichia coli* BL21 (DE3) + pETDuet1-omp-CRS; omp-GDH, *Escherichia coli* C41 (DE3) + pETDuet1-omp-CRS; omp-GDH and *Escherichia coli* C43(DE3) + pETDuet1-omp-CRS; omp-GDH expressing both carbonyl reductase and glucose dehydrogenase on the surface.

Using the recombinant plasmid pET23(a)-CRS constructed in example 2, the *Escherichia coli* BL21(DE3) competent cells was transformed to obtain *Escherichia coli* BL21 (DE3) + pET23(a)-CRS expressing carbonyl reductase in cytoplasm of the cell.

Using the recombinant plasmid pET23(a)-crs constructed in example 2 and pET 29(a)-GDH constructed as given below, the *Escherichia coli* BL21(DE3) competent cells were co-transformed to obtain *Escherichia coli* BL21 (DE3) + pET23(a)-CRS, pET 29(a)-GDH expressing carbonyl reductase and glucose dehydrogenase in cytoplasm of the cell. *Escherichia coli* BL21 (DE3) + pET23(a)-CRS, pET 29(a)-gdh was constructed by using the art disclosed in Kizaki, N. et al. *Applied Microbiology and Biotechnology* **2001,** *55*, 590-595.

Construction of recombinant plasmid pET29(a)-GDH: gdhF (SEQ ID NO 38) and gdhR (SEQ ID NO 40) primers for cloning were synthesized based on the nucleotide sequence of glucose dehydrogenase gene represented by SEQ ID NO 26 of the Sequence Listing, which code a polypeptide has an amino acid sequence of SEQ ID NO 25 of the Sequence Listing. The custom synthesized pUC 19-omp-gdh plasmid was subjected to polymerase chain reaction (PCR) for 30 cycles of a reaction. The PCR conditions were, initial denaturing at 95 °C for 5 min followed by 30 cycles of 95 °C for 60 s, 52 °C for 60 s and 72 °C for 60 s as well as a final extension step of 72 °C for 5 min. The purified PCR product was digested with NdeI & XhoI, and was run on the 1% agarose gel. The 0.81 Kb NdeI and XhoI digested fragment was excised from the gel and purified using *Qiaquick kit* (Qiagen). Plasmid pET 29(a) was separately digested with NdeI & XhoI, followed by the dephosphorylation with calf intestinal alkaline phosphatase to avoid self-ligation. NdeI and XhoI digested 0.81 Kb has nucleotide sequence of SEQ ID NO 26 of the Sequence Listing and pET 29(a) were ligated with T4 DNA Ligase and transformed in *Escherichia coli* DH5α. The resultant clones were grown in LB broth containing kanamycin and plasmid were isolated, which were then double digested with NdeI and XhoI. Plasmid gave 5.4 Kb *pET 29(a)* backbone and 0.81 Kb insert after double digestion, suggesting

0.81 Kb gene was cloned downstream of the *lac* promoter of *pET 29(a)* giving 6.0 Kb plasmid *pET 29(a)-GDH.*

Example 5

Expression of gene in Transformant

Fresh culture of recombinant *Escherichia coli* harboring plasmid was grown in 10 ml Luria Bertani HiVeg Broth media (1% HiVeg hydrolase, 0.5% Yeast Extract, 1% Sodium Chloride, pH-7.5) containing antibiotic (ampicillin, kanamycin, chloramphenicol etc. alone or in combination) at 37 °C, after 6 hr of growth 1 ml of the culture was inoculated in 100 ml fresh LB media containing antibiotic (ampicillin, kanamycin, chloramphenicol etc. alone or in combination) and grown at 37 °C under 200 rpm shaking condition. When the OD at 600 nm was reached to 0.5-0.6, the culture was induced with final concentration of 0.2 mM IPTG and it was further grown for 16 hr at 20 °C under 200 rpm shaking condition.

Example 6

Demonstration of the expression of carbonyl reductase on the surface of transformant *Escherichia coli* BL21(DE3) + pET 23(a)-omp-CRS (CRS, Sequence ID No 1)

The transformant Escherichia coli BL21(DE3) + pET23(a)-omp-CRS was grown as described in example 5 above, in 100 ml culture media. The cells were isolated by centrifugation and washed with 50 mM phosphate buffer (pH 7.0). The cells were then suspended in 5 ml lysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, 1mg ml^{-1} Lysozyme, pH 8.0) for 30 min at 4 °C. The cell suspension was then sonicated 30 sec pulse on and 30 sec pulse off at 4 °C for 20 min. The cell debris was removed by centrifugation at 14,000 rpm for 30 min. The supernatant (cell-free extract) was then subjected to ultra-centrifugation at 1,00,000*g* for 2 hr at 4 °C for separation of membrane fraction and soluble fraction. The sediment containing the membrane fraction was washed with the same buffer and re-suspended in membrane solublization buffer (25 mM Tris HCl, 20% Glycerol and 2% Triton X100, pH 7.5). All the three fractions, cell-free extract, membrane fraction and soluble protein fraction were assayed for their activity to reduce ethyl 4-chloro-3-oxobutyrate to ethyl 4-chloro-3-hydroxybutyrate, like, the standard reaction mixture (1 ml) in 50 mM phosphate buffer pH 7.0, containing 0.2 mM nicotinamide adenine dinucleotide phosphate, reduced (NADPH), 2.0 mM ECOB and 1-50 µl of the sample was incubated at 30°C and the total activity determined. The results have been summarized in Table 1. As expected, most of the activity was recovered from membrane fraction. Significantly, membrane fractions of the *Escherichia coli* BL21 (DE3) + *pET23(a)* (negative control) was devoid of any activity.

Table 1: Carbonyl reductase (CRS) activity of various fractions obtained from *Escherichia coli* BL21(DE3) + *pET23(a)* and *Escherichia coli* BL21 + *pET 23(a)-omp-CRS* (CRS, Sequence ID No 1).

Entry	**Fractions of *Escherichia coli***	***Escherichia coli* BL21(DE3) + *pET 23(a)*, Total activity (nmol/min)**	***Escherichia coli* BL21(DE3) + *pET 23(a)-omp-CRS*, Total activity (nmol/min)**
1	Cell-free extract	376	1673
2	Soluble fraction	76	350
3	Membrane fraction	0.0	1192

The presence of CRS on the surface of *Escherichia coli* BL21(DE3) + *pET 23(a)-omp-CRS* was further confirmed by EM immunogold labeling studies carried out with ultrathin sections of *Escherichia coli* BL21(DE3) + *pET 23(a)-omp-cr1* cells. Anti-CRS polyclonal antibody was raised against the purified CRS in rabbit and was assayed for their specificity by Western blotting. The purified CRS was run on SDS-PAGE under the reducing condition and after electro-blotting on to nitrocellulose membrane was probed with rabbit anti-CRS polyclonal antibody which was further probed with alkaline phosphatase conjugated goat *anti-rabbit IgG* (whole molecule) secondary antibody. The blot was then developed by dipping it in the substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 0.15 mg/ml), nitro blue tetrazolium (NBT, 0.30 mg/ml), Tris HCl (100 mM) and $MgCl_2$ (5 mM), pH 9.5 for 10 minute. The polyclonal antibody that specifically labeled pure CRS was also able to specifically label omp-CRS.

After several dehydration steps, recombinant *Escherichia coli* BL21(DE3) + *pET 23(a)* (negative control) and *Escherichia coli BL21(DE3) + pET 23(a)-omp-CRS* were embedded in LR white resin, which was then dehydrated in several steps using 0.2% glutaraldehyde as fixative. Thin sections cut using an ultramicrotome were incubated with rabbit anti-*CRS* polyclonal antibody followed by nanogold-labeled goat *anti-rabbit IgG* (whole molecule) secondary antibody and visualized under the transmission electron microscope. Different fields were observed and the gold particles were found to be exclusively present on the surface of the cells (Figure 7). No labeling occurred with cells as the negative control.

