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(54) Title: PHOTOCATALYTIC HYDROGEN PRODUCTION AND POLYPEPTIDES CAPABLE OF SAME

(57) Abstract: An isolated polypeptide comprising a hydrogen generating enzyme attached to a heterologous ferredoxin is disclosed, as well as polynucleotides encoding same, nucleic acid constructs capable of expressing same and cells expressing same. A method for generating hydrogen using the isolated polypeptide is also disclosed.

PHOTOCATALYTIC HYDROGEN PRODUCTION AND POLYPEPTIDES CAPABLE OF SAME

FIELD AND BACKGROUND OF THE INVENTION

5 The present invention relates to hydrogen production and, more particularly, to polypeptides capable of same.

 The development of a clean, sustainable and economically viable energy supply for the future is one of the most urgent challenges of our generation. Oil production is expected to peak in the near future and economically viable oil reserves are expected to be largely depleted by 2050. A
10 viable hydrogen economy requires clean, sustainable and economic ways of generating hydrogen. Current hydrogen production depends almost entirely on the use of non-renewable resources (i.e. steam reformation of natural gas, coal gasification and nuclear power driven electrolysis of water). Although these approaches are initially likely to drive a transition towards a hydrogen economy, the hydrogen produced is more expensive and contains less energy than the non-renewable energy
15 source from which it is derived. In addition, the use of fossil fuels and nuclear power is unsustainable. Therefore, there is a clear need to establish economically viable means of hydrogen production.

 A particularly desirable option is the production of hydrogen using photosynthetic machinery, since the ultimate energy source is solar energy. The twin hearts of the photosynthetic
20 machinery in plants, algae, and cyanobacteria are the two photochemical reaction centers known as Photosystem I (PSI) and Photosystem II (PSII). PSII drives the most highly oxidizing reaction known to occur in biology, splitting water into oxygen, protons and electrons. Oxygen is released into the atmosphere and is responsible for maintaining aerobic life on Earth. The derived electrons are passed along the photosynthetic electron transport chain from PSII via Plastoquinone (PQ) to
25 Cytochrome b_{6f} (cyt b_{6f}) and Photosystem I (PSI). From PSI, most of the negative redox potential is stabilized in the form of reduced ferredoxin (Fd) that serves as an electron donor to ferredoxin-NADP⁺-reductase (FNR) enzyme. Under normal physiological conditions, Fd reduces NADP⁺ to NADPH via the Fd-FNR complex. In a parallel process (photophosphorylation), protons are released into the thylakoid lumen where they generate a proton gradient that is used to drive ATP
30 production via ATP synthase. NADPH and ATP are subsequently used to produce starch and other biomolecules.

 Some green algae and cyanobacteria have evolved the ability to channel the protons and electrons stored in starch into hydrogen production under anaerobic conditions by expressing a hydrogenase enzyme. [Wunschiers, Stangier et al. 2001, *Curr Microbiol* 42(5): 353-60; Happe and
35 Kaminski 2002, *Eur J Biochem* 269(3): 1022-32]. The hydrogenase enzyme is localized in the chloroplast stroma and obtains electrons from ferredoxin or flavodoxin that is reduced by Photosystem I and thus competes with FNR for the PSI generated electrons (Figure 1). However, oxygen is a powerful inhibitor of the hydrogenase enzyme and thus, the generation of hydrogen in these organisms is only transient and also inefficient.

40 Efforts to generate oxygen-tolerant algal hydrogenases have not met with much success [Seibert et al. 2001, *Strategies for improving oxygen tolerance of algal hydrogen production.*

Biohydrogen II. J. M. Miyake, T.; San Pietro, A., eds, Oxford, UK: Pergamon 67-77]. McTavish et al [J Bacteriol 177(14): 3960-4, 1995] have shown that site-directed mutagenesis of *Azotobacter vinelandii* hydrogenase can render hydrogen production insensitive to oxygen inhibition, but with a substantial (78 %) loss of hydrogen evolution activity.

5 Melis (U.S. Patent Application No. 2001/005343) teaches a process in which the inhibition was lifted by temporally separating the oxygen generating water splitting reaction, catalyzed by PSII, from the oxygen sensitive hydrogen production catalyzed by the chloroplast Hydrogenase (HydA). This separation was achieved by culturing green algae first in the presence of sulfur to build stores of an endogenous substrate and then in the absence of sulfur. This led to inactivation
10 of Photosystem II so that cellular respiration led to anaerobiosis, the induction of hydrogenase, and sustained hydrogen evolution in the light.

 The Melis process is, however, subject to considerable practical constraints. The actual rate of hydrogen gas accumulation is at best 15 to 20 % of the photosynthetic capacity of the cells [Melis and Happe 2001, Plant Physiol. Nov;127(3):740-8] and suffers the inherent limitation that
15 hydrogen production by sulfur deprivation of the algae cannot be continued indefinitely. The yield begins to level off and decline after about 40-70 hours of sulfur deprivation, and after about 100 hours of sulfur deprivation the algae need to revert to a phase of normal photosynthesis to replenish endogenous substrates.

 International Publication No. WO 03/067213 describes a process for hydrogen production
20 using *Chlamydomonas reinhardtii* wherein the algae has been genetically modified to down regulate expression of a sulfate permease, CrpSulP, through insertion of an antisense sequence. This is said to render obsolete prior art sulfur deprivation techniques, as it obviates the need to physically remove sulfur nutrients from growth media in order to induce hydrogen production. The reduced sulfur uptake by the cell using this technique not only results in a substantial lowering of
25 the levels of the major chloroplast proteins such as Rubisco, D1 and the LHCII, but also deprives the cell of sulfur for use in the biosynthesis of other proteins.

 Ihara et al (Ihara, Nakamoto et al. 2006; Ihara, Nishihara et al. 2006) teach a fusion protein comprising membrane bound [NiFe] hydrogenase (from the α -proteobacterium *Ralstonia eutropha* H16) and the peripheral PSI subunit PsaE of the cyanobacterium *Thermosynechococcus elongatus*
30 as a direct light-to-hydrogen conversion system. The isolated hydrogenase-PSI isolated complex displayed light-driven hydrogen production at a rate of [0.58 μ mol H₂] / [mg chlorophyll] h *in vitro*. The inefficiency of this system is thought to be derived from the mismatched ability of the hydrogenase to accept electrons compared to the ability of PSI to donate electrons.

 Peters et al [Science, 282, 4 Dec, 1998], teach isolation of an Fe-only hydrogenase from
35 *clostridium pasteurianum* which naturally comprises ferredoxin-like structures. Although this hydrogenase is potentially capable of directly generating hydrogen under illuminated conditions, it can not accept electron from PSI since it lacks the native plant structural docking site to do so.

 There is thus a widely recognized need for, and it would be highly advantageous to have, a sustainable and efficient process for photosynthetic hydrogen production devoid of the above
40 limitations.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided an isolated polypeptide comprising a hydrogen generating enzyme attached to a heterologous ferredoxin.

5 According to another aspect of the present invention there is provided an isolated polynucleotide encoding a polypeptide comprising a hydrogen generating enzyme attached to a heterologous ferredoxin via a peptide bond.

According to yet another aspect of the present invention there is provided a nucleic acid construct, comprising an isolated polynucleotide encoding a polypeptide comprising a hydrogen generating enzyme attached to a heterologous ferredoxin via a peptide bond.

10 According to still another aspect of the present invention there is provided a cell comprising a nucleic acid construct, comprising an isolated polynucleotide encoding a polypeptide comprising a hydrogen generating enzyme attached to a heterologous ferredoxin via a peptide bond.

15 According to an additional aspect of the present invention there is provided a method of generating hydrogen, the method comprising combining an isolated polypeptide comprising a hydrogen generating enzyme attached to a heterologous ferredoxin with an electron donor so as to generate an electron transfer chain, wherein the electron transfer chain is configured such that the electron donor is capable of donating electrons to the polypeptide thereby generating hydrogen.

20 According to yet an additional aspect of the present invention there is provided a system comprising an isolated polypeptide comprising a hydrogen generating enzyme attached to a heterologous ferredoxin and an electron donor.

According to still an additional aspect of the present invention there is provided a bioreactor for producing hydrogen, comprising:

a vessel **321**, holding a hydrogen producing system, the system comprising a suspension of hydrogen generating enzyme attached to a heterologous ferredoxin and PSI;

25 a light providing apparatus comprising an optic fiber, the light providing apparatus being configured to provide light of a selected spectrum to the system; and

a gas liquid separation membrane for separating gas leaving the suspension from the suspension.

30 According to further features in the embodiments of the invention described below, the hydrogen generating enzyme is a hydrogenase.

According to still further features in the described embodiments, the hydrogen generating enzyme is a nitrogenase.

35 According to still further features in the described embodiments, the hydrogenase enzyme is selected from the group consisting of an Fe only hydrogenase, a Ni-Fe hydrogenase and a non-metal hydrogenase.

According to still further features in the described embodiments, the polypeptide further comprises a linker, capable of linking the hydrogen generating enzyme to the ferredoxin.

According to still further features in the described embodiments, the linker is a covalent linker.

According to still further features in the described embodiments, the linker is a non-covalent linker.

According to still further features in the described embodiments, the covalent linker is a peptide bond.

5 According to still further features in the described embodiments, the polypeptide is as set forth in SEQ ID NOs: 24 or 25.

According to still further features in the described embodiments, the polynucleotide comprises a nucleic acid sequence as set forth in SEQ ID NOs: 1-6.

10 According to still further features in the described embodiments, wherein the nucleic acid construct further comprises a cis-regulatory element.

According to still further features in the described embodiments, the cis-regulatory element is a promoter.

According to still further features in the described embodiments, the promoter is an inducible promoter.

15 According to still further features in the described embodiments, the cell is a prokaryotic cell.

According to still further features in the described embodiments, the cell is a eukaryotic cell.

20 According to still further features in the described embodiments, the prokaryotic cell is a cyanobacteria cell.

According to still further features in the described embodiments, the cell is an algae cell.

According to still further features in the described embodiments, the eukaryotic cell is part of a higher plant.

25 According to still further features in the described embodiments, the generating hydrogen is effected under anaerobic conditions.

According to still further features in the described embodiments, the electron donor is selected from the group consisting of a biomolecule, a chemical, water, an electrode and a combination of the above.

30 According to still further features in the described embodiments, the electron donor comprises a biomolecule.

According to still further features in the described embodiments, the biomolecule is light sensitive.

According to still further features in the described embodiments, the light sensitive biomolecule comprises a photocatalytic unit of a photosynthetic organism.

35 According to still further features in the described embodiments, the photocatalytic unit comprises Photosystem I (PSI).

According to still further features in the described embodiments, a ratio of a polypeptide comprising a hydrogen generating enzyme attached to a heterologous ferredoxin: PSI is greater than 100:1.

According to still further features in the described embodiments, the light sensitive biomolecule comprises rhodopsin.

According to still further features in the described embodiments, the biomolecule is immobilized to a solid support.

5 According to still further features in the described embodiments, the chemical is selected from the group consisting of dithiothreitol, ascorbic acid, N, N, N', N'-tetramethyl-p-phenyldiamine (TMPD), 2,6-dichlorophenol indophenol and a combination of any of the above.

According to still further features in the described embodiments, the method further comprises illuminating the light sensitive biomolecule following or concomitant with the combining.

10 According to still further features in the described embodiments, the method further comprises harvesting the hydrogen following the generating.

According to still further features in the described embodiments, the combining is effected in a cell-free system.

15 According to still further features in the described embodiments, the cell-free system is selected from the group consisting of polymeric particles, microcapsules liposomes, microspheres, microemulsions, nano-plates, nanoparticles, nanocapsules and nanospheres.

According to still further features in the described embodiments, the combining is effected in a cellular system.

20 According to still further features in the described embodiments, the cellular system is selected from the group consisting of a cyanobacteria, an alga and a higher plant.

According to still further features in the described embodiments, the method further comprises down-regulating an expression of endogenous ferredoxin in the cellular system.

According to still further features in the described embodiments, the biomolecule is comprised in particles.

25 According to still further features in the described embodiments, the particles are selected from the group consisting of polymeric particles, microcapsules liposomes, microspheres, microemulsions, nanoparticles, nanocapsules, nano-plates and nanospheres.

According to still further features in the described embodiments, the biomolecule is encapsulated within the particle.

30 According to still further features in the described embodiments, the biomolecule is embedded within the particle.

According to still further features in the described embodiments, the biomolecule is adsorbed on a surface of the particle.

35 According to still further features in the described embodiments, the system is expressed in cells.

According to still further features in the described embodiments, the cells are selected from the group consisting of cyanobacteria cells, algae cells and higher plant cells.

According to still further features in the described embodiments, the system comprises a suspension of cells.

According to still further features in the described embodiments, the system comprises a suspension of liposomes.

According to still further features in the described embodiments, the spectrum is selected as not to damage the system.

5 According to still further features in the described embodiments, the spectrum is selected to include activating wavelengths only.

The present invention successfully addresses the shortcomings of the presently known configurations by providing novel polypeptides capable of generating photocatalytically induced hydrogen production.

10 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials,
15 methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that
20 the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the
25 description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 is a schematic diagram illustrating the overall process of native light dependent hydrogen production in algae.

30 FIGs. 2A-B is a schematic diagram illustrating the position of PSI in multilamellar (Figure 2A) and unilamellar (Figure 2B) liposomes.

FIG. 3 is a schematic representation of a system for producing hydrogen to an embodiment of the present invention.

FIG. 4 is a schematic representation of an optic fiber bioreactor according to an
35 embodiment of the present invention.