Example 7

Relative expression levels of cytoplasmic and surface expressed CRS in transformant *Escherichia coli* BL21(DE3) + pET 23(a)-omp-CRS (CRS, Sequence ID No 1)

Fresh culture of recombinant *Escherichia coli BL21(DE3) + pET 23(a)-CRS* (CRS, Sequence ID No 1) and *Escherichia coli BL21(DE3) + pET 23(a)-omp-CRS* (CRS, Sequence ID No 1) were grown and whole cell proteome of each clone was prepared. The proteome obtained from various concentrations of cells was run on 12.5% SDS-PAGE under reducing conditions. After electro blotting on to nitrocellulose membrane, it was probed with rabbit *anti-CRS* polyclonal antibody, which was further probed with alkaline phosphatase conjugated goat-*anti rabbit IgG* antibody. Probing with antibody was followed by developing the blot by dipping it in the substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 0.15 mg ml^{-1}), nitro blue tetrazolium (NBT, 0.30 mg ml^{-1}), Tris HCl (100 mM) and $MgCl_2$ (5 mM), pH 9.5 for 10 min. The expression of the CRS was determined by analyzing the band intensity by software Scion Image of the corresponding clone. The intensity was plotted against amount of the cells taken and the slope (dy/dx) for the intracellular expression and surface expression compared. The expression of CRS on the surface of the cells as omp-CRS fusion protein was found to be 17.9-fold less as compared to CRS expressed in the cytoplasm.

Example 8

Surface expressed CRS showed 275-fold increased activity compared to cytoplasmic CRS for reduction of ethyl 4-chloro-3-oxobutyrate

Fresh culture of recombinant *Escherichia coli BL21(DE3) + pET 23(a)-CRS* (CRS Sequence ID No 1) and *Escherichia coli BL21(DE3) + pET 23(a)-omp-CRS* (CRS Sequence ID No 1) were grown and used to determine the carbonyl reductase activity. The assay was done using ethyl 4-chloro-3-oxobutyrate as substrate and nicotinamide adenine dinucleotide phosphate, reduced (NADPH) as cofactor. The initial rate of carbonyl reductase activity was determined by monitoring the decrease in absorbance of nicotinamide adenine dinucleotide phosphate, reduced (NADPH) at λ_{340}. The standard reaction mixture (1 ml) in 50 mM phosphate buffer pH 7.0 contained 0.2 mM nicotinamide adenine dinucleotide phosphate, reduced (NADPH), 10^6-10^8 recombinant *Escherichia coli* cells and 2.0 mM ethyl (*S*)-4-chloro-3-oxobutanoate. One unit of activity was defined as the amount of dry cell weight that catalyzed the oxidation of 1 μmol nicotinamide adenine dinucleotide phosphate, reduced (NADPH) per minute under specified conditions. The activity of recombinant *Escherichia coli* BL21(DE3) +

pET 23(a)-omp-CRS (CRS Sequence ID No 1) expressing omp-CRS on the surface was determined to be 156.20 x 10^3 nmol/min/gm dry cell weight. In comparison recombinant *Escherichia coli* BL21(DE3) + *pET 23(a)-CRS* (CRS Sequence ID No 1) expressing CRS in cytoplasm showed activity of 10.16 x 10^3 nmol/min/gm dry cell weight. Thus, for equal amount of cells, the surface displayed CRS showed 15.4-fold higher activity. However, we have shown that for equal amount of cells, the recombinant *Escherichia coli* BL21(DE3) + *pET 23(a)-CRS* expressed 17.9-fold more protein as compared to recombinant *Escherichia coli* BL21 + *pET23(a)-omp-CRS*. Thus, activity per unit protein for recombinant *Escherichia coli* BL21(DE3) + *pET23(a)-omp-CRS* was 275-fold higher than recombinant *Escherichia coli* BL21(DE3) + *pET23(a)-omp-CRS*. The result has been summarized in Table 2.

Table 2: Relative activity of recombinant *Escherichia coli* expressing CRS (CRS Sequence ID No 1) on surface as omp-CRS fusion protein and recombinant *Escherichia coli* expressing CRS (CRS Sequence ID No 1) in cytoplasm.

Clone used for the experiment	**Activity (nmole/min/gm DCW*)**	**Relative concentration of CRS**	**Activity per unit of CRS**	**Fold increase in activity**
Escherichia coli Bl21(DE3) + *CRS-pET23(a)*	10.16 x10^3	17.9	0.57 x 10^3	1
Escherichia coli Bl21(DE3) + *omp-CRS-pET23(a)*	156.20 x10^3	1	156.20 x 10^3	275.19

* DCW = Dry cell weight.

Negative control *Escherichia coli* BL21(DE3) + *pET23(a)* did not given any activity.

Example 9

Surface expressed CRS showed 50-fold to 275-fold increased activity compared to cytoplasmic CRS for reduction of variant ketones

The assays were done in 96-well ELISA plate. The reaction mixture consisting of 0.2 mM nicotinamide adenine dinucleotide phosphate, reduced (NADPH), 2.0 mM substrate, 50µg/ml *Escherichia coli* cells (for calculation of cell mass, OD_{600} = 1 of cell suspension was taken as equivivalent to 0.25 mg/ml dry cell weight) in 50 mM phosphate buffer, pH 7.0 was incubated at 30 °C for about 30 min to 12 hr depending upon the consumption of the substrate. The consumption of nicotinamide adenine dinucleotide phosphate, reduced (NADPH) was monitored by decrease in the absorbance at λ_{340}. The results have been summarized in Table 3 and 4. As expected surface expressed CRS (CRS Sequence ID no 1) was much more efficient than

intracellularly expressed CRS (Sequence ID no 1) in reduction of a variety of aliphatic and aromatic ketones. In general, the increase in activity was in the range of 50-fold and 275-fold per unit CRS protein under these conditions. The relative concentration of CRS was determined as described in Example 7.

Table 3: Relative activity of recombinant *Escherichia coli* expressing CRS (Sequence ID No 1) on surface as omp-CRS fusion protein and recombinant *Escherichia coli* expressing CRS (Sequence ID No 1) in cytoplasm for variant ketones[a].

Entry	Substrate	*Escherichia coli* BL21 + *pET23(a)-CRS* (nmol/min/ gm DCW*)	*Escherichia coli* BL21 + *pET23(a)-omp-CRS* (nmol/min/ gm DCW*)	Fold increase in activity per unit DCW*	Fold increase in activity per unit protein#
1	Ethyl 4-chloro-3-oxobutyrate	10.16×10^3	156.20×10^3	15.37	275.19
2	Ethyl 2-chloro-3-oxobutyrate	30.06×10^3	161.48×10^3	5.37	96.12
3	Ethyl -3-oxobutyrate	3.60×10^3	12.01×10^3	3.34	59.80
4	Ethyl 4-methyl-3-oxopentanoate	3.52×10^3	43.12×10^3	12.25	219.27
5	Octyl 4-chloro-3-oxobutanoate	1.72×10^3	10.12×10^3	5.88	105.25
6	ethyl 4,4,4-trifluoro-3-oxobutyate	1.94×10^3	5.54×10^3	2.86	51.19
8	Acetophenone	1.54×10^3	5.19×10^3	3.37	60.3
9	4-Chloroacetophenone	3.43×10^3	12.28×10^3	3.58	64.09
10	4-Bromoacetophenone	3.79×10^3	18.48×10^3	4.88	87.28
11	4-Fluoroacetophenone	2.56×10^3	22.88×10^3	8.94	159.98
12	4-Methylacetophenone	1.94×10^3	5.45×10^3	2.82	50.29
13	4-Methoxyacetophenone	1.06×10^3	3.60×10^3	3.40	60.79
14	4-trifluoromethylacetophenone	3.0×10^3	30.10×10^3	10.03	179.60
15	4-Nitroacetophenone	6.16×10^3	49.63×10^3	8.06	142.22

[a] External GDH was added to assay mixture for cofactor recycle

* DCW = Dry cell weight

The CRS expression per g DCW was 17.9-fold lower for surface expressed CRS compared to cytoplasmically expressed CRS (Example 7).

Table 4: Relative activity of recombinant *Escherichia coli* co-expressing CRS (Sequence ID No 1) and GDH (Sequence ID No 9) on surface as omp-CRS fusion protein and omp-GDH protein and recombinant *Escherichia coli* co-expressing CRS (Sequence ID No 1) and GDH (Sequence ID No 1) in cytoplasm for variant ketones.

Entry	Substrate	*Escherichia coli C41(DE3) + pETDuet1-CRS, GDH* (nmol/min/ gm DCW*)	*Escherichia coli C41(DE3) + pETDuet1-omp-CRS, omp-GDH* (nmol/min/ gm DCW*)	Fold increase in activity per unit DCW*	Fold increase in activity per unit protein#
1	Ethyl 4-chloro-3-oxobutyrate	$14.42x10^3$	$217.36 x10^3$	15.08	269.87
2	Ethyl 2-chloro-3-oxobutyrate	$37.26 x10^3$	$165.88 x10^3$	4.45	79.69
3	Ethyl -3-oxobutyrate	$4.03 x10^3$	$12.76 x10^3$	3.17	56.74
4	Ethyl 4-methyl-3-oxopentanoate	$4.06 x10^3$	$53.02 x10^3$	13.06	233.90
5	Octyl 4-chloro-3-oxobutanoate	$1.65 x10^3$	$9.50 x10^3$	5.74	102.78
6	ethyl 4,4,4-trifluoro-3-oxobutyate	$1.65 x10^3$	$5.48 x10^3$	3.31	59.33
7	Acetophenone	$2.53 x10^3$	$5.79 x10^3$	2.29	41.03
8	4-Chloroacetophenone	$3.26 x10^3$	$11.07 x10^3$	3.40	60.78
9	4-Bromoacetophenone	$2.44 x10^3$	$19.54 x10^3$	8.0	143.26
10	4-Fluoroacetophenone	$2.96 x10^3$	$22.66 x10^3$	7.64	136.82
11	4-Methylacetophenone	$1.97 x10^3$	$6.53 x10^3$	3.31	59.19
12	4-Methoxyacetophenone	$1.22 x10^3$	$3.32x10^3$	2.73	48.81
13	4-trifluoromethylacetophenone	$3.37 x10^3$	$29.37 x10^3$	8.71	155.99
14	4-Nitroacetophenone	$7.07 x10^3$	$46.31 x10^3$	6.55	117.28

* DCW = Dry cell weight

The CRS expression per g DCW was 17.9-fold lower for surface expressed CRS compared to cytoplasmically expressed CRS.

Example 10

Demonstration of the expression of glucose dehydrogenase on the surface of transformant *Escherichia coli C41(DE3) + pETDuet1-omp-CRS, omp-GDH* (CRS, Sequence ID No 1; GDH, Sequence ID No 9)

The transformant *Escherichia coli* C41(DE3) + *pETDuet1-omp-CRS; omp-GDH* (CRS, Sequence ID No 1; GDH, Sequence ID No 9) was grown as described in example 5 above, in 100 ml culture media. The cells were isolated by centrifugation and washed with 50 mM phosphate buffer (pH 7.0). The cells were then suspended in 5 ml lysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, 1 mg ml^{-1} Lysozyme, pH 8.0) for 30 min at 4 °C. The cell suspension was then sonicated with 30 sec pulse on and 30 sec pulse off at 4 °C for 20 min. The cell debris was removed by centrifugation at 14000 rpm for 30 min and supernatant (cell free extract) was taken for further analysis. Membrane fraction and soluble protein fraction from the supernatant (cell-free extract) were separated as described earlier. All the three fractions, cell-free extract, membrane fraction and soluble protein fraction were assayed for their ability to oxidize glucose to gluconate, like the standard reaction mixture (1 ml) in 100 mM tris-HCl buffer, pH 8.0, containing 0.5 mM nicotinamide adenine dinucleotide phosphate (NADP) (50µl of 7.78 mg ml^{-1}), 5 mM glucose (25 µl of 180 mg ml^{-1}) and 1-50 µl of the sample was incubated at 30 °C and the activity was determined by monitoring the formation of nicotinamide adenine dinucleotide phosphate, reduced (NADPH) at λ_{340}. The results have been summarized in Table 5. As expected most of the activity was recovered from membrane fraction. The membrane fraction of *Escherichia coli* C41(DE3) + *pETDuet1* (negative control) was devoid of any activity.

Table 5: Glucose dehydrogenase (GDH) activity of various fractions from *Escherichia coli* C41(DE3) + *pETDuet1-omp-CRS, omp-GDH* (CRS, Sequence ID No 1; GDH, Sequence ID No 9) and *Escherichia coli* C41(DE3) + *pETDuet1*

Entry	**Fraction of *Escherichia coli***	***Escherichia coli* C41(DE3) + pETDuet1 Total activity (nmol/min)**	***Escherichia coli* C41(DE3) + pETDuet1-omp-CRS, omp-GDH Total activity (nmol/min)**
1	Cell-free extract	632.26	6275.02
2	Soluble fraction	438.71	1055.72
3	Membrane fraction	0.0	4290.32

Example 11

Relative expression levels for cytoplasmic and surface expressed GDH (Sequence ID No 9) in *Escherichia coli* C41(DE3)

Glucose dehydrogenase activity of recombinant *Escherichia coli* C41(DE3) + *pET 29(a)-CRS* + *pET 29(a)-GDH* (CRS, Sequence ID No 1; GDH, Sequence ID No 9) and *Escherichia coli* C41(DE3) + *pETDuet1-omp-CRS, omp-GDH* (CRS, Sequence ID No 1; GDH, Sequence ID No 9) was determined by monitoring the increase in the absorbance of nicotinamide adenine dinucleotide phosphate, reduced (NADPH) at 340 nm spectrophotometrically using glucose as a substrate. The reaction mixture (1 ml) in 50 mM Tris-HCl buffer pH 8.0, contained 0.5 mM nicotinamide adenine dinucleotide phosphate $(NADP)^+$, 10^6 to 10^8 cells and 5.0 mM glucose. One unit of activity was defined as the amount of cells (dry cell weight) that catalyzed the reduction of 1 µmol NADP per minute under these conditions. Recombinant *Escherichia coli* expressing GDH on the surface of the cells as omp-GDH fusion protein showed activity of 683.52 x 10^3 nmol/min/gm dry cell weight, whereas recombinant *Escherichia coli* expressing GDH in the cytoplasm showed activity of 41.87 x 10^3 nmol/min/gm dry cell weight. The relative expression level of GDH expressed on the surface and in cytoplasm was estimated from SDS PAGE. The cytoplasmic expression was estimated to be about 13.8-fold higher compared to surface expressed protein of recombinant *Escherichia coli Escherichia coli* C41(DE3) + *pETDuet1-omp-CRS, omp-GDH* was 225-fold higher compared to recombinant *Escherichia coli* C41(DE3) + *pET 29(a)-CRS* + *pET 29(a)-GDH.* The results are summarized in Table 6.

Table 6: Relative activity of recombinant *Escherichia coli* expressing GDH on surface as omp-GDH (Sequence ID No 9) fusion protein and recombinant *Escherichia coli* expressing GD (Sequence ID No 9). in cytoplasm

Clone used for the experiment	**Activity (nmole/min/gm DCW*)**	**Relative concentration of GDH**	**Activity per unit of GDH**	**Fold increase in activity**
Escherichia coli C41(DE3) + *pET 29(a)-CRS* + *pET 29(a)-GDH*	41.87 x10^3	13.8	3.03 x 10^3	1
Escherichia coli C41(DE3) + *pETDuet1-omp-CRS, omp-GDH*	683.52 x10^3	1	683.52 x 10^3	225.58

* DCW = Dry cell weight

Negative control *Escherichia coli* BL21(DE3) + *pET29(a)* did not given any activity

Example 12

Production of ethyl (*S*)-4-chloro-3-hydroxybutyrate using transformant *Escherichia coli* BL21(DE3) + *pET 23(a)-omp-CRS* (CRS, Sequence ID No 1) or C41(DE3) + *pET 23(a)-omp-CRS* (CRS, Sequence ID No 1) as biocatalyst

The reaction was done in 2-phase system using dibutyl ether as co-solvent in an autotitrator (718 STAT Titrino, Metrohm, Switzerland). *Escherichia coli* BL21(DE3) + *pET 23(a)-omp-CRS* cells or C41(DE3) + *pET 23(a)-omp-CRS* (1.38 g; dry cell weight basis) were suspended in 20 ml phosphate buffer, 100 mM, pH 6.5. Glucose (9 g, 50 mmol), glucose dehydrogenase (3600 U), nicotinamide adenine dinucleotide phosphate (NADP) (18 mg, 0.02 mmol) and ECOB (6 g, 36.5 mmol) in 15 ml dibutyl ether were added to the suspension and contents gently stirred on magnetic stirrer at 30 °C. The pH of the reaction was maintained at 6.5 with 5 M NaOH. The progress of the reaction was monitored at one hr interval as follows. An aliquots of 0.2 ml each was withdrawn and extracted with 2 ml of ethyl acetate. The organic phase was separated by centrifugation, dried over sodium sulphate and solvents removed to leave a residue, which was analyzed by ^{1}H NMR. In ^{1}H NMR methylene group corresponding to CH_2Cl in ECOB appeared as a singlet at δ 3.65, whereas it appeared as dd at 3.60 in reduced product ECHB. Since, these peaks appear in well resolved segment of ^{1}H NMR, their relative concentrations in the mixture of two compounds can be easily calculated by comparing the relative integral values of the resonance at δ 3.60 and 3.65.