FIG. 5 is a photograph illustrating generation of chimeric fusions of HydA1 and petF genes. 1HydFd- direct linkage of HydA1 and petF; 2HydFd- short linker of four glycine and 1 serine between HydA1 and petF; 3HydFd- medium linker of two repeats of: four glycine and 1 serine between HydA1 and petF; 4HydFd- direct linkage of C-terminus truncated HydA1 and N-terminus

truncated petF; 5HydFd- direct linkage of C-terminus truncated HydA1 and petF; 6HydFd- direct linkage of HydA1 and N-terminus truncated petF.

FIGs. 6A-B are maps of exemplary expression constructs used for the expression of the constructs of the present invention.

5 FIGs. 7A-B are photographs illustrating expression of the polypeptides of the present invention. Figure 7A – Western analysis using the monoclonal anti StrpTagII (IBA®). In addition, the lower band of native HydA1 was used as internal control. It can be seen that native HydA1 is expressed 10X more than fusion proteins.

10 Figure 7B - the 12 % polyacrylamide gel shows the accessory proteins HydG/F/E expression pattern which are expressed for all of the chimeras as well as the native hydA1 protein.

FIG. 8 is a bar graph illustrating hydrogen generation from a total cell extract prepared from *E. coli* cells that express HydA1, Pet F and HydFd chimera. Dithionite was used as electron donor. The experiment was performed in argon atmosphere. Light gray bars: Hydrogen generation by the hydrogenase component alone, as measured by addition of methyl viologen as an electron mediator. Dark gray bars: Ferredoxin-mediated hydrogen production following elimination of methyl viologen from the system. The displayed values of hydrogen production were based on H₂ gas production measured by gas chromatography and corrected according to the relative expression level of the proteins according to Figures 7A-B.

20

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of a biological method of generating hydrogen and polypeptides capable of catalyzing this reaction.

25 Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

30 Molecular hydrogen is a candidate for replacing or supplementing fossil fuels and as a source of clean energy. Natural biological production of hydrogen is based on the presence of hydrogenase enzymes present in certain green algae and photosynthetic bacteria which are capable of accepting electrons from photosystem I (PSI) and conversion thereof into hydrogen gas. The yield of molecular hydrogen from this process is limited because the endogenous electron carriers donate their electrons to destinations other than hydrogenase. For example, reduced electron carriers, such as ferredoxin also donate electrons to ferredoxin-NADP⁺-reductase (FNR) enzyme.

40 The present inventors have deduced that in order to increase hydrogen production, electrons must be encouraged to shuttle towards hydrogenase (or other hydrogen generating enzymes, such as nitrogenase) at the expense of the competing processes. The present inventors have contemplated a novel hydrogenase polypeptide which is artificially linked to a heterologous

ferredoxin. Such a polypeptide would force the flow of electrons from an electron donor such as photosystem I (PSI) directly to the hydrogenase at the expense of FNR. This novel polypeptide may be expressed in cellular or cell-free systems in order to generate hydrogen gas. Furthermore, the present inventors have conceived that in order to further up-regulate hydrogen production in photosynthetic organisms, the competing process (i.e. the endogenous Fd-FNR complex) is preferably down-regulated.

Whilst reducing the present invention to practice, the present inventors have generated a number of hydrogenase polypeptides, artificially linked to heterologous ferredoxins (Figures 7A-B). Such polypeptides were shown to generate hydrogen (Figure 8) and may also comprise a reduced sensitivity to oxygen.

Thus, according to one aspect of the present invention, there is provided an isolated polypeptide comprising a hydrogen generating enzyme attached to a heterologous ferredoxin.

The phrase "hydrogen generating enzyme" refers to a protein capable of catalyzing a reaction where at least one of the end-products is hydrogen. According to one embodiment the hydrogen generating enzyme is a hydrogenase.

As used herein, the phrase "hydrogenase enzyme" refers to an amino acid sequence of a hydrogenase enzyme with the capability of catalyzing hydrogen oxidation/reduction. Thus the present invention contemplates full-length hydrogenase as well as active fragments thereof. According to one embodiment, the hydrogenase enzyme is a Fe only hydrogenase. According to another embodiment, the hydrogenase is a Ni-Fe hydrogenase. According to yet another embodiment, the hydrogenase is a non-metal hydrogenase. Exemplary hydrogenase enzymes which may be used in accordance with the present invention are set forth by EC 1.12.1.2, EC 1.12.1.3, EC 1.12.2.1, EC 1.12.7.2, EC 1.12.98.1, EC 1.12.99.6, EC 1.12.5.1, EC 1.12.98.2 and EC 1.12.98.3.

Other examples of hydrogenases that may be used according to the teaching of the present invention are listed below in Table 1 together with their source organisms.

Table 1

<i>Source Organism</i>	<i>Protein accession number</i>
<i>Chlamydomonas reinhardtii</i>	AY055756
<i>Desulfovibrio vulgaris hydrogenase</i>	CA26266.1
<i>Megasphaera elsdenii</i>	AF120457
<i>Anabaena variabilis</i>	CAA55878
<i>Desulfovibrio Desulfuricans</i>	1E3D A
<i>Clostridium Pasteurianum</i>	1FEH A
<i>Chlamydomonas reinhardtii</i>	AAR04931

According to another embodiment the hydrogen generating enzyme is a nitrogenase enzyme.

As used herein, the phrase "nitrogenase enzyme" refers to an amino acid sequence of a nitrogenase enzyme (EC 1.18.6.1) with the capability of generating hydrogen as a byproduct in a nitrogen fixation reaction. Thus the present invention contemplates full-length nitrogenase as well

as active fragments thereof. Examples of nitrogenases that may be used according to the teaching of the present invention are listed below in Table 2 together with their source organisms.

Table 2

<i>Source Organism</i>	<i>Protein accession number</i>
<i>Azotobacter Vinelandii</i>	1MIN_A
<i>Clostridium Pasteurianum</i>	1MIO_A
<i>Anabaena variabilis</i>	AAX82499

5 As mentioned the polypeptide of the present invention, comprises a hydrogenase attached to a heterologous ferredoxin.

As used herein, the term "ferredoxin" refers to an amino acid sequence of the iron sulfur protein that is capable of mediating electron transfer to hydrogenase. Thus the present invention contemplates full-length ferredoxin as well as active fragments thereof. According to a preferred
10 embodiment of this aspect of the present invention, the ferredoxin is a plant-type ferredoxin.

Exemplary ferredoxin polypeptides that may be used in accordance with the present invention include, but are not limited to cyanobacterial ferredoxins, algae ferredoxins and non photosynthetic organism ferredoxins.

The qualifier "heterologous" when relating to the ferredoxin indicates that the ferredoxin is not naturally associated with (i.e. endogenous to) the hydrogenase of the present invention. Thus,
15 for example, the phrase "hydrogenase attached to a heterologous ferredoxin" does not comprise the Fe-only hydrogenase from *clostridium pasteurianum*.

The present invention envisages attachment of the heterologous ferredoxin at any position to the hydrogen generating enzyme so long as the hydrogen generating enzyme is capable of
20 generating hydrogen from electrons donated thereto from the attached ferredoxin. The hydrogen generating enzyme and ferredoxin may be linked via bonding at their carboxy (C) or amino (N) termini, or via bonding to internal chemical groups such as straight, branched or cyclic side chains, internal carbon or nitrogen atoms, and the like. Methods of linking the hydrogen generating enzyme to the ferredoxin are further described herein below.

25 Amino acid sequences of exemplary polypeptides of the present invention are set forth in SEQ ID NOs: 23-28.

The term "polypeptide" as used herein encompasses native polypeptides (either degradation products, synthetically synthesized polypeptides or recombinant polypeptides) and peptidomimetics (typically, synthetically synthesized polypeptides), as well as as peptoids and
30 semipeptoids which are polypeptide analogs, which may have, for example, modifications rendering the polypeptides more stable while in a body or more capable of penetrating into cells. Such modifications include, but are not limited to N terminus modification, C terminus modification, polypeptide bond modification, including, but not limited to, CH₂-NH, CH₂-S, CH₂-S=O, O=C-NH, CH₂-O, CH₂-CH₂, S=C-NH, CH=CH or CF=CH, backbone modifications, and residue modification.
35 Methods for preparing peptidomimetic compounds are well known in the art and are specified, for example, in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992), which is incorporated by reference as if fully set forth herein. Further details in this

respect are provided hereinunder. Polypeptide bonds (-CO-NH-) within the polypeptide may be substituted, for example, by N-methylated bonds (-N(CH₃)-CO-), ester bonds (-C(R)H-C-O-O-C(R)-N-), ketomethylen bonds (-CO-CH₂-), α -aza bonds (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba bonds (-CH₂-NH-), hydroxyethylene bonds (-CH(OH)-CH₂-), thioamide bonds (-CS-NH-), olefinic double bonds (-CH=CH-), retro amide bonds (-NH-CO-), polypeptide derivatives (-N(R)-CH₂-CO-), wherein R is the "normal" side chain, naturally presented on the carbon atom.

These modifications can occur at any of the bonds along the polypeptide chain and even at several (2-3) at the same time.

Natural aromatic amino acids, Trp, Tyr and Phe, may be substituted for synthetic non-natural acid such as Phenylglycine, TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr.

In addition to the above, the polypeptides of the present invention may also include one or more modified amino acids or one or more non-amino acid monomers (e.g. fatty acids, complex carbohydrates etc).

As used herein in the specification and in the claims section below the term "amino acid" or "amino acids" is understood to include the 20 naturally occurring amino acids; those amino acids often modified post-translationally *in vivo*, including, for example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual amino acids including, but not limited to, 2-aminoadipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. Furthermore, the term "amino acid" includes both D- and L-amino acids.

Tables 3 and 4 below list naturally occurring amino acids (Table 3) and non-conventional or modified amino acids (Table 4) which can be used with the present invention.

Table 3

Amino Acid	Three-Letter abbreviation	One-letter Symbol
alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
glycine	Gly	G
Histidine	His	H
isoleucine	Ile	I
leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

Table 4

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α -aminobutyric acid	Abu	L-N-methylalanine	Nmala
α -amino- α -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
aminoisobutyric acid	Aib	L-N-methylaspartic acid	Nmasp
aminonorbornyl-carboxylate	Norb	L-N-methylcysteine	Nmcys
cyclohexylalanine	Chexa	L-N-methylglutamine	Nmgin
cyclopentylalanine	Cpen	L-N-methylglutamic acid	Nmglu
D-alanine	Dal	L-N-methylhistidine	Nmhis
D-arginine	Darg	L-N-methylisoleucine	Nmile
D-aspartic acid	Dasp	L-N-methylleucine	Nmleu
D-cysteine	Dcys	L-N-methyllysine	Nmlys
D-glutamine	Dgln	L-N-methylmethionine	Nmmet
D-glutamic acid	Dglu	L-N-methylnorleucine	Nmnle
D-histidine	Dhis	L-N-methylnorvaline	Nmnva
D-isoleucine	Dile	L-N-methylornithine	Nmorn
D-leucine	Dleu	L-N-methylphenylalanine	Nmphe
D-lysine	Dlys	L-N-methylproline	Nmpro
D-methionine	Dmet	L-N-methylserine	Nmser
D-ornithine	Dorn	L-N-methylthreonine	Nmthr
D-phenylalanine	Dphe	L-N-methyltryptophan	Nmtrp
D-proline	Dpro	L-N-methyltyrosine	Nmtyr
D-serine	Dser	L-N-methylvaline	Nmval
D-threonine	Dthr	L-N-methylethylglycine	Nmetg
D-tryptophan	Dtrp	L-N-methyl-t-butylglycine	Nmtbug
D-tyrosine	Dtyr	L-norleucine	Nle
D-valine	Dval	L-norvaline	Nva
D- α -methylalanine	Dmala	α -methyl-aminoisobutyrate	Maib
D- α -methylarginine	Dmarg	α -methyl- γ -aminobutyrate	Mgab
D- α -methylasparagine	Dmasn	α ethylcyclohexylalanine	Mchexa
D- α -methylaspartate	Dmasp	α methylcyclopentylalanine	Mcpen
D- α -methylcysteine	Dmcys	α -methyl- α -naphthylalanine	Manap
D- α -methylglutamine	Dmgln	α -methylpenicillamine	Mpen
D- α -methylhistidine	Dmhis	N-(4-aminobutyl)glycine	Nglu
D- α -methylisoleucine	Dmile	N-(2-aminoethyl)glycine	Naeg
D- α -methylleucine	Dmleu	N-(3-aminopropyl)glycine	Norn
D- α -methyllysine	Dmlys	N-amino- α -methylbutyrate	Nmaabu
D- α -methylmethionine	Dmmet	α -naphthylalanine	Anap
D- α -methylornithine	Dmorn	N-benzylglycine	Nphe
D- α -methylphenylalanine	Dmphe	N-(2-carbamylethyl)glycine	Ngln
D- α -methylproline	Dmpro	N-(2-carboxyethyl)glycine	Nasn
D- α -methylserine	Dmser	N-(carbamylmethyl)glycine	Nglu
D- α -methylthreonine	Dmthr	N-(carboxymethyl)glycine	Nasp
D- α -methyltryptophan	Dmtrp	N-cyclobutylglycine	Ncbut
D- α -methyltyrosine	Dmtyr	N-cycloheptylglycine	Nchep
D- α -methylvaline	Dmval	N-cyclohexylglycine	Nchex
D- α -methylalanine	Dmala	N-cyclodecylglycine	Ncdec
D- α -methylarginine	Dmarg	N-cyclododecylglycine	Ncdod
D- α -methylasparagine	Dnmasn	N-cyclooctylglycine	Ncoct
D- α -methylaspartate	Dnmasp	N-cyclopropylglycine	Ncpro
D- α -methylcysteine	Dnmcys	N-cycloundecylglycine	Ncund
D-N-methylleucine	Dnmleu	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methyllysine	Dnmlys	N-(3,3-diphenylpropyl)glycine	Nbhe
N-methylcyclohexylalanine	Nmchexa	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methylornithine	Dnmorn	N-methyl- γ -aminobutyrate	Nmgabu
N-methylglycine	Nala	D-N-methylmethionine	Dnmmet
N-methylaminoisobutyrate	Nmaib	N-methylcyclopentylalanine	Nmcpen
N-(1-methylpropyl)glycine	Nile	D-N-methylphenylalanine	Dnmphe
N-(2-methylpropyl)glycine	Nile	D-N-methylproline	Dnmpro
		D-N-methylserine	Dnmser
		D-N-methylserine	Dnmser