When the reaction mixture showed absence of the starting material, contents were extracted in ethyl acetate. The organic phase was separated by centrifugation, dried over sodium sulphate and solvents removed to give ethyl (*S*)-4-chloro-3-hydroxybutyrate. The optical purity of ethyl (S)-4-chloro-3-hydroxybutyrate is measured by high performance liquid chromatography by using Chiracel OB-H, product of Daicel Chemical Industries; eluent: hexane/isopropanol =96/4; flow rate: 1 ml/min; detection: 217 nm; column temperature: room temperature.

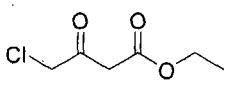
Example 13

Production of ethyl (*S*)-4-chloro-3-hydroxybutyrate using transformant *Escherichia coli* BL21(DE3) + *pET Duet1-omp-CRS, omp-GDH* (CRS, Sequence ID No 1; GDH, Sequence ID No 9) or *Escherichia coli* C41(DE3) + *pET Duet1-omp-CRS, omp-GDH* (CRS, Sequence ID No 1; GDH, Sequence ID No 9) as biocatalyst

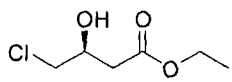
The production method was same as described in Example 12 except that no glucose dehydrogenase was added to the reaction.

We claim:

1. A designer cell that expresses a non-naturally occurring carbonyl reductase polypeptide of sequence selected from SEQ ID NO: 1, 3, 5 or 7 of the Sequence Listing on the surface of cell having 250-fold to 300-fold higher activity per unit mass of CRS polypeptide compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 or 15 or 17 or 19 of the sequence listing in cytoplasm of cell for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2.



Formula 1

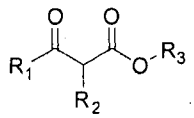


Formula 2

2. A designer cell as claimed in claim 1 that expresses a non-naturally occurring carbonyl reductase polypeptide SEQ ID NO: 1 of the Sequence Listing on the surface of cell having about 275-fold higher activity per unit mass of CRS polypeptide compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 of the sequence listing in cytoplasm of cell for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2.
3. A designer cell as claimed in claim 1 that expresses a non-naturally occurring carbonyl reductase polypeptide of sequence selected from SEQ ID NO: 1, 3, 5 or 7 of the Sequence Listing on the surface of cell having 15-fold to 26-fold higher activity per unit cell mass compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 or 15 or 17 or 19 of the Sequence Listing in cytoplasm of cell for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2.
4. A designer cell as claimed in claim 1 that expresses a non-naturally occurring carbonyl reductase polypeptide of SEQ ID NO: 1 of the Sequence Listing on the surface of cell having at least 15-fold higher activity per unit cell mass compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13

of the sequence listing in cytoplasm of cell for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2.

5. A designer cell as claimed in claim 1 that expresses a non-naturally occurring carbonyl reductase polypeptide of SEQ ID NO: 1 of the Sequence Listing on the surface of cell having 50-fold to 275-fold higher activity per unit mass of CRS polypeptide compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 of the Sequence Listing in cytoplasm of cell for reduction of compound of formula 3



Formula 3

wherein $R_1 = CH_3$, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$

R_2 = H, X or $CH_3(CH_2)_n$;

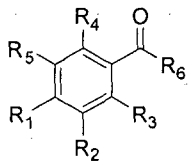
R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8;

6. A designer cell as claimed in claim 1 that expresses a non-naturally occurring carbonyl reductase polypeptide of SEQ ID NO: 1 of the Sequence Listing on the surface of cell having 50-fold to 180-fold higher activity per unit mass of CRS polypeptide compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 of the Sequence Listing in cytoplasm of cell for reduction of compound of formula 4



Formula 4

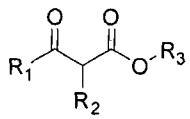
wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

7. A designer cell as claimed in claim 1 that expresses a non-naturally occurring carbonyl reductase polypeptide of SEQ ID NO: 1 of the Sequence Listing on the

surface of cell having about 3-fold to 15-fold higher activity per unit cell mass compared to the designer cell that expresses corresponding carbonyl reductase of SEQ ID NO: 13 in cytoplasm of cell for reduction of compound of formula 3 or compound of formula 4



Formula 3

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$

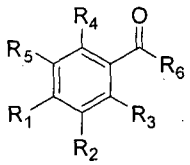
R_2 = H, X or $CH_3(CH_2)_n$;

R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8;



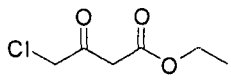
Formula 4

wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

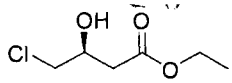
R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

8. A designer cell that simultaneously expresses a non-naturally occurring CRS polypeptide of sequence selected from SEQ ID NO: 1, 3, 5 or 7 and a non-naturally occurring GDH polypeptide of sequence selected from SEQ ID NO: 9 or 11 of the Sequence Listing on the surface of cell that has 250-fold to 300-fold higher activity for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 per unit mass of CRS polypeptide and 200-fold to 250-fold enhanced activity for oxidation of glucose to gluconate per unit mass of GDH polypeptide compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 or 15 or 17 or 19 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 or 23 of the Sequence Listing in cytoplasm of cell.



Formula 1



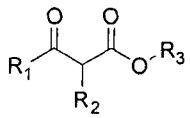
Formula 2

9. A designer cell as claimed in claim 8 that simultaneously expresses a non-naturally occurring CRS polypeptide SEQ ID NO: 1 and a non-naturally occurring GDH polypeptide SEQ ID NO: 9 of the Sequence Listing on the surface of cell that has about 270-fold higher activity for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 per unit mass of CRS polypeptide and about 225-fold enhanced activity for oxidation of glucose to gluconate per unit mass of GDH polypeptide compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 of the Sequence Listing in cytoplasm of cell.

10. A designer cell as claimed in claim 8 that simultaneously expresses a non-naturally occurring CRS polypeptide of sequence selected from SEQ ID NO: 1, 3, 5 or 7 and a non-naturally occurring GDH polypeptide of sequence selected from SEQ ID NO: 9, 11, 13 or 15 of the Sequence Listing on the surface of cell that has about 11-fold to 24-fold higher activity for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 per unit cell mass and 9-fold to 31-fold enhanced activity for oxidation of glucose to gluconate per unit cell mass compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 or 15 or 17 or 19 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 or 23 of the Sequence Listing in cytoplasm of cell.

11. A designer cell as claimed in claim 8 that simultaneously expresses a non-naturally occurring CRS polypeptide of SEQ ID NO: 1 and a non-naturally occurring GDH polypeptide of SEQ ID NO: 9 of the Sequence Listing on the surface of cell that has about 15-fold higher activity for conversion of ethyl 4-chloro-3-oxobutyrate of formula 1 to ethyl 4-chloro-3-hydroxybutyrate of formula 2 per unit cell mass and about 16-fold enhanced activity for oxidation of glucose to gluconate per unit cell mass compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 of the Sequence Listing in cytoplasm of cell.

12. A designer cell as claimed in claim 8 that simultaneously expresses a non-naturally occurring CRS polypeptide of SEQ ID NO: 1 and a non-naturally occurring GDH polypeptide of SEQ ID NO: 9 of the Sequence Listing on the surface of cell having about 55-fold to 270-fold higher activity per unit mass of CRS polypeptide compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 of the Sequence Listing in cytoplasm of cell for reduction of compound of formula 3



Formula 3

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$

R_2 = H, X or $CH_3(CH_2)_n$;

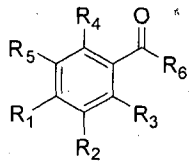
R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8

13. A designer cell as claimed in claim 8 that simultaneously expresses a non-naturally occurring CRS polypeptide of SEQ ID NO: 1 and a non-naturally occurring GDH polypeptide of SEQ ID NO: 9 of the Sequence Listing on the surface of cell having about 40-fold to 156-fold higher activity per unit mass of CRS polypeptide compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 of the Sequence Listing in cytoplasm of cell for reduction of compound of formula 4



Formula 4

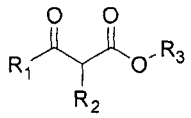
wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

14. A designer cell as claimed in claim 8 that simultaneously expresses a non-naturally occurring CRS polypeptide of SEQ ID NO: 1 and a non-naturally occurring GDH polypeptide of SEQ ID NO: 9 of the Sequence Listing on the surface of cell having

about 2-fold to 15-fold higher activity per unit cell mass compared to the designer cell that simultaneously expresses corresponding CRS of SEQ ID NO: 13 of the Sequence Listing and corresponding GDH of SEQ ID NO: 21 of the Sequence Listing in cytoplasm of cell for reduction of compound of compound of formula 3 or compound of formula 4



Formula 3

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$;

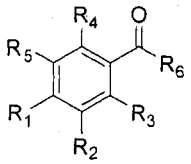
R_2 = H, X or $CH_3(CH_2)_n$;

R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8



Formula 4

wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

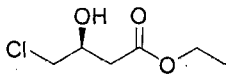
n= 1 to 5.

15. A designer cell as claimed in claim 1 to 14, wherein designer cell is recombinant *Escherichia coli* BL21(DE3) or recombinant *Escherichia coli* C41(DE3) or recombinant *Escherichia coli* C43(DE3).

16. A recombinant expression vector comprising polynucleotide encoding the polypeptide having the amino acid sequence of SEQ ID NO 1 or 3 or 5 or 7 showing carbonyl reductase activity.

17. A recombinant expression vector comprising polynucleotides encoding the polypeptide having the amino acid sequence of SEQ ID NO 1 or 3 or 5 or 7 showing carbonyl reductase activity and the polypeptide having the amino acid sequence of SEQ ID NO 9 or 11 showing GDH activity.

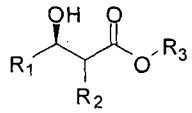
18. Use of the designer cell as claimed in claim 1 for the production of ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 in about 100% enantiomeric excess



Formula 2

19. Use of the designer cell as claimed in claim 8 for the production of ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 in about 100% enantiomeric excess

20. Use of the designer cell as claimed in claim 1 for the production of compound of formula 5



Formula 5

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$;

R_2 = H, X or $CH_3(CH_2)_n$;

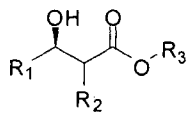
R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8

21. Use of the designer cell as claimed in claim 8 for the production of compound of formula 5



Formula 5

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$;

R_2 = H, X or $CH_3(CH_2)_n$;

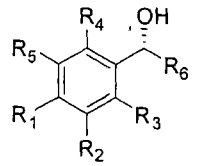
R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8

22. Use of the designer cell as claimed in claim 1 for the production of compound of formula 6



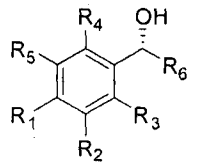
Formula 6

wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

23. Use of designer cell as claimed in claim 8 for the production of compound of formula 6



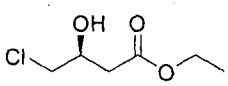
Formula 6

wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

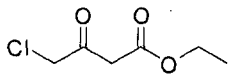
24. A process for production of ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 in about 100% enantiomeric excess,



Formula 2

and the said process comprising the steps of;

f. providing ethyl 4-chloro-3-oxobutyrate of formula 1;



Formula 1

g. contacting the ethyl 4-chloro-3-oxobutyrate with designer cell as claimed in claim 1 and 0.005 to 0.02 mol % nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and 100 to 500 units glucose dehydrogenase and a buffer solution of pH 5.0 to 9.0;

h. adding to the reaction mixture obtained in step (b) an organic solvent such as ethyl acetate, butyl acetate, diethylether, methyl n-butyl ether or di-n-butyl ether in the ratio ranging between 10:1 to 1:1;

i. energetically mixing the reaction mixture on magnetic stirrer at constant temperature of 20 to 40 °C;

j. extracting the product obtained in step (d) in ethyl acetate followed by isolating the product ethyl (*S*)-4-chloro-3-hydroxybutyrate.

25. The process as claimed in claim 24 wherein pH is preferably 6.5.

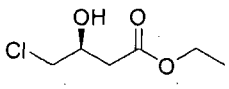
26. The process as claimed in claim 24 wherein temperature is preferably 30 °C.

27. The process as claimed in claim 24 wherein organic solvent is preferably di-n-butyl ether.

28. The process as claimed in claim 24 wherein designer cell is a strain of recombinant *Escherichia coli* selected from recombinant *Escherichia coli* BL21(DE3), recombinant *Escherichia coli* C41(DE3) and recombinant *Escherichia coli* C43(DE3).

29. The process as claimed in claim 24 wherein designer cell is preferably recombinant *Escherichia coli* C41(DE3).

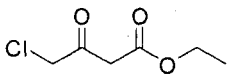
30. A process for production of ethyl (*S*)-4-chloro-3-hydroxybutyrate of formula 2 in about 100% enantiomeric excess



Formula 2

and the said process comprising of steps of;

a. providing ethyl 4-chloro-3-oxobutyrate;



Formula 1

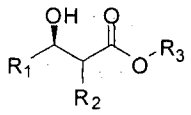
b. contacting the ethyl 4-chloro-3-oxobutyrate with designer cell as claimed in claim 8 and 0.005 to 0.02 mol % nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and a buffer solution of pH 5.0 to 9.0;

c. adding to the reaction mixture obtained in step (b) an organic solvent such as ethyl acetate, butyl acetate, diethylether, methyl n-butyl ether or di-n-butyl ether in the ratio ranging between 10:1 to 1:1;

d. energetically mixing the reaction mixture on magnetic stirrer at constant temperature of 20 to 40 °C;

e. extracting the product obtained in step (d) in ethyl acetate followed by isolating the product ethyl (*S*)-4-chloro-3-hydroxybutyrate.

31. The process as claimed in claim 30 wherein pH is preferably 6.5.
32. The process as claimed in claim 30 wherein temperature is preferably 30 °C.
33. The process as claimed in claim 30 wherein organic solvent is preferably di-n-butyl ether.
34. The process as claimed in claim 30 wherein designer cell is a strain of recombinant *Escherichia coli* selected from recombinant *Escherichia coli* BL21(DE3), recombinant *Escherichia coli* C41(DE3) and recombinant *Escherichia coli* C43(DE3).
35. The process as claimed in claim 30 wherein designer cell is recombinant *Escherichia coli* BL21(DE3).
36. The process as claimed in claim 30 wherein designer cell is preferably recombinant *Escherichia coli* C41(DE3).
37. A process for the production of optically enriched aliphatic alcohols of formula 5



Formula 5

wherein $R_1 = CH_3$, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$;

R_2 = H, X or $CH_3(CH_2)_n$;

R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

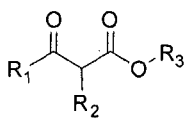
X = Cl or Br;

n = 1-4 and

m = 1-8

and the said process comprising the steps of;

f. providing a ketone of formula 3



Formula 3

wherein $R_1 = CH_3$, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$;

R_2 = H, X or $CH_3(CH_2)_n$;

R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8

g. contacting the ketone of formula 3 as provided in step (a) with designer cell as claimed in claim 1 and 0.005 to 0.02 mol % nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and 100 to 500 units glucose dehydrogenase and buffer solution of pH 5.0 to 9.0 to form the reaction mixture;

h. adding to the reaction mixture obtained in step (b) an organic solvent such as ethyl acetate, butyl acetate, diethylether, methyl n-butyl ether or di-n-butyl ether in the ratio ranging between 10:1 to 1:1;

i. energetically mixing the reaction mixture on magnetic stirrer at constant temperature of 20 to 40 °C;

j. extracting the product obtained in step (d) in ethyl acetate followed by isolating the compound of formula 5.

38. The process as claimed in claim 37 wherein pH is preferably 6.5.

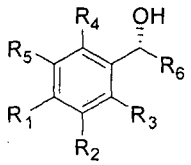
39. The process as claimed in claim 37 wherein temperature is preferably 30 °C.

40. The process as claimed in claim 37 wherein organic solvent is preferably di-n-butyl ether.

41. The process as claimed in claim 37 wherein designer cell is a strain of recombinant *Escherichia coli* selected from recombinant *Escherichia coli* BL21(DE3), recombinant *Escherichia coli* C41(DE3) and recombinant *Escherichia coli* C43(DE3).