N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nva
D-N-methyltyrosine	Dnmtyr	N-methyl-naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- α -methylalanine	Mala
L- α -methylarginine	Marg	L- α -methylasparagine	Masn
L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
L- α thylglutamine	Mgln	L- α -methylglutamate	Mglu
L- α -methylhistidine	Mhis	L- α -methylhomo phenylalanine	Mhphe
L- α -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmt
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyl-naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- α -methylalanine	Mala
L- α -methylarginine	Marg	L- α -methylasparagine	Masn
L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
L- α -methylglutamine	Mgln	L- α -methylglutamate	Mglu
L- α ethylhistidine	Mhis	L- α -methylhomophenylalanine	Mhphe
L- α thylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L- α -methylleucine	Mleu	L- α -methyllysine	Mlys
L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
L- α -methylserine	mser	L- α -methylthreonine	Mthr
L- α ethylvaline	Mtrp	L- α -methyltyrosine	Mtyr
L- α -methylleucine	Mval	L-N-methylhomophenylalanine	Nmhph
N-(N-(2,2-diphenylethyl) carbamylmethyl-glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl(1)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl aylamino)cyclopropane	Nmbc		

Table 4 Cont.

The polypeptides of the present invention are preferably utilized in a linear form, although it will be appreciated that in cases where cyclization does not severely interfere with polypeptide characteristics (e.g. electron transfer), cyclic forms of the polypeptide can also be utilized.

5 The polypeptide of present invention can be synthesized biochemically. Alternatively, the polypeptide of present invention can be generated using recombinant techniques in order to generate a fusion protein wherein the hydrogen generating enzyme amino acid sequence is

attached to the ferredoxin amino acid sequence via a peptide bond or a substituted peptide bond as further described herein above. It will be appreciated that the attachment of the hydrogen generating enzyme to the ferredoxin may also be effected following the independent synthesis (either biochemically, or using recombinant techniques) of hydrogenase (or nitrogenase) and ferredoxin. It addition, the hydrogen generating enzyme and/or ferredoxin may also be isolated from their natural environment and subsequently linked. Each alternative method will be further described herein below.

Biochemical synthesis

Standard solid phase techniques may be used to biochemically synthesize the polypeptides of the present invention. These methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis. These methods are preferably used when the polypeptide cannot be produced by recombinant techniques (i.e., not encoded by a nucleic acid sequence) and therefore involves different chemistry.

Solid phase polypeptide synthesis procedures are well known in the art and further described by John Morrow Stewart and Janis Dillaha Young, *Solid Phase Polypeptide Syntheses* (2nd Ed., Pierce Chemical Company, 1984).

Synthetic polypeptides can be purified by preparative high performance liquid chromatography [Creighton T. (1983) *Proteins, structures and molecular principles*. WH Freeman and Co. N.Y.] and the composition of which can be confirmed via amino acid sequencing.

Recombinant techniques

Recombinant techniques are preferably used to generate the polypeptides of the present invention since these techniques are better suited for generation of relatively long polypeptides (e.g., longer than 20 amino acids) and large amounts thereof, as long as no modified amino acids are included in the sequence. As mentioned, recombinant techniques may be used to generate the hydrogen generating enzyme and ferredoxin independently or alternatively to generate a fusion protein where the hydrogen generating enzyme is attached to the ferredoxin via a peptide bond.

Such recombinant techniques are described by Bitter et al., (1987) *Methods in Enzymol.* 153:516-544, Studier et al. (1990) *Methods in Enzymol.* 185:60-89, Brisson et al. (1984) *Nature* 310:511-514, Takamatsu et al. (1987) *EMBO J.* 6:307-311, Coruzzi et al. (1984) *EMBO J.* 3:1671-1680 and Brogli et al., (1984) *Science* 224:838-843, Gurley et al. (1986) *Mol. Cell. Biol.* 6:559-565 and Weissbach & Weissbach, 1988, *Methods for Plant Molecular Biology*, Academic Press, NY, Section VIII, pp 421-463.

To produce the polypeptides of the present invention using recombinant technology, a polynucleotide encoding the polypeptides of the present invention is ligated into a nucleic acid expression vector, which comprises the polynucleotide sequence under the transcriptional control of a cis-regulatory sequence (e.g., promoter sequence) suitable for directing constitutive, tissue specific or inducible transcription of the polypeptides of the present invention in the host cells.

Thus, the present invention contemplates isolated polynucleotides encoding the fusion protein of the present invention.

The phrase "an isolated polynucleotide" refers to a single or double stranded nucleic acid sequence which is isolated and provided in the form of an RNA sequence, a complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence and/or a composite polynucleotide sequences (e.g., a combination of the above).

5 As used herein the phrase "complementary polynucleotide sequence" refers to a sequence, which results from reverse transcription of messenger RNA using a reverse transcriptase or any other RNA dependent DNA polymerase. Such a sequence can be subsequently amplified *in vivo* or *in vitro* using a DNA dependent DNA polymerase.

10 As used herein the phrase "genomic polynucleotide sequence" refers to a sequence derived (isolated) from a chromosome and thus it represents a contiguous portion of a chromosome.

As used herein the phrase "composite polynucleotide sequence" refers to a sequence, which is at least partially complementary and at least partially genomic. A composite sequence can include some exon sequences required to encode the polypeptide of the present invention, as well
15 as some intronic sequences interposing therebetween. The intronic sequences can be of any source, including of other genes, and typically will include conserved splicing signal sequences. Such intronic sequences may further include cis acting expression regulatory elements.

Exemplary nucleic acid sequences of the polynucleotides of the present invention are set forth in SEQ ID NOs: 1-6.

20 As mentioned hereinabove, polynucleotide sequences of the present invention are inserted into expression vectors (i.e., a nucleic acid construct) to enable expression of the recombinant polypeptide. The expression vector of the present invention includes additional sequences which render this vector suitable for replication and integration in prokaryotes, eukaryotes, or preferably both (e.g., shuttle vectors). Typical cloning vectors contain transcription and translation initiation
25 sequences (e.g., promoters, enhances) and transcription and translation terminators (e.g., polyadenylation signals).

A variety of prokaryotic or eukaryotic cells can be used as host-expression systems to express the polypeptides of the present invention. These include, but are not limited to, microorganisms, such as bacteria transformed with a recombinant bacteriophage DNA, plasmid
30 DNA or cosmid DNA expression vector containing the polypeptide coding sequence; yeast transformed with recombinant yeast expression vectors containing the polypeptide coding sequence; plant cell systems infected with recombinant virus expression vectors or transformed with recombinant plasmid expression vectors, such as Ti plasmid, containing the polypeptide coding sequence.

35 According to one embodiment of this aspect of the present invention, the polynucleotides of the present invention are expressed in a photosynthetic organism. (e.g. higher plant, alga, cyanobacteria) which endogenously express PSI and/or PSII. Advantages thereof are discussed herein below.

40 Examples of constitutive plant promoters include, but are not limited to CaMV35S and CaMV19S promoters, tobacco mosaic virus (TMV), FMV34S promoter, sugarcane bacilliform

badnavirus promoter, CsVMV promoter, Arabidopsis ACT2/ACT8 actin promoter, Arabidopsis ubiquitin UBQ 1 promoter, barley leaf thionin BTH6 promoter, and rice actin promoter.

An inducible promoter is a promoter induced by a specific stimulus such as stress conditions comprising, for example, light, temperature, chemicals, drought, high salinity, osmotic shock, oxidant conditions or in case of pathogenicity. Examples of inducible promoters include, but are not limited to, the light-inducible promoter derived from the pea rbcS gene, the promoter from the alfalfa rbcS gene, the promoters DRE, MYC and MYB active in drought; the promoters INT, INPS, prxEa, Ha hsp17.7G4 and RD21 active in high salinity and osmotic stress, and the promoters hsr2O3J and str246C active in pathogenic stress.

These constructs can be introduced into plant cells using Ti plasmid, Ri plasmid, plant viral vectors, direct DNA transformation, microinjection, electroporation, Biolistics (gene gun) and other techniques well known to the skilled artisan. See, for example, Weissbach & Weissbach [Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463 (1988)]. Other expression systems such as insects and mammalian host cell systems, which are well known in the art, can also be used by the present invention.

It will be appreciated that other than containing the necessary elements for the transcription and translation of the inserted coding sequence (encoding the polypeptide), the expression construct of the present invention can also include sequences engineered to optimize stability, production, purification, yield or activity of the expressed polypeptide.

Various methods can be used to introduce the expression vector of the present invention into the host cell system. Such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et al. [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

Transformed cells are cultured under effective conditions, which allow for the expression of high amounts of recombinant polypeptide. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An effective medium refers to any medium in which a cell is cultured to produce the recombinant polypeptide of the present invention. Such a medium typically includes an aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. In addition, cells of the current invention can be cultured under field conditions such as open ponds, covered ponds, plastic bags (see for example "A Look Back at the U.S.

Department of Energy's Aquatic Species Program - Biodiesel from Algae, July 1998, U.S. Department of Energy's Office of Fuels Development, incorporated herein by reference). Such culturing conditions are within the expertise of one of ordinary skill in the art.

Depending on the vector and host system used for production, resultant polypeptides of the present invention may either remain within the recombinant cell, secreted into the fermentation medium, secreted into a space between two cellular membranes, such as the periplasmic space in *E. coli*; or retained on the outer surface of a cell or viral membrane.

It will be appreciated that if the polypeptide of the present invention is to be used in a cell free system, following a predetermined time in culture, recovery of the recombinant polypeptide is effected.

The phrase "recovering the recombinant polypeptide" used herein refers to collecting the whole fermentation medium containing the polypeptide and need not imply additional steps of separation or purification.

Thus, polypeptides of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, salting out (as in ammonium sulfate precipitation), affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

To facilitate recovery, the expressed coding sequence can be engineered to encode the polypeptide of the present invention and fused cleavable moiety. Such a fusion protein can be designed so that the polypeptide can be readily isolated by affinity chromatography; e.g., by immobilization on a column specific for the cleavable moiety. Where a cleavage site is engineered between the polypeptide and the cleavable moiety, the polypeptide can be released from the chromatographic column by treatment with an appropriate enzyme or agent that specifically cleaves the fusion protein at this site [e.g., see Booth *et al.*, Immunol. Lett. 19:65-70 (1988); and Gardella *et al.*, J. Biol. Chem. 265:15854-15859 (1990)].

The polypeptide of the present invention is preferably retrieved in "substantially pure" form.

As used herein, the phrase "substantially pure" refers to a purity that allows for the effective use of the protein in the applications described herein.

In addition to being synthesizable in host cells, the polypeptide of the present invention can also be synthesized using *in vitro* expression systems. These methods are well known in the art and the components of the system are commercially available.

Site-directed linkage of hydrogenase to ferredoxin

Non-natural amino acids may be added to specific places within a recombinant protein followed by chemical conjugation at these specific positions [Chin JW, Cropp TA, Anderson JC, Mukherji M, Zhang Z, Schultz PG. Science. 2003 Aug 15;301(5635):964-7; Dieterich DC, Link AJ, Graumann J, Tirrell DA, Schuman EM.. Proc Natl Acad Sci U S A. 2006 Jun 20;103(25):9482-7].

Non-recombinant linkage of hydrogenase to ferredoxin

As mentioned, the hydrogen generating enzyme and the ferredoxin may be generated (e.g. synthesized) or isolated independently and chemically linked one to the other via a covalent (e.g. peptide) or non-covalent linker either directly or via bonding to an intervening linker element, such as a linker peptide or other chemical moiety, such as an organic polymer.

Exemplary chemical crosslinking methods for conjugating the hydrogen generating enzyme with ferredoxin are described herein below:

Thiol-amine crosslinking:

In this scheme, the amine group of the hydrogen generating enzyme is indirectly conjugated to a thiol group on the ferredoxin or vice versa, usually by a two- or three-step reaction sequence. The high reactivity of thiols and their relative rarity in most polypeptides make thiol groups ideal targets for controlled chemical crosslinking. Thiol groups may be introduced into one of the two polypeptides using one of several thiolation methods including SPDP. The thiol-containing biomolecule is then reacted with an amine-containing biomolecule using a heterobifunctional crosslinking reagent.

Amine - amine crosslinking:

Conjugation of the hydrogen generating enzyme with ferredoxin can be accomplished by methods known to those skilled in the art using amine-amine crosslinkers including, but not limited to glutaraldehyde, bis(imido esters), bis(succinimidyl esters), diisocyanates and diacid chlorides.