42. The process as claimed in claim 37 wherein designer e cell is preferably recombinant *Escherichia coli* C41(DE3).

43. A process for production of optically enriched aryl alcohols of formula 6



Formula 6

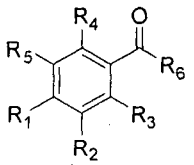
wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

and the said process comprising the steps of;

f. providing a ketone of formula 4



Formula 4

Wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5.

g. contacting the ketone of formula 3 as provided in step (a) with designer cell as claimed in claim 1 and 0.005 to 0.02 mol % nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and 100 to 500 units glucose dehydrogenase and buffer solution of pH 5.0 to 9.0 to form the reaction mixture;

h. adding to the reaction mixture obtained in step (b) an organic solvent such as ethyl acetate, butyl acetate, diethylether, methyl n-butyl ether or di-n-butyl ether in the ratio ranging between 10:1 to 1:1;

i. energetically mixing the reaction mixture on magnetic stirrer at constant temperature of 20 to 40 °C;

j. extracting the product obtained in step (d) in ethyl acetate followed by isolating the compound of formula 6.

44. The process as claimed in claim 43 wherein pH is preferably 6.5.

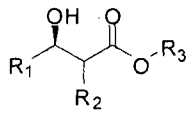
45. The process as claimed in claim 43 wherein temperature is preferably 30 °C.

46. The process as claimed in claim 43 wherein organic solvent is preferably di-n-butyl ether.

47. The process as claimed in claim 43 wherein designer cell is a strain of recombinant *Escherichia coli* selected from recombinant *Escherichia coli* BL21(DE3), recombinant *Escherichia coli* C41(DE3) and recombinant *Escherichia coli* C43(DE3).

48. The process as claimed in claim 43 wherein designer cell is preferably recombinant *Escherichia coli* C41(DE3).

49. A process for the production of optically enriched aliphatic alcohols of formula 5



Formula 5

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$;

R_2 = H, X or $CH_3(CH_2)_n$;

R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

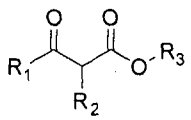
X = Cl or Br;

n = 1-4 and

m = 1-8;

and the said process comprising the steps of;

a. providing a ketone of formula 3



Formula 3

wherein R_1 = CH_3, CH_2X, $(CH_3)_2CH$, CF_3 or $CH_3(CH_2)_n$;

R_2 = H, X or $CH_3(CH_2)_n$;

R_3 = alkyl group such as CH_3 or $CH_3(CH_2)_m$;

X = Cl or Br;

n = 1-4 and

m = 1-8;

b. contacting the ketone of formula 3 as provided in step (a) with designer cell as claimed in claim 8 and 0.005 to 0.02 mol % nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and buffer solution of pH 5.0 to 9.0 to form the reaction mixture;

c. adding to the reaction mixture obtained in step (b) an organic solvent such as ethyl acetate, butyl acetate, diethylether, methyl n-butyl ether or di-n-butyl ether in the ratio ranging between 10:1 to 1:1;

d. energetically mixing the reaction mixture on magnetic stirrer at constant temperature of 20 to 40 °C;

e. extracting the product obtained in step (d) in ethyl acetate followed by isolating the compound of formula 5.

50. The process as claimed in claim 49 wherein pH is preferably 6.5.

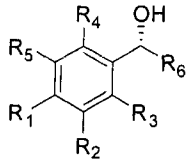
51. The process as claimed in claim 49 wherein temperature is preferably 30 °C.

52. The process as claimed in claim 49 wherein organic solvent is preferably di-n-butyl ether.

53. The process as claimed in claim 49 wherein designer cell is a strain of recombinant *Escherichia coli* selected from recombinant *Escherichia coli* BL21(DE3), recombinant *Escherichia coli* C41(DE3) and recombinant *Escherichia coli* C43(DE3).

54. The process as claimed in claim 49 wherein designer cell is preferably recombinant *Escherichia coli* C41 (DE3).

55. A process for production of optically enriched aryl alcohols of formula 6



Formula 6

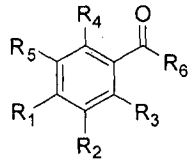
wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5;

and the said process comprising the steps of;

a. providing a ketone of formula 4



Formula 4

wherein R_1=R_2=R_3=R_4=R_5=H, CH_3, F, Cl, Br, I, CF_3, NO_2 or OCH_3;

R_6= alkyl group such as CH_3 or $CH_3(CH_2)_n$;

n= 1 to 5;

b. contacting the ketone of formula 3 as provided in step (a) with designer cell as claimed in claim 8 and 0.005 to 0.02 mol % nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and buffer solution of pH 5.0 to 9.0 to form the reaction mixture;

c. adding to the reaction mixture obtained in step (b) an organic solvent such as ethyl acetate, butyl acetate, diethylether, methyl n-butyl ether or di-n-butyl ether in the ratio ranging between 10:1 to 1:1;

d. energetically mixing the reaction mixture on magnetic stirrer at constant temperature of 20 to 40 °C;

e. extracting the product obtained in step (d) in ethyl acetate followed by isolating the compound of formula 6.

56. The process as claimed in claim 55 wherein pH is preferably 6.5.

57. The process as claimed in claim 55 wherein temperature is preferably 30 °C.

58. The process as claimed in claim 55 wherein organic solvent is preferably di-n-butyl ether.

59. The process as claimed in claim 55 wherein designer cell is a strain of recombinant *Escherichia coli* selected from recombinant *Escherichia coli* BL21(DE3), recombinant *Escherichia coli* C41(DE3) and recombinant *Escherichia coli* C43(DE3).

60. The process as claimed in claim 55 wherein designer cell is preferably recombinant *Escherichia coli* C41 (DE3).

61. Designer cells having Accession No. MTCC No.5806, MTCC No. 5807, MTCC No. 5808 and MTCC No. 5809.

62. The designer cells as claimed in claim 61, wherein the designer cell having Accession Nos. MTCC 5806-5809 expresses amino acid sequences having SEQ ID No. 1, 3, 5 and 7.

63. The designer cells as claimed in claims 61-62, wherein the amino acid sequences having SEQ ID Nos. 1, 3, 5 and 7 correspond to nucleotide sequences having SEQ ID No. 2, 4, 6, 8.

64. The designer cells as claimed in claims 61-63, wherein the SEQ ID Nos. 1-8, are non-naturally occurring sequences of carbony reductase enzyme.

65. The designer cells as claimed in claims 61-64, capable of expressing high activity of carbonyl reductase on the cell surface.

66. A recombinant vector construct comprising and capable of expressing SEQ ID Nos. 1-8.

67. The recombinant vector construct as claimed in claim 66, comprising non-naturally occurring sequences of carbony reductase enzyme.

68. The recombinant vector construct as claimed in claim 66-67, capable of expressing high activity of carbonyl reductase on the cell surface.

69. Designer cells having Accession No. MTCC No.5810, MTCC No. 5811, MTCC No. 5812, MTCC No. 5813; MTCC No. 5814. MTCC No. 5815, MTCC No. 5816 and MTCC No. 5817.

70. The designer cells as claimed in claim 69, wherein designer cells having accession no. having MTCC No.5810 expressig amino acid sequences having SEQ ID NO. 1 and SEQ ID NO.9; wherein designer cells having accession no. having MTCC No.5811 expressig amino acid sequences having SEQ ID NO. 1 and SEQ ID NO.11; wherein designer cells having accession no. having MTCC No.5812 expressig amino acid sequences having SEQ ID NO. 3 and SEQ ID NO. 9; wherein designer cells having accession no. having MTCC No.5813 expressig amino acid sequences having SEQ ID NO. 3 and SEQ ID NO.11; wherein designer cells having accession no. having MTCC No.5814 expressig amino acid sequences having SEQ ID NO. 5 and SEQ ID NO.9; wherein designer cells having accession no. having MTCC No.5815 expressig amino acid sequences having SEQ ID NO. 5 and SEQ ID NO.11; wherein designer cells having accession no. having MTCC No.5816 expressig amino acid sequences

having SEQ ID NO. 7 and SEQ ID NO.9; wherein designer cells having accession no. having MTCC No.5817 expressig amino acid sequences having SEQ ID NO. 7 and SEQ ID NO.11.