Carbodiimide conjugation:

Conjugation of the hydrogen generating enzyme with ferredoxin can be accomplished by methods known to those skilled in the art using a dehydrating agent such as a carbodiimide. Most preferably the carbodiimide is used in the presence of 4-dimethyl aminopyridine. As is well known to those skilled in the art, carbodiimide conjugation can be used to form a covalent bond between a carboxyl group of one polypeptide and an hydroxyl group of a second polypeptide (resulting in the formation of an ester bond), or an amino group of a second polypeptide (resulting in the formation of an amide bond) or a sulfhydryl group of a second polypeptide (resulting in the formation of a thioester bond).

Likewise, carbodiimide coupling can be used to form analogous covalent bonds between a carbon group of a first polypeptide and an hydroxyl, amino or sulfhydryl group of a second polypeptide. See, generally, J. March, *Advanced Organic Chemistry: Reaction's, Mechanism, and Structure*, pp. 349-50 & 372-74 (3d ed.), 1985. By means of illustration, and not limitation, the hydrogen generating enzyme may be conjugated to the ferredoxin via a covalent bond using a carbodiimide, such as dicyclohexylcarbodiimide or EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride. See generally, the methods of conjugation by B. Neises et al. (1978, *Angew Chem., Int. Ed. Engl.* 17:522; A. Hassner et al. (1978, *Tetrahedron Lett.* 4475); E.P. Boden et al. (1986, *J. Org. Chem.* 50:2394) and L.J. Mathias (1979, *Synthesis* 561).

Crosslinking of cysteine residues

The hydrogen generating enzyme and ferredoxin of the present invention may be linked during a programmed cross linking by the addition of cysteine residues within the sequence of one of the proteins or both. Gentle reduction and oxidation, would then allow the formation of a viable, disulfide linked hydrogenase-ferredoxin complex.

As mentioned, the fusion polypeptide of the present invention is artificially engineered such that the hydrogen generating enzyme comprised within is in an optimal environment for receiving electrons and therefore for generating hydrogen.

Thus, according to another aspect of the present invention, there is provided a method of generating hydrogen. The method comprises combining the fusion polypeptide of the present invention (i.e. the polypeptide comprising the hydrogen generating enzyme and ferredoxin) with an electron donor so as to generate an electron transfer chain.

The phrase "electron donor" as used herein, refers to any biological or non-biological component that is capable of donating electrons. Thus, according to one embodiment of this aspect of the present invention, the electron donor is an electrode. Thus the present invention contemplates a system **10**, illustrated herein in Figure 3 comprising an electrode **12** in contact with the polypeptide of the present invention **14**. The polypeptide **14** may be attached to the electrode **12** for direct bioelectrocatalysis using any method known in the art such as for example the modification of electrode surfaces by redox mediators or hydrophilic adsorption. Exemplary material that may be used for generating electrode **12** is carbon covered with viologen substituted poly(pyrrole), pyrolytic carbon paper (PCP) and packed graphite columns (PGC). In order to generate hydrogen, the electrode **12** is attached to an electrical source **16**.

According to another embodiment of this aspect of the present invention the electron donor comprises a light-sensitive biomolecule in combination with an electron source.

The term "biomolecule" as used herein refers to a molecule that is or can be produced by a living system as well as structures derived from such molecules. Biomolecules include, for example, proteins, glycoproteins, carbohydrates, lipids, glycolipids, fatty acids, steroids, purines, pyrimidines, and derivatives, analogs, and/or combinations thereof.

According to a preferred embodiment of this aspect of the present invention, the biomolecular electron donor comprises a photocatalytic unit of a photosynthetic organism.

As used herein, the phrase "photocatalytic unit" refers to a complex of at least one polypeptide and other small molecules (e.g. chlorophyll and pigment molecules), which when integrated together work as a functional unit converting light energy to chemical energy. The photocatalytic units of the present invention are present in photosynthetic organisms (i.e. organisms that convert light energy into chemical energy). Examples of photosynthetic organisms include, but are not limited to green plants, cyanobacteria, red algae, purple and green bacteria.

Thus examples of photocatalytic units which can be used in accordance with this aspect of the present invention include biological photocatalytic units such as PS I and PS II, bacterial light-sensitive proteins such as bacteriorhodopsin, photocatalytic microorganisms, pigments (e.g.,

proflavine and rhodopsin), organic dyes and algae. Preferably, the photocatalytic unit of the present invention is photosystem I (PS I).

PSI is a protein-chlorophyll complex, present in green plants and cyanobacteria, that is part of the photosynthetic machinery within the thylakoid membrane. It is ellipsoidal in shape and has dimensions of about 9 by 15 nanometers. The PS I complex typically comprises chlorophyll molecules which serve as antennae which absorb photons and transfer the photon energy to P700, where this energy is captured and utilized to drive photochemical reactions. In addition to the P700 and the antenna chlorophylls, the PSI complex contains a number of electron acceptors. An electron released from P700 is transferred to a terminal acceptor at the reducing end of PSI through intermediate acceptors, and the electron is transported across the thylakoid membrane.

Examples of PSI polypeptides are listed below in Table 5 together with their source organisms.

Table 5

<i>Source Organism</i>	<i>Protein accession number</i>
Amphidinium carterae	CAC34545
Juniperus chinensis	CAC87929
Cedrus libani	CAC87143
Spathiphyllum sp. SM328	CAC87924
Persea americana	CAC87920
Zamia pumila	CAC87935
Ophioglossum petiolatum	CAC87936
Taxus brevifolia	CAC87934
Afrocarpus gracilior	CAC87933
Pinus parviflora	CAC87932
Picea spinulosa	CAC87931
Phyllocladus trichomanoides	CAC87930
Serenoa repens	CAC87923
Saururus cernuus	CAC87922
Platanus racemosa	CAC87921
Pachysandra terminalis	CAC87919
Nymphaea sp. cv. Paul Harriot	CAC87918
Nuphar lutea	CAC87917
Nelumbo nucifera	CAC87916
Acer palmatum	CAD23045
Cupressus arizonica	CAC87928
Cryptomeria japonica	CAC87927
Abies alba]	CAC87926
Gnetum gnemon	CAC87925
Magnolia grandiflora	CAC87915
Liquidambar styraciflua	CAC87914
Lilium brownii	CAC87913
Isomeris arborea	CAC87912
Fagus grandifolia	CAC87911
Eupomatia laurina	CAC87910
Enkianthus chinensis	CAC87909
Coptis laciniata	CAC87908
Chloranthus spicatus	CAC87907
Calycanthus occidentalis	CAC87906
Austrobaileya scandens]	CAC87905
Amborella trichopoda	CAC87904
Acorus calamus	CAC87142

The photosystem I complex may be in the native cellular membrane along with photosystem II and the rest of the photosynthetic electron transport chain, or it can be provided in a detergent-solubilized form. Methods for isolating native membranes from photosynthetic organisms are known in the art and a preferred method is provided in the publication of (Murata 1982, Plant Cell Physiol 23: 533-9). Purified thylakoids may be quantitated and expressed as a particular amount of chlorophyll. Methods for quantitating chlorophyll are known for example as set forth by (Arnon 1949, Plant Physiol 24(1): 1-15). Methods for obtaining isolated photosystem I in a detergent solubilized form is also known and an exemplary method is disclosed by (Evans, Sihra et al. 1977, Biochem J 162(1): 75-85).

According to one embodiment, the light sensitive biomolecule is immobilized on a solid support. WIPO PCT Application WO2006090381, incorporated herein by reference, teaches immobilization of PS-I on a solid supports such as metal surfaces by genetic manipulation thereof.

As mentioned hereinabove, in order for light sensitive biomolecules to act as electron donors they typically act in combination with an electron source. Exemplary electron sources that may be used in combination with PSI include sodium dithionite (e.g. at about 5 mM), dithiothreitol (e.g. at about 50 mM) and a combination of dithiothreitol plus ascorbic acid (e.g. at about 2 mM ascorbic acid). According to one embodiment PSI is used in combination with PSII, where the latter serves as an intermediate, passing electrons from the electron source to PSI. In this case, the electron source may be water. Other electron sources that may be used according to the teachings of the present invention include, but are not limited to N, N, N', N'-tetramethyl-p-phenyldiamine (TMPD) and 2,6-dichlorophenol indophenol.

As mentioned, the ferredoxin-hydrogenase (or ferredoxin-nitrogenase) fusion protein of the present invention is preferably not inhibited by the presence of dissolved oxygen (or at least, comprise a reduced sensitivity to oxygen). Such fusion proteins may survive in the atmosphere for approximately ~300 seconds (IC_{50}) in comparison to 1 sec of native *Chlamydomonas reinhardtii* hydrogenase. However, if the ferredoxin-hydrogenase fusion protein of the present invention is inhibited by dissolved oxygen, it may be necessary to remove oxygen from the reaction mixture. The removal of oxygen can be performed in a number of ways. For example, if dithionite or high concentrations of dithiothreitol (e.g., 50 mM dithiothreitol) are used as electron donors, these will react with dissolved oxygen to remove it. If water is used as an electron donor in cellular systems, oxygen will be produced by photosystem II and 5 mM glucose plus the over-expression/ or the external addition of glucose oxidase (3 μ g/ml, Sigma, St. Louis, Mo.) can be included in the reaction mix to rapidly remove oxygen as it is produced.

As mentioned herein above, the method of the present invention envisages combining the fusion protein of the present invention with an electron donor in such a way so as to promote electron transfer from the donor to the fusion protein.

As used herein, the term "combining" refers to any method where the fusion protein and the electron donor are in close enough proximity that electron transfer from the latter to the former occurs. Thus, the term "combining" incorporates such methods as co-expressing and co-solubilizing the fusion protein and electron donor of the present invention.

According to one embodiment, the fusion protein and electron donor are combined in a cellular system. Thus, for example the fusion protein may be expressed in a photosynthetic organism where PSI and optionally PSII are endogenously expressed.

Preferably, the amount of fusion protein is adjusted for maximal optimization of the system.

5 Thus, according to an embodiment of this aspect of the present invention, a ratio of fusion protein:PSI is greater than 100:1, more preferably greater than 500:1 and even more preferably greater than 1000:1. Preferably, the cellular system is forced to respire under anaerobic conditions so as to avoid the generation of oxygen.

10 In order for PSI to transfer electrons to the ferredoxin unit of the polypeptide of the present invention, preferably PSI is energized using light energy. The illuminating may proceed following or concomitant with the expression of the fusion protein of the present invention. A bioreactor may be used which excludes high energy wavelength such as UV radiation, and enables the entrance of the visible red which exclusively feeds and ignite the PSI. An exemplary bioreactor that may be used in accordance with the teachings of the present invention is illustrated in Figure 3.

15 Figure 4 is a schematic illustration of a reactor **300** for producing hydrogen according to an embodiment of the invention. Reactor **300** comprises a vessel **321**, in which the hydrogen producing system comprising PSI, and the ferredoxin unit of the polypeptide of the present invention, are held in a suspension **322**. The suspension **322** may also comprise other components such as sodium citrated and TMPD. The suspension may include the hydrogen
20 producing systems, that is, the PSI and the ferredoxin unit, in any of the above-mentioned ways, for instance, in liposomes or in cell culture. Suspension **322** is constantly stirred with stirring blades **324** by rotor **325**. The rotor and stirring blade are operated as not to damage the cells or liposomes, but only homogenize them within vessel **321**.

A temperature control **326** controls the temperature to optimize the activity of the cells or
25 liposomes, for instance, 37°C.

An optic fiber **323**, provides light to the cells or liposomes. Preferably, the light provided by fiber **323** is free of damaging wavelengths, such as UV. Optionally, the light is also free of non-activating wavelengths, for instance, green light. Hydrogen produced by the hydrogen producing systems bubbles out of the suspension, through a gas-liquid separation membrane **328**. From the
30 gas side of membrane **328**, the hydrogen is optionally pumped with pump **329** to a hydrogen tank **330**.

As mentioned herein above, the endogenous electron transport system in all photosynthetic organisms comprises donation of electrons from ferredoxin to ferredoxin-NADP⁺-reductase (FNR). In order to divert the flow of electrons away from this competing enzyme, the present invention
35 contemplates down-regulation thereof.

The phrase "ferredoxin-NADP⁺-reductase" as used herein refers to the enzyme as set forth by EC 1.18.1.2. present in photosynthetic organisms.

Downregulation of FNR may be effected on the genomic level (using classical genetic approaches) and/or the transcript level. This may be achieved using a variety of molecules which
40 interfere with transcription and/or translation (e.g., antisense, siRNA, Ribozyme, DNAzyme).

Following is a list of agents capable of downregulating expression level of FNR.

One agent capable of downregulating a FNR is a small interfering RNA (siRNA) molecule. RNA interference is a two step process. The first step, which is termed as the initiation step, input dsRNA is digested into 21-23 nucleotide (nt) small interfering RNAs (siRNA), probably by the action of Dicer, a member of the RNase III family of dsRNA-specific ribonucleases, which processes (cleaves) dsRNA (introduced directly or via a transgene or a virus) in an ATP-dependent manner. Successive cleavage events degrade the RNA to 19-21 bp duplexes (siRNA), each with 2-nucleotide 3' overhangs [Hutvagner and Zamore *Curr. Opin. Genetics and Development* 12:225-232 (2002); and Bernstein *Nature* 409:363-366 (2001)].