71. The designer cells as claimed in claims 69-70, wherein the amino acid sequences having SEQ ID Nos. 1, 3, 5, 7, 9 and 11 correspond to nucleotide sequences having SEQ ID Nos. 2, 4, 6, 8, 10 and 12.

72. The designer cells as claimed in claims 65-67, capable of co-expressing enzyme carbonyl reductase and gluconase dehydrogenase together on cell surface.

73. A recombinant vector comprising and capable of expressing sequences as claimed in claim 71.

74. The recombinant vector as claimed in claim 73, capable of co-expressing enzyme carbonyl reductase and gluconase dehydrogenase together on cell surface.

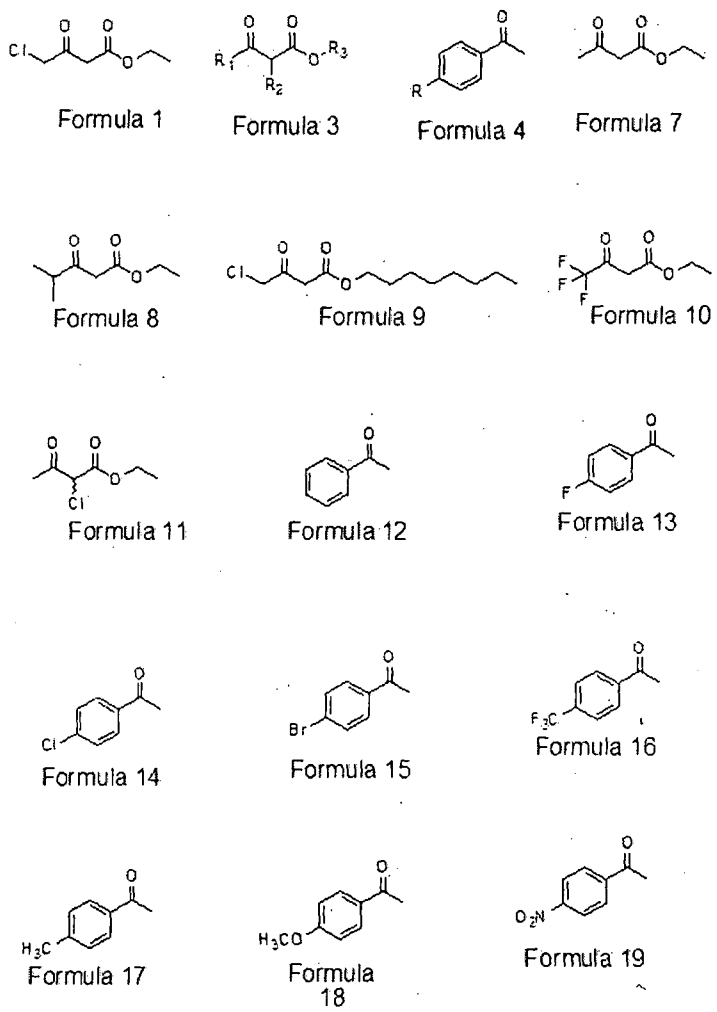


Figure 1: Examples of aliphatic and aromatic compounds serving as substrates

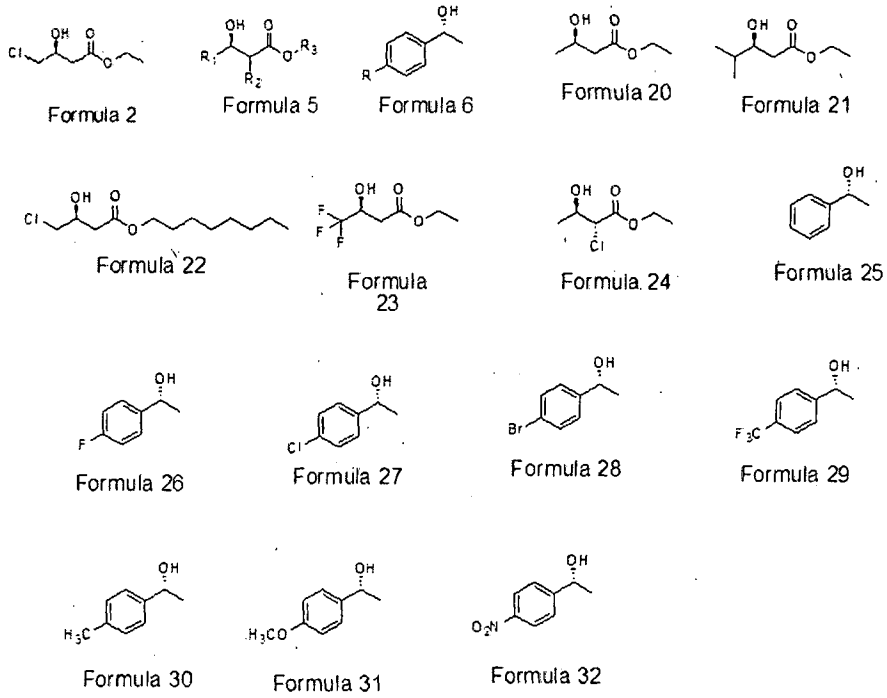


Figure 2: Examples of compounds serving as products

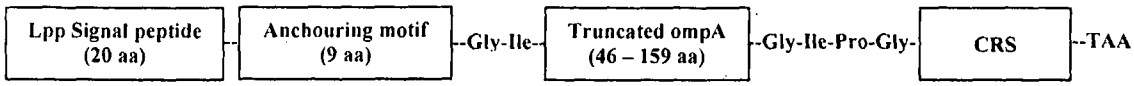


Figure 3: Schematic representation of 'CRS polypeptide' corresponding to SEQ ID No.1, 3, 5 or 7.

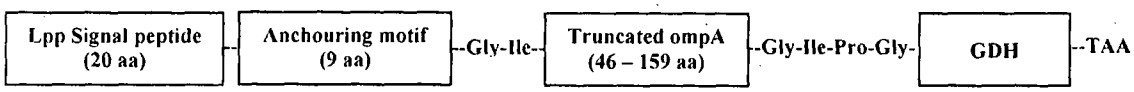


Figure 4: Schematic representation of 'CRS polypeptide' corresponding to SEQ ID No.9, or 11.