In the effector step, the siRNA duplexes bind to a nuclease complex to form the RNA-induced silencing complex (RISC). An ATP-dependent unwinding of the siRNA duplex is required for activation of the RISC. The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA into 12 nucleotide fragments from the 3' terminus of the siRNA [Hutvagner and Zamore *Curr. Opin. Genetics and Development* 12:225-232 (2002); Hammond et al. (2001) *Nat. Rev. Gen.* 2:110-119 (2001); and Sharp *Genes. Dev.* 15:485-90 (2001)]. Although the mechanism of cleavage is still to be elucidated, research indicates that each RISC contains a single siRNA and an RNase [Hutvagner and Zamore *Curr. Opin. Genetics and Development* 12:225-232 (2002)].

Because of the remarkable potency of RNAi, an amplification step within the RNAi pathway has been suggested. Amplification could occur by copying of the input dsRNAs which would generate more siRNAs, or by replication of the siRNAs formed. Alternatively or additionally, amplification could be effected by multiple turnover events of the RISC [Hammond et al. *Nat. Rev. Gen.* 2:110-119 (2001), Sharp *Genes. Dev.* 15:485-90 (2001); Hutvagner and Zamore *Curr. Opin. Genetics and Development* 12:225-232 (2002)]. For more information on RNAi see the following reviews Tuschl *ChemBiochem.* 2:239-245 (2001); Cullen *Nat. Immunol.* 3:597-599 (2002); and Brantl *Biochem. Biophys. Act.* 1575:15-25 (2002).

Synthesis of RNAi molecules suitable for use with the present invention can be effected as follows. First, the FNR mRNA sequence is scanned downstream of the AUG start codon for AA dinucleotide sequences. Occurrence of each AA and the 3' adjacent 19 nucleotides is recorded as potential siRNA target sites. Preferably, siRNA target sites are selected from the open reading frame, as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex [Tuschl *ChemBiochem.* 2:239-245]. It will be appreciated though, that siRNAs directed at untranslated regions may also be effective, as demonstrated for GAPDH wherein siRNA directed at the 5' UTR mediated about 90 % decrease in cellular GAPDH mRNA and completely abolished protein level (www.ambion.com/techlib/tn/91/912.html).

Second, potential target sites are compared to an appropriate genomic database (e.g., plant or bacteria etc.) using any sequence alignment software, such as the BLAST software available from the NCBI server (www.ncbi.nlm.nih.gov/BLAST/). Putative target sites which exhibit significant homology to other coding sequences are filtered out.

Qualifying target sequences are selected as template for siRNA synthesis. Preferred sequences are those including low G/C content as these have proven to be more effective in mediating gene silencing as compared to those with G/C content higher than 55 %. Several target sites are preferably selected along the length of the target gene for evaluation. For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative control siRNA preferably include the same nucleotide composition as the siRNAs but lack significant homology to the genome. Thus, a scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene.

RNAi has been successfully used in plants for down-regulation of proteins- see for example Moritoh et al., *Plant and Cell Physiology* 2005 46(5):699-715.

Another agent capable of downregulating FNR is a DNAzyme molecule capable of specifically cleaving an mRNA transcript or DNA sequence of the FNR. DNAzymes are single-stranded polynucleotides which are capable of cleaving both single and double stranded target sequences (Breaker, R.R. and Joyce, G. *Chemistry and Biology* 1995;2:655; Santoro, S.W. & Joyce, G.F. *Proc. Natl. Acad. Sci. USA* 1997;94:4262) A general model (the "10-23" model) for the DNAzyme has been proposed. "10-23" DNAzymes have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. This type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro, S.W. & Joyce, G.F. *Proc. Natl. Acad. Sci. USA* 1999; for rev of DNAzymes see Khachigian, LM [*Curr Opin Mol Ther* 4:119-21 (2002)].

Examples of construction and amplification of synthetic, engineered DNAzymes recognizing single and double-stranded target cleavage sites have been disclosed in U.S. Pat. No. 6,326,174 to Joyce et al.

Downregulation of a FNR can also be effected by using an antisense polynucleotide capable of specifically hybridizing with an mRNA transcript encoding the FNR. Design of antisense molecules which can be used to efficiently downregulate a FNR must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide which specifically binds the designated mRNA within cells in a way which inhibits translation thereof.

Algorithms for identifying those sequences with the highest predicted binding affinity for their target mRNA based on a thermodynamic cycle that accounts for the energetics of structural alterations in both the target mRNA and the oligonucleotide are also available [see, for example, Walton et al. *Biotechnol Bioeng* 65: 1-9 (1999)].

In addition, several approaches for designing and predicting efficiency of specific oligonucleotides using an in vitro system were also published (Matveeva et al., *Nature Biotechnology* 16: 1374 - 1375 (1998)].

For more information pertaining to the inhibition of gene expression in plant cells by expression of antisense RNA, see for example Joseph R. Ecker and Ronald W. Davis, *PNAS*, 1986, vol. 83, no. 15, 5372-5376.

Thus, the current consensus is that recent developments in the field of antisense technology which, as described above, have led to the generation of highly accurate antisense design algorithms and a wide variety of oligonucleotide delivery systems, enable an ordinarily skilled artisan to design and implement antisense approaches suitable for downregulating expression of known sequences without having to resort to undue trial and error experimentation.

Another agent capable of downregulating a FNR is a ribozyme molecule capable of specifically cleaving an mRNA transcript encoding FNR. Ribozymes are being increasingly used for the sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding proteins of interest [Welch et al., *Curr Opin Biotechnol.* 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications.

Yet another agent capable of downregulating FNR would be any molecule which binds to and/or cleaves FNR. Such molecules can be FNR antagonists, or FNR inhibitory peptide.

It will be appreciated that a non-functional analogue of at least a catalytic or binding portion of FNR can be also used as an agent which downregulates FNR.

Another agent which can be used along with the present invention to downregulate FNR is a molecule which prevents FNR activation or substrate binding.

As mentioned, the fusion protein and electron donor may also be combined in a non-cellular system.

In one embodiment the components of the present invention are suspended in a buffered aqueous solution at a pH at which both the photosynthetic components (e.g. PSI) and ferredoxin-hydrogenase fusion protein are active (for example, in a solution of about 2 mM to about 500 mM Tris-HCl, pH 8.0, preferably 30 mM Tris-HCl to 100 mM Tris-HCl, and preferably about 40 mM Tris-HCl), at a temperature at which both the fusion protein of the present invention and the photosynthetic components are active (generally about 10 °C to about 40 °C), and with an appropriate electron donor.

In one embodiment, the fusion protein of the present invention and the electron donor are encapsulated in a carrier system (i.e., encapsulating agent) of desired properties. In a specific embodiment, the encapsulating agent is a liposome.

As used herein and as recognized in the art, the term "liposome" refers to a synthetic (i.e., not naturally occurring) structure composed of lipid bilayers, which enclose a volume. Exemplary liposomes include, but are not limited to emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. The liposomes may be prepared by any of the known methods in the art [Monkkonen, J. *et al.*, 1994, *J. Drug Target*, 2:299-308; Monkkonen, J. *et al.*, 1993, *Calcif. Tissue Int.*, 53:139-145; Lasic D D., *Liposomes Technology Inc.*, Elsevier, 1993, 63-105. (chapter 3); Winterhalter M, Lasic D D, *Chem Phys Lipids*, 1993 September;64(1-3):35-43]. The liposomes may be positively charged, neutral, or, negatively charged.

The liposomes may be a single lipid layer or may be multilamellar. In the case of PS-I as the electron donor, multilamellar vesicles may be advantageous. Alternatively, it may

advantageous to increase the surface area of the liposome and adsorb the PS-I on the surface thereof. An exemplary liposomal system for the polypeptides of the present invention includes PS-I constructed within the lipid bilayer, and the fusion protein of the present invention constructed in the enclosed volume as illustrated in Figures 2A-B. Surfactant peptide micelles are also contemplated.

In another embodiment, the PSI and fusion protein of the present invention are embedded in a carrier (i.e., embedding agent) of desired properties. In specific embodiments, the embedding agent (or carrier) is a microparticle, nanoparticle, nanosphere, microsphere, nano-plate, microcapsule, or nanocapsule [M. Donbrow in: *Microencapsulation and Nanoparticles in Medicine and Pharmacy*, CRC Press, Boca Raton, Fla., 347, 1991]. The term carrier includes both polymeric and non-polymeric preparations. According to a specific embodiment, the embedding agent is a nanoparticle. The polypeptides of the present invention may be embedded in the nanoparticle, dispersed uniformly or non-uniformly in the polymer matrix, adsorbed on the surface, or in combination of any of these forms. Polymers which may be used for fabricating the nanoparticles include, but are not limited to, PLA (polylactic acid), and their copolymers, polyanhydrides, polyalkyl-cyanoacrylates (such as polyisobutylcyanoacrylate), polyethyleneglycols, polyethyleneoxides and their derivatives, chitosan, albumin, gelatin and the like.

It will be appreciated that the fusion protein of the present invention and the electron donor need not be encapsulated. Thus, according to yet another embodiment, the fusion protein and the electron donor of the present invention are free in solution.

Hydrogen gas can be harvested from the system of the present invention by direct or indirect biophotolysis:

Direct biophotolysis has been demonstrated under conditions where the resulting oxygen and hydrogen are flushed from the system using inert gas [Greenbaum 1988, *Biophysical Journal* 54: 365-368].

Indirect biophotolysis intends to circumvent the oxygen sensitivity of the hydrogenases by temporally separating the hydrogen-producing reactions from the oxygen evolving ones. According to one embodiment plant cells (e.g. algae) may be grown in open ponds to evolve oxygen and store carbohydrates. The plant cells may then be harvested, and placed in an anaerobic reactor. (For whole plants an anaerobic or a semi-anaerobic environment would be created in greenhouses by flushing nitrogen inside at night only). Induction of expression of the fusion protein of the present invention may then ensue concomitant with the inactivation of Photosystem II. Illumination then oxidizes the stored carbohydrate, lipid, and produces hydrogen, either directly or after an anaerobic dark fermentation [Hallenbeck PC 2002, *International Journal of Hydrogen Energy* 27: 1185-1193]. Since ideally only hydrogen and carbon dioxide are produced in the photobioreactor, gas handling is simpler and less hazardous.

As used herein the term "about" refers to $\pm 10\%$.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are

not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in

Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Cloning, expression, purification and analysis of hydrogenase-ferredoxin recombinant fusion proteins

MATERIALS AND METHODS

Cloning

A clone of ferredoxin (petF) was amplified from *Synechocystis pcc 6803* genome using the following primers:

Forward: ATCTATGGCATCCTATAACCG (SEQ ID NO: 7); and

Reverse to plant: TTATGCGGTGAGCTCTTCTTTGTGGTTTCAATG (SEQ ID NO: 8)

In addition the C-terminus of the native cyanobacterial ferredoxin was replaced with the higher plant ferredoxin C-terminus using the "reverse to plant primer – SEQ ID NO: 8".

A HydA1 gene of *Chlamydomonas reinhardtii* was expressed essentially as described in (King et al., 2006, J.Bacteriology: 2163–2172).

The ferredoxin petF was fused to the C-terminus of the target hydrogenase HydA1 according to the free space available in the PSI ferredoxin binding site of plant PSI utilizing the published structures of PSI, ferredoxin and hydrogenase [Peters et al., 1998, Science, 282, 4 Dec; Nicolet et al., 1999, Structure 7, 13-23; Asada et al., 2000, Biochim Biophys Acta 1490, 269-78; Ben-Shem et al., 2003, Nature 426, 630-5; Amunts et al., 2007, Nature 447, 58-63].

Different linkers as well as several deletions were prepared using the following primers:

Primary For' (Hyd For'): gatata CATATGGGCTGG (SEQ ID NO: 9)

Primary Rev' (Fd Rev'): accaga CTCGAGttatgcggtgagctcttc (SEQ ID NO: 10)

1) Hyd A1 Cr Fd no linker for construction of SEQ ID NO: 1, a direct fusion of HydA1 C-terminus to petF N-terminus.

Hyd-Fd Rev: GGTATAGGATGCCATTTTTTTTTCATCTTTTCTTCCAC (SEQ ID NO: 11).

Hyd-Fd For: gtggaagaaaaagatgaaaaaaaATGGCATCCTATAACCG (SEQ ID NO: 12).

2) Hyd A1 Cr Fd short linker for construction of SEQ ID NO: 2, a short linker of four glycine a single serine used to create a fusion of HydA1 C-terminus to petF N-terminus.

Hyd-Fd Rev: ggatccgccccaccTTTTTTTTCATCTTTTCTTCCAC (SEQ ID NO: 13).

Hyd-Fd For: ggtggcggcgatccATGGCATCCTATAACCG (SEQ ID NO: 14).

3) Hyd A1 Cr Fd medium linker for construction of SEQ ID NO: 3, a medium linker of two repeat of the short linker (composed from four glycine and a single serine) used to create a fusion of HydA1 C-terminus to petF N-terminus.

Hyd-Fd Rev: ggagccgccgcccggatcctcctcctcTTTTTTTTCATCTTTTCTTCCAC (SEQ ID NO: 15).

Hyd-Fd For: ggaggaggaggatccggcggcggcggctccATGGCATCCTATACCG (SEQ ID NO: 16).

4) Hyd A1 Cr C truncated Fd N truncated no linker for construction of SEQ ID NO: 4, a direct fusion of truncated (11aa were deleted at C-terminus) HydA1 C-terminus to truncated (30aa were deleted at N-terminus) petF N-terminus.

Hyd-Fd Rev: gaggataggtatcgtccacataatgggtatgcag (SEQ ID NO: 17).

Hyd-Fd

For: ctgcataccattatgtggacgatacctatacctc (SEQ ID NO: 18).

5) Hyd A1 Cr C truncated Fd no linker for construction of SEQ ID NO: 5, a direct fusion of truncated (11aa were deleted at C-terminus) HydA1 C-terminus to petF N-terminus.

Hyd-Fd Rev: cggtaggtatgcatcacataatgggtatgcag (SEQ ID NO: 19).

Hyd-Fd For: ctgcataccattatgtgatggcatcctataccg (SEQ ID NO: 20).

6) Hyd A1 Cr Fd N truncated no linker for construction of SEQ ID NO: 6, a direct fusion of HydA1 C-terminus to truncated (30aa were deleted at N-terminus) petF N-terminus.

Hyd-Fd Rev: gaggataggtatcgtcttttttcatcttttctTCCAC (SEQ ID NO: 21).

Hyd-Fd For: gtggaagaaaaagatgaaaaaaagacgatacctatacctc (SEQ ID NO: 22).

HydA1 and petF genes were assembled using two PCR steps. In the first PCR, each gene HydA1 and petF were amplified separately with the appropriate primers set, which include overlapping region with the product of each other (at sense primers of petF and at antisense primers of HydA1). In the next step the two products of these PCRs were mixed at 1:1 proportion and amplified in a second PCR reaction using only the edges primer set e.g sense primer of hydA1 and antisense primer of petF. The assembly gene fusion product was introduced into the pETDuet system and transformed together with the HydF+G in pCDFduet as described essentially in (King et al., 2006, J.Bacteriology: 2163–2172).

Protein Expression

Ferredoxin-hydrogenase fusion proteins were expressed in *E. coli*. (BL-21 DE3 Rosetta). Essentially, an overnight starter of 5 ml TB + ampicillin (Amp) (100 g/ml) 15 µl + 5 streptomycin (Sm) (50 g/ml) was washed twice with fresh media (to remove traces of β-lactamases). 2 ml of the washed starter was diluted into 100ml TB and 30mg (300µl of 100 mg/ml stock solution) Amp and 5 mg Sm (100µl of 50 mg/ml stock solution) was added. The bacteria were grown in 250 ml flask for ~2.5 hr at 37°C; 250 RPM to reach O.D₆₀₀=0.5-0.7. Next, Fe citrate 10 mg/100ml was added and bacteria were grown for an additional 10 minutes at 37°C; 250 RPM. Induction was initiated by the addition of IPTG 37.5 mg/100ml. The bacteria were then aerobically grown for 1 hour at 30°C; 250 RPM, and then transferred to a 100 ml bottle. Argon gas was added directly into

the growing media to create bubbles for an additional 6 hours at 30 °C to maintain anaerobic conditions.

Activity assay

The activity assay was carried as described (King et al., 2006, J. Bacteriol: 2163–2172) as follows: Activities of hydrogenases alone was measured as the evolution of H₂ gas from reduced methyl viologen (MV). Activity assays of whole cells extracts were performed with argon-flushed vials that contained an anaerobically prepared whole-cell extract comprising reaction buffer (50 mM potassium phosphate, pH 7; 10 mM MV; 20 mM sodium dithionite; 6 mM NaOH; 0.2 % Triton X-100) and 1 ml of anaerobically grown and induced cells.

The fusion protein was analyzed for the ability to produce hydrogen directly from dithionite as an electron donor using their fused ferredoxin part as the electron mediator replacing the function of the chemical mediator methyl viologen (native hydrogenase can not accept electrons directly from dithionite).

Gas chromatography

Hydrogen production was measured by gas chromatography using Varian 3600 machine equipped with thermal conductivity detector (TCD) and hydrogen column. Nitrogen was used as a carrier gas.

100 µl of gas sample was injected and the area of the specific Hydrogen peak, eluted ~1.25 minutes post injection was calculated using the area of 5.15 % hydrogen standard as reference.

RESULTS

The genes of *petF* and *HydA1* was successfully amplified using the primers and assembled together to form the chimera genes as can be seen in Figure 5.

Next these Hyd-Fd chimeras were cloned into pETDuet plasmids that contained the *HydE* structural gene as previously described (King et al., 2006, J. Bacteriol: 2163–2172) which is crucial for recombinant expression of hydrogenases in *E. coli*. Figures 6A-B show the two expression plasmids. Figure 6A is a map of the pETDuet plasmid which contains the Hyd-Fd chimeric genes between the restriction sites *NdeI* and *XhoI* of Multiple cloning site II. In addition, the plasmid contains the *HydE* structural gene. Figure 6B is a map of the plasmid CDFduet that contains the coding enquences for the two other structural proteins *HydF* and *HydG*, both of which are crucial for the recombinant expression of hydrogenase protein in *E.coli*.

Following preparation of DNA constructs, the protein expression pattern was tested by Western blot analysis using monoclonal antibodies against the StrepTag II (IBA, Gottingen, Germany) (which in our plasmids is located at the N-terminus of the *HydA1* protein or the Hyd-Fd chimera). All beside 6HydFd- SEQ ID NO: 6 (direct linkage of *HydA1* and N-terminus truncated *petF*) were well expressed (Figures 7A-B).

The hydrogen generated by the expressed chimeras was tested sequentially. First, the hydrogenase component of each chimera was tested separately using a well-known procedure by which dithionite donates electrons to the chemical electron mediator methyl viologen which feeds electrons directly to hydrogenase as described by (King et al., 2006, J. Bacteriol: 2163–2172).

Feredoxin electron mediating ability was tested by elimination of the chemical mediator methyl viologen. Under such conditions, hydrogen production by native hydrogenase is negligible while chimera protein produced at least 50 % of full activity with the chemical electron mediator methyl viologen. Figure 8 illustrates hydrogen generation by three selected chimera.

5 All the tested chimeras generated at least 4 fold more hydrogen than the native hydrogenase.

WHAT IS CLAIMED IS:

1. An isolated polypeptide comprising a hydrogen generating enzyme attached to a heterologous ferredoxin.
2. The isolated polypeptide of claim 1, wherein said hydrogen generating enzyme is a hydrogenase.
3. The isolated polypeptide of claim 2, as set forth in SEQ ID NOs: 23, 26, 27 or 28.
4. The isolated polypeptide of claim 1, wherein said hydrogen generating enzyme is a nitrogenase.
5. The isolated polypeptide of claim 2, wherein said hydrogenase enzyme is selected from the group consisting of an Fe only hydrogenase, a Ni-Fe hydrogenase and a non-metal hydrogenase.
6. The isolated polypeptide of claim 1, further comprising a linker, capable of linking said hydrogen generating enzyme to said ferredoxin.
7. The isolated polypeptide of claim 6, wherein said linker is a covalent linker.
8. The isolated polypeptide of claim 6, wherein said linker is a non-covalent linker.
9. The isolated polypeptide of claim 7, wherein said covalent linker is a peptide bond.
10. The isolated polypeptide of claim 9, as set forth in SEQ ID NOs: 24 or 25.
11. An isolated polynucleotide encoding the polypeptide of claim 1.
12. The isolated polynucleotide of claim 11, comprising a nucleic acid sequence as set forth in SEQ ID NOs: 1-6.
13. A nucleic acid construct, comprising the polynucleotide of claim 11.
14. The nucleic acid construct of claim 13, further comprising a cis-regulatory element.
15. The nucleic acid construct of claim 14, wherein said cis-regulatory element is a promoter.

16. The nucleic acid construct of claim 15, wherein said promoter is an inducible promoter.
17. A cell comprising the isolated polynucleotide of claim 11.
18. The cell of claim 17, being a prokaryotic cell.
19. The cell of claim 17, being a eukaryotic cell.
20. The cell of claim 18, wherein said prokaryotic cell is a cyanobacteria cell.
21. The cell of claim 17, wherein said cell is an algae cell.
22. The cell of claim 17, wherein said eukaryotic cell is part of a higher plant.
23. A method of generating hydrogen, the method comprising combining the polypeptide of claim 1 with an electron donor so as to generate an electron transfer chain, wherein said electron transfer chain is configured such that said electron donor is capable of donating electrons to the polypeptide of claim 1, thereby generating hydrogen.
24. The method of claim 23, wherein the generating hydrogen is effected under anaerobic conditions.
25. The method of claim 23, wherein said electron donor is selected from the group consisting of a biomolecule, a chemical, water, an electrode and a combination of the above.
26. The method of claim 23, wherein said electron donor comprises a biomolecule.
27. The method of claim 26, wherein said biomolecule is light sensitive.
28. The method of claim 27, wherein said light sensitive biomolecule comprises a photocatalytic unit of a photosynthetic organism.
29. The method of claim 28, wherein said photocatalytic unit comprises PSI.
30. The method of claim 29, wherein a ratio of the polypeptide of claim 1: said PSI is greater than 100:1.
31. The method of claim 27, wherein said light sensitive biomolecule comprises rhodopsin.

32. The method of claim 26, wherein said biomolecule is immobilized to a solid support.
33. The method of claim 26, wherein said chemical is selected from the group consisting of dithiothreitol, ascorbic acid, N, N, N', N'-tetramethyl-p-phenyldiamine (TMPD), 2,6-dichlorophenol indophenol and a combination of any of the above.
34. The method of claim 27, further comprising illuminating said light sensitive biomolecule following or concomitant with said combining.
35. The method of claim 23, further comprising harvesting the hydrogen following the generating.
36. The method of claim 23, wherein said combining is effected in a cell-free system.
37. The method of claim 35, wherein said cell-free system is selected from the group consisting of polymeric particles, microcapsules liposomes, microspheres, microemulsions, nanoplates, nanoparticles, nanocapsules and nanospheres.
38. The method of claim 21, wherein said combining is effected in a cellular system.
39. The method of claim 38, wherein said cellular system is selected from the group consisting of a cyanobacteria, an algae and a higher plant.
40. The method of claim 38, further comprising down-regulating an expression of endogenous ferredoxin in said cellular system.
41. A system comprising the polypeptide of claim 1 and an electron donor.
42. The system of claim 41, wherein said electron donor comprises an agent selected from the group consisting of a biomolecule, a chemical, water, an electrode and any combination of the above.
43. The system of claim 41, wherein said electron donor comprises a biomolecule.
44. The system of claim 43, wherein said biomolecule is light sensitive.
45. The system of claim 44, wherein said light sensitive biomolecule comprises a photocatalytic unit of a photosynthetic organism.
46. The system of claim 45, wherein said photocatalytic unit comprises PS-I.

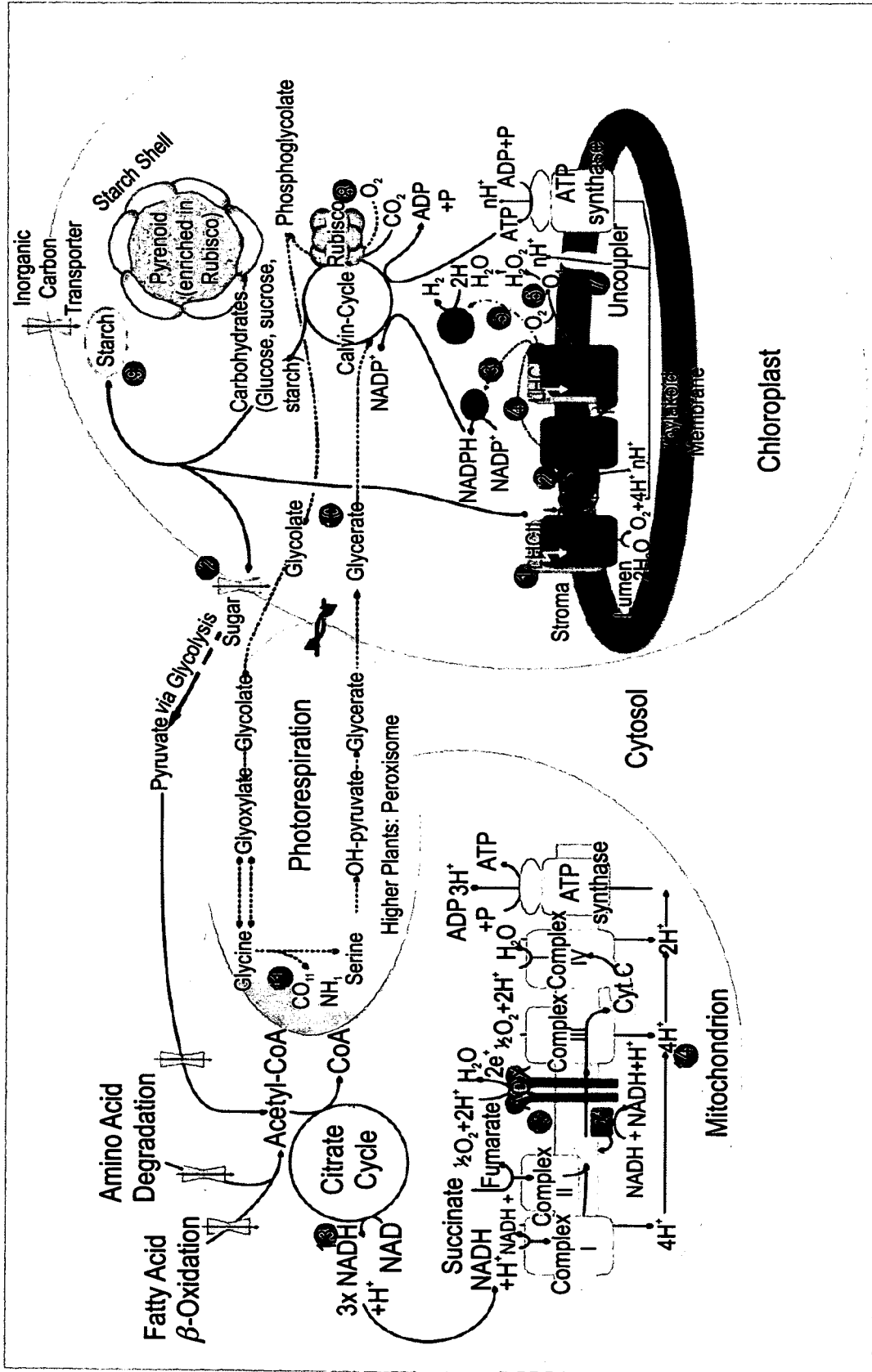
47. The system of claim 44, wherein said light sensitive biomolecule comprises rhodopsin.
48. The system of claim 43, wherein said biomolecule is immobilized to a solid support.
49. The system of claim 42, wherein said chemical is selected from the group consisting of dithiothreitol, ascorbic acid, N, N, N', N'-tetramethyl-p-phenyldiamine (TMPD), 2,6-dichlorophenol indophenol and a combination of at least any two of the above.
50. The system of claim 43, wherein a ratio of the polypeptide of claim 1: said biomolecule is greater than 100:1.
51. The system of claim 43, wherein said biomolecule is comprised in particles.
52. The system of claim 51, wherein said particles are selected from the group consisting of polymeric particles, microcapsules liposomes, microspheres, microemulsions, nanoparticles, nanocapsules, nano-plates and nanospheres.
53. The system of claim 43, wherein said biomolecule is encapsulated within said particle.
54. The system of claim 43, wherein said biomolecule is embedded within said particle.
55. The system of claim 43, wherein said biomolecule is adsorbed on a surface of said particle.
56. The system of claim 43, being expressed in cells.
57. The system of claim 56, wherein said cells are selected from the group consisting of cyanobacteria cells, algae cells and higher plant cells.
58. A bioreactor for producing hydrogen, comprising:
a vessel **321**, holding a hydrogen producing system, said system comprising a suspension of hydrogen generating enzyme attached to a heterologous ferredoxin and PSI;
a light providing apparatus comprising an optic fiber, said light providing apparatus being configured to provide light of a selected spectrum to said system; and
a gas liquid separation membrane for separating gas leaving the suspension from said suspension.
59. The bioreactor of claim 58, wherein said system comprises a suspension of cells.