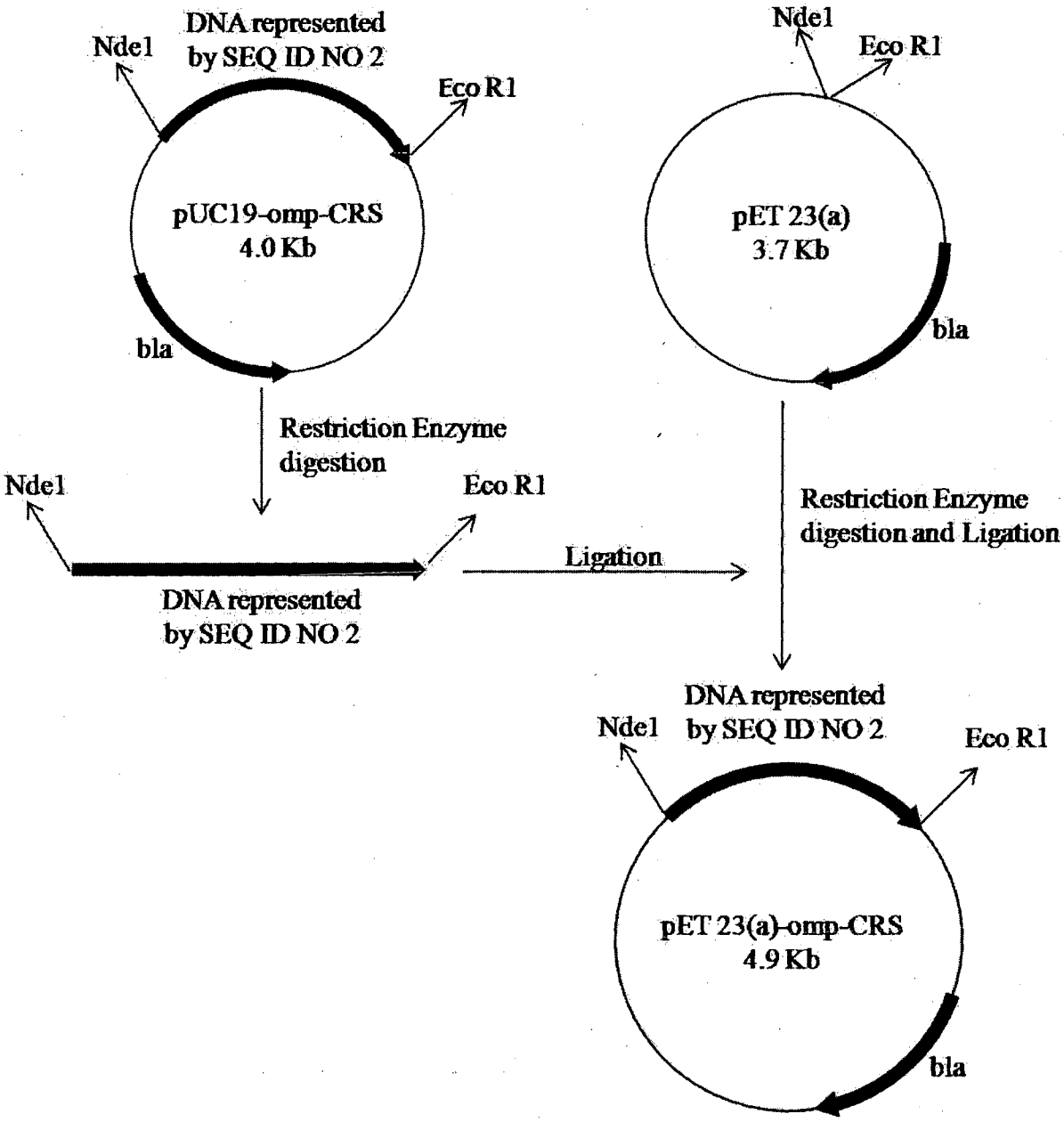


Figure 5: Recombinant Vector pET239a)-omp-CRS

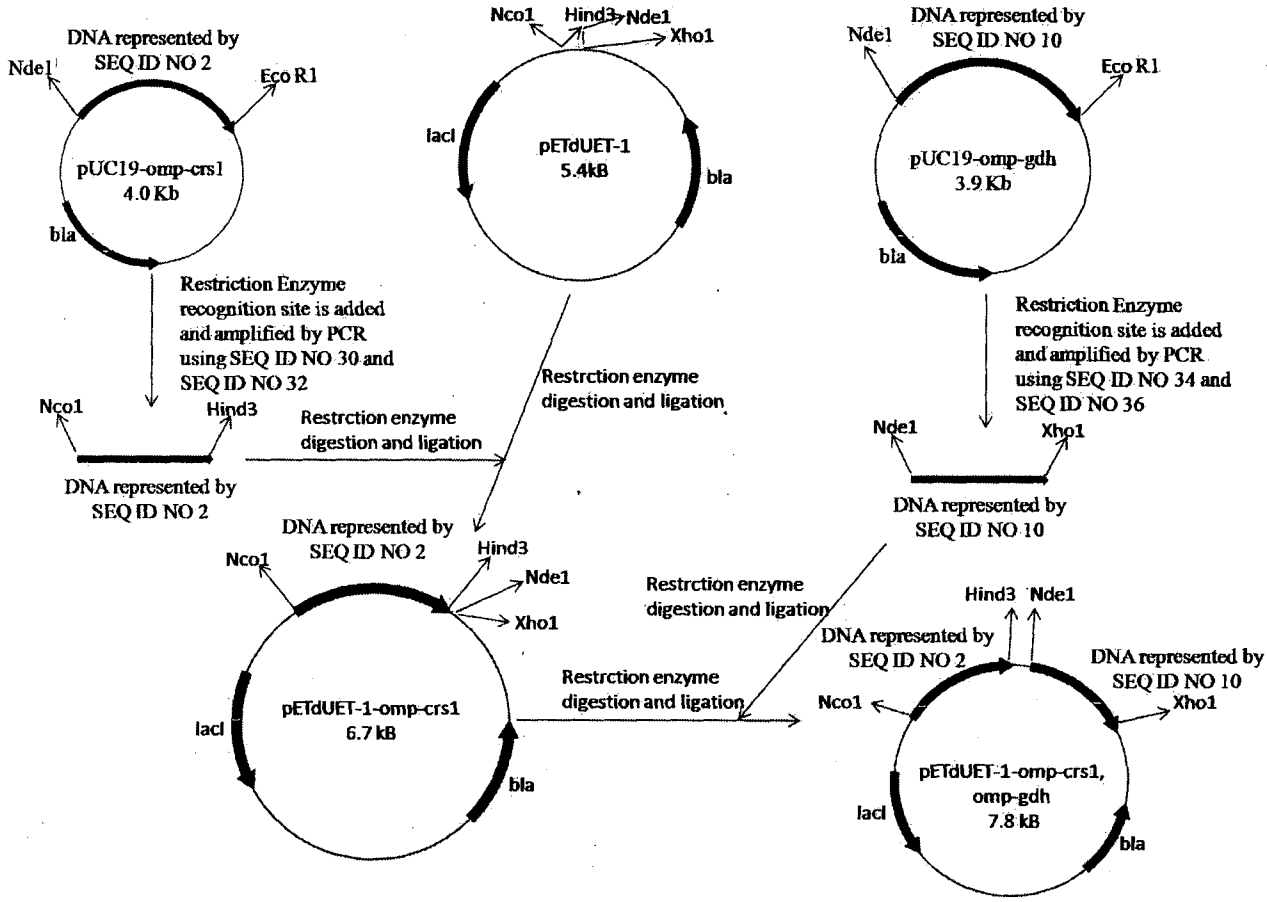


Figure 6: Recombinant Vectors pETDuet1-omp-CRS, omp-GDH

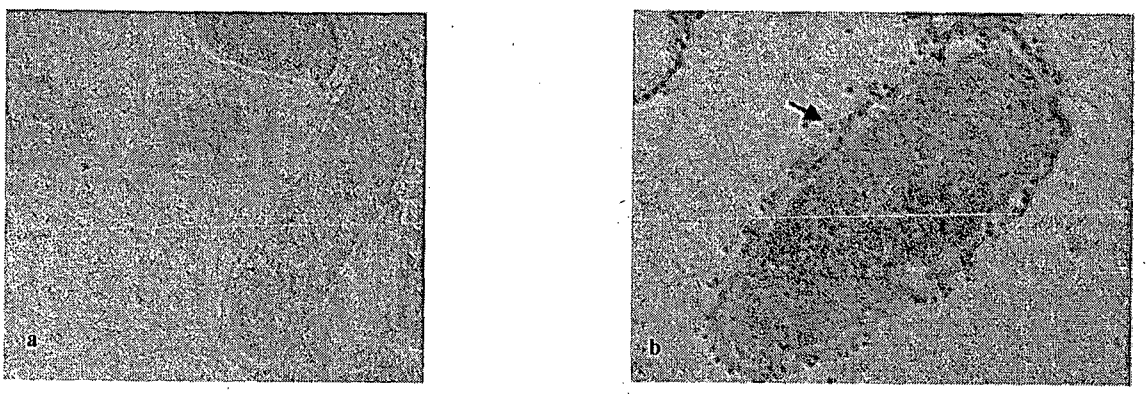


Figure 7: TEM of 'Designer whole-cell biocatalyst'.

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2014/000247

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/00
ADD.

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

B. FIELDS SEARCHED

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

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YUNING SU ET AL: "Two-Enzyme Coexpressed Recombinant Strain for Asymmetric Synthesis of Ethyl (R)-2-Hydroxy-4-phenylbutyrate", CHINESE JOURNAL OF CATALYSIS, vol. 33, no. 9-10, 1 September 2012 (2012-09-01), pages 1650-1660, XP055142325, ISSN: 1872-2067, DOI: 10.1016/S1872-2067(11)60436-1 page 1652 item 1.8	1-74

Y	US 2007/254368 A1 (LEE SANG Y [KR] ET AL LEE SANG YUP [KR] ET AL) 1 November 2007 (2007-11-01) cited in the application page 1, [0008] and [0010]; claims	1-74

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 September 2014	07/10/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hennard, Christophe

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2014/000247

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 2010/151593 A1 (UNIV RUTGERS [US]; UNIV JIANGNAN [CN]; MONTELIONE GAETANO T [US]; XIAO) 29 December 2010 (2010-12-29) example 1	1-74

A	US 2009/004720 A1 (HUA LING [US] ET AL) 1 January 2009 (2009-01-01) example 4	1-74

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IN2014/000247

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