60. The bioreactor of claim 58 or 59, wherein said system comprises a suspension of liposomes.

61. The bioreactor of claims 58-60, wherein said spectrum is selected as not to damage said system.

62. The bioreactor of claims 58-61, wherein said spectrum is selected to include activating wavelengths only.

FIG. 1



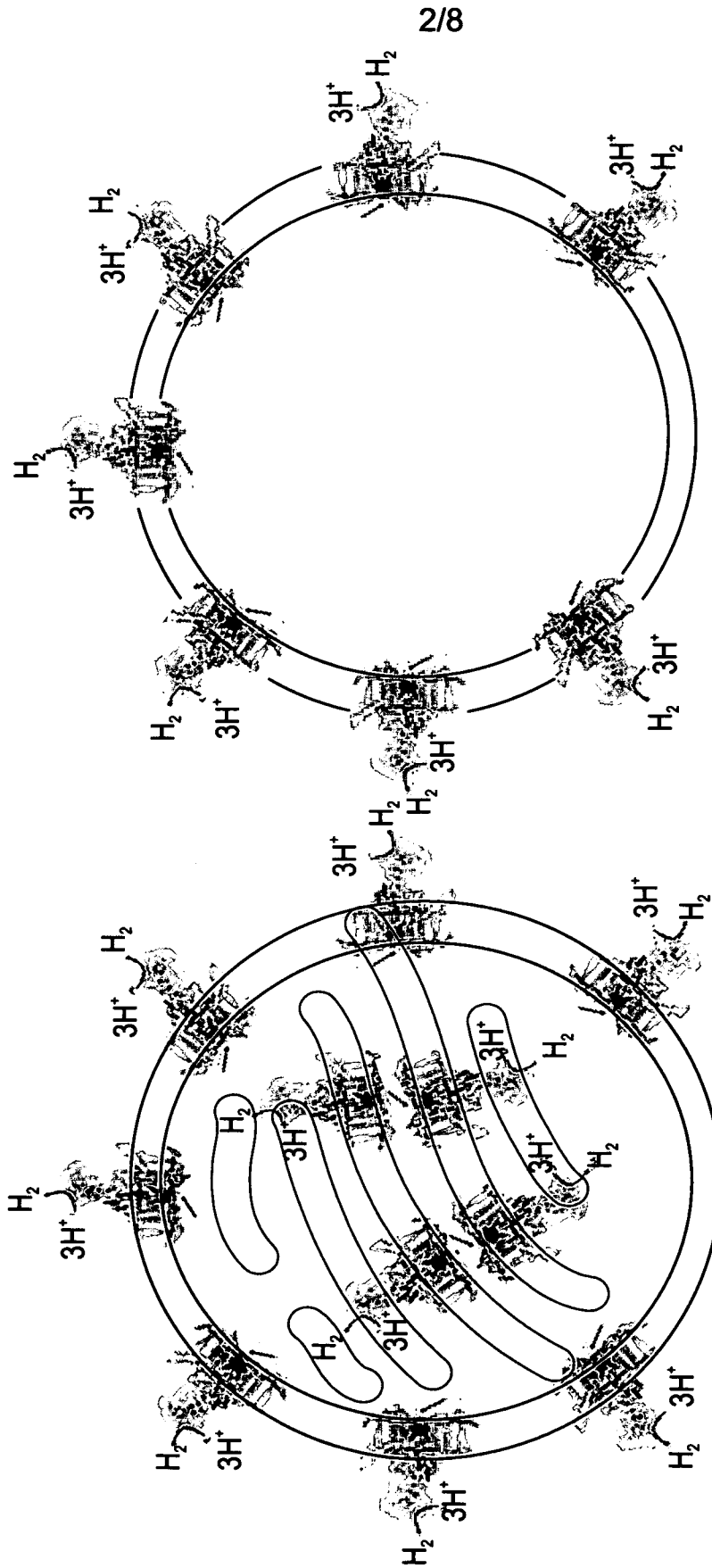
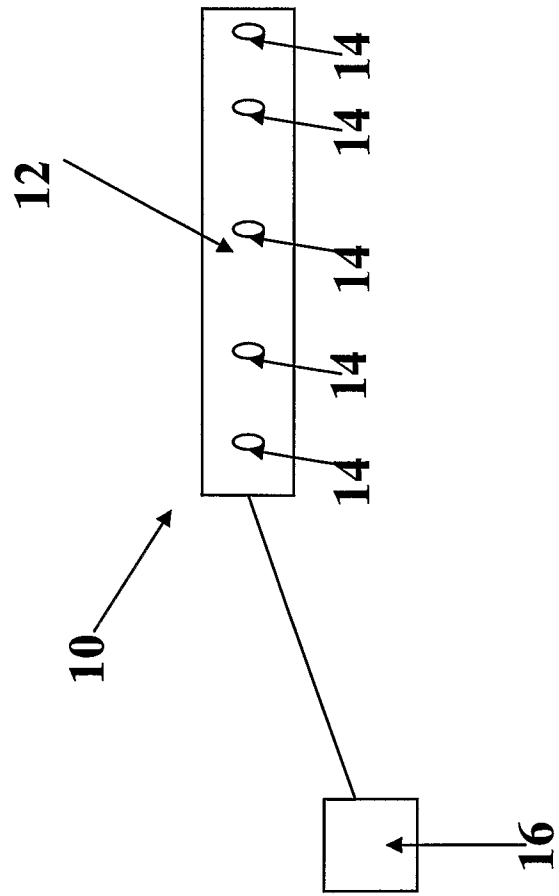


FIG. 2B

FIG. 2A

FIG. 3



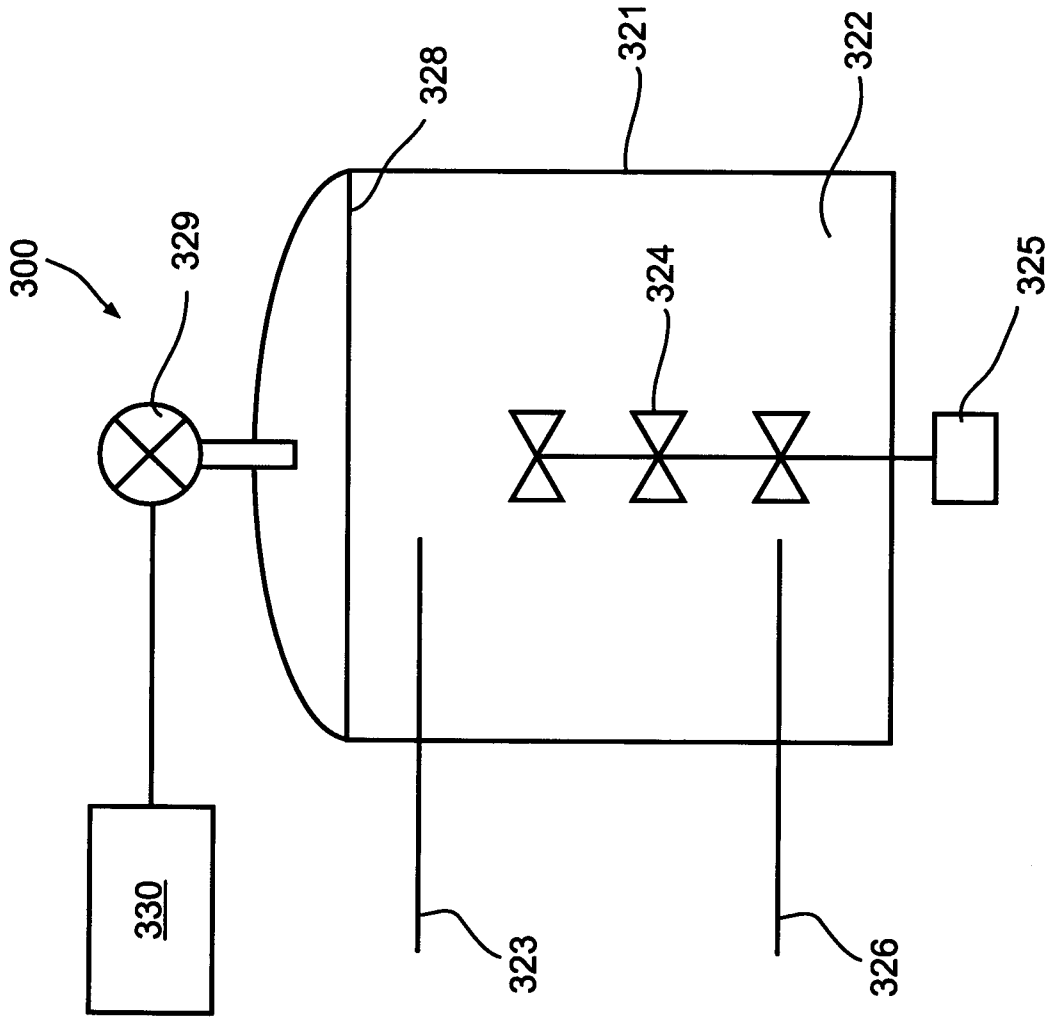
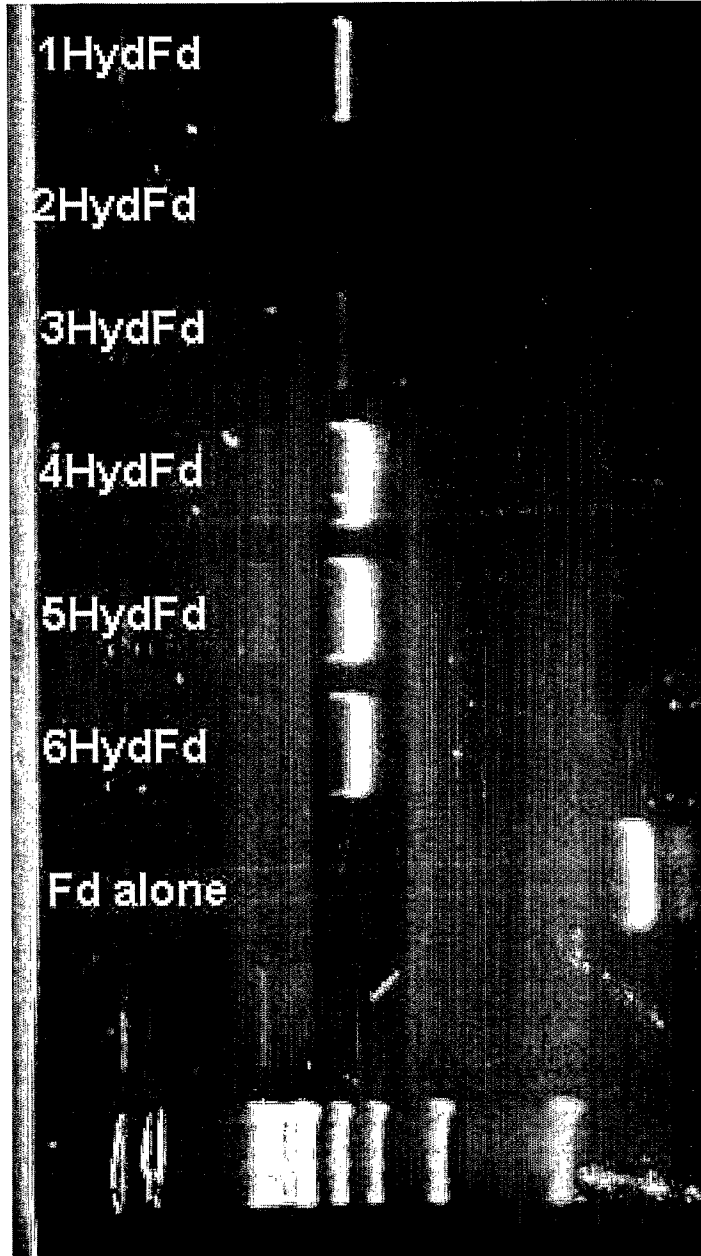


FIG. 4

FIG. 5



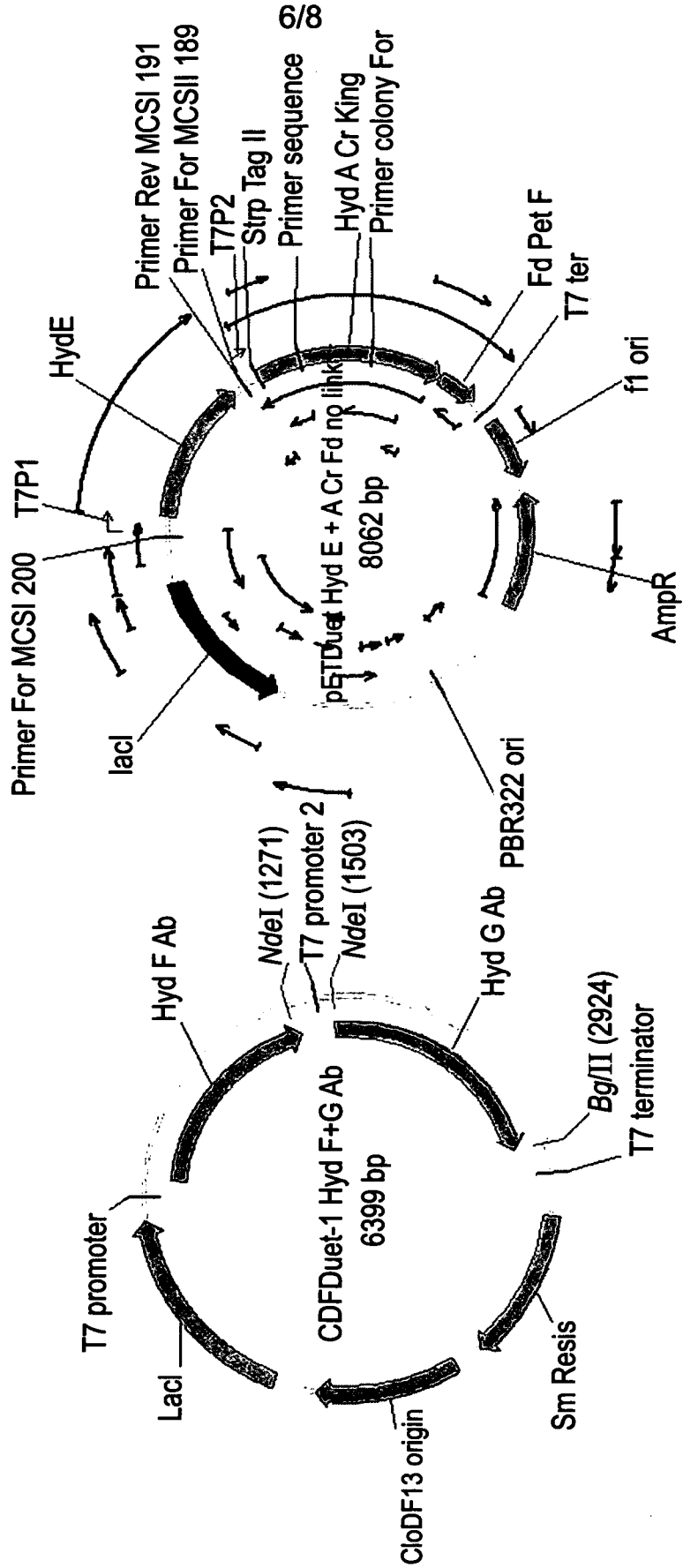


FIG. 6B

FIG. 6A

Expression of hydrogenase-ferredoxin fusion enzymes

FIG. 7A

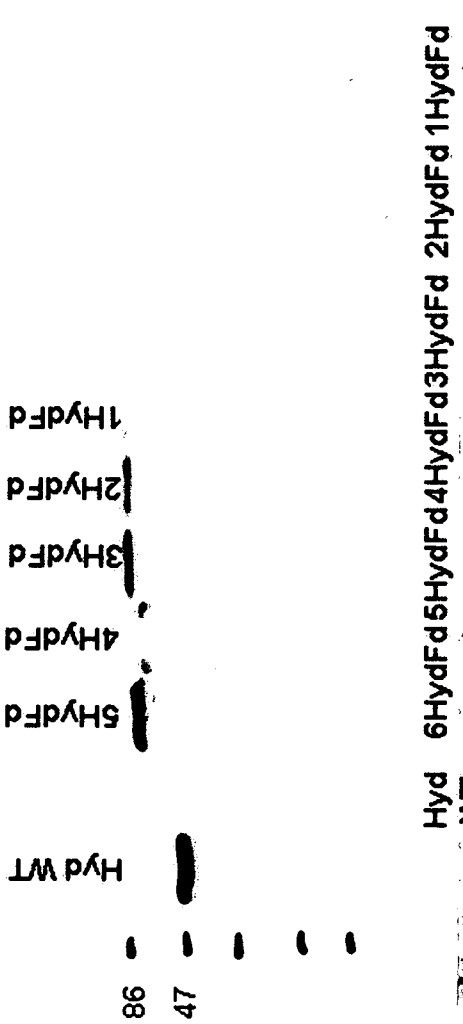


FIG. 7B

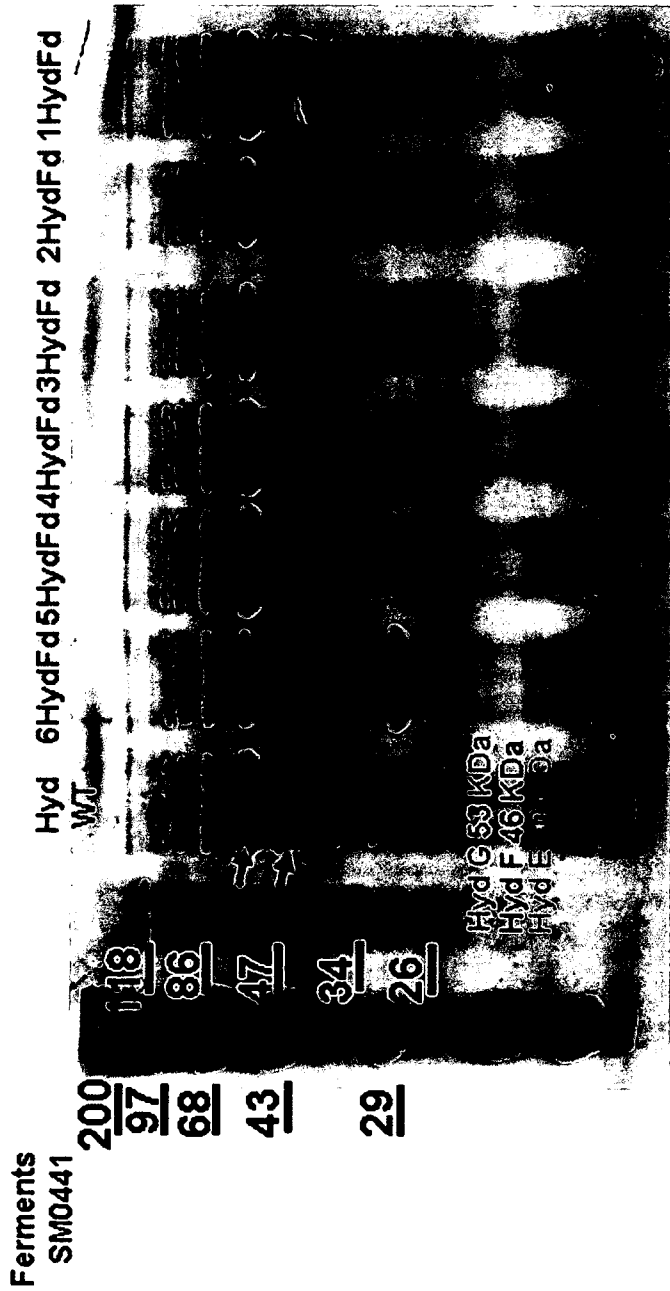
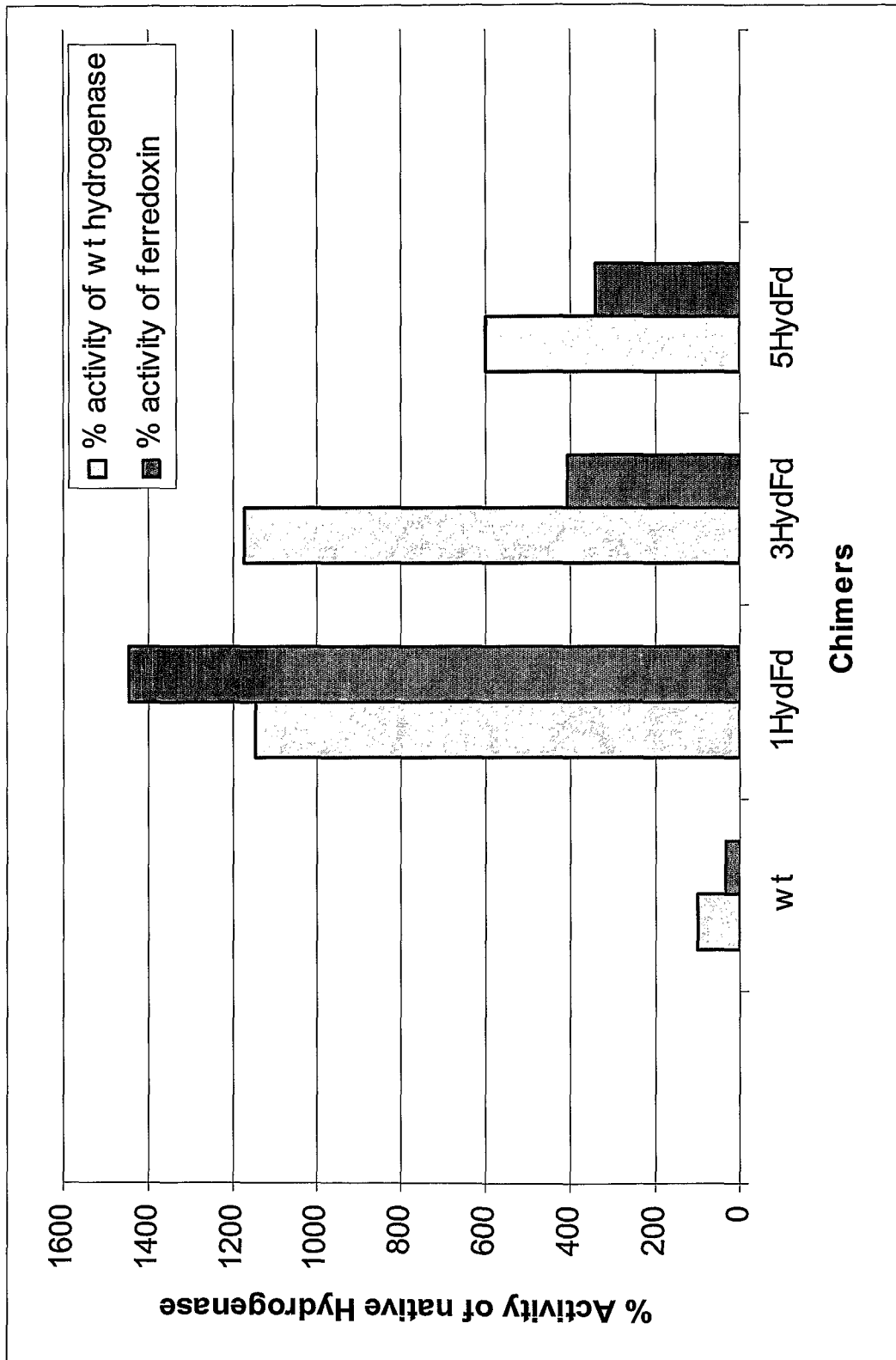


FIG. 8



INTERNATIONAL SEARCH REPORT

International application No

PCT/IL2008/001018

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N9/02 C12N15/62 C07K14/00

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 C07K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, FSTA, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>IHARA MASAKI ET AL: "Photoinduced hydrogen production by direct electron transfer from photosystem I cross-linked with cytochrome c3 to [NiFe]-hydrogenase." PHOTOCHEMISTRY AND PHOTOBIOLOGY 2006 NOV-DEC, vol. 82, no. 6, November 2006 (2006-11), pages 1677-1685, XP002497931 ISSN: 0031-8655 cited in the application the whole document</p> <p style="text-align: center;">----- -/--</p>	1-62



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

14 October 2008

Date of mailing of the international search report

30/10/2008

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INTERNATIONAL SEARCH REPORT

International application No

PCT/IL2008/001018

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	BASHTON ET AL: "The Generation of New Protein Functions by the Combination of Domains" STRUCTURE, CURRENT BIOLOGY LTD., PHILADELPHIA, PA, US, vol. 15, no. 1, 16 January 2007 (2007-01-16), pages 85-99, XP005827821 ISSN: 0969-2126	

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Information on patent family members

International application No

PCT/IL2008/001018

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		EP 1853716 A2	14-11-2007
		JP 2008531055 T	14-08-2008
		KR 20080011274 A	01-02-2008