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(71) Applicants: **DEPARTMENT OF BIOTECHNOLOGY MINISTRY OF SCIENCE & TECHNOLOGY** [IN/IN]; Block 2, 6-8th Floors, C.G.O. Complex, Lodi Road, New Delhi 110 003 (IN). **JAWAHARLAL NEHRU UNIVERSITY** [IN/IN]; New Mehrauli Road, New Delhi 110 067 (IN).

(72) Inventor: **DAS, Hirendra, Kumar**; B-9/6324, Vasant Kunj, New Delhi 110 070 (IN).

(74) Agents: **GROVER, Sri Vivek** et al.; Krishna & Saurastri Associates, 74/F, Venus, Worli Sea Face, Mumbai 400 018, Maharashtra (IN).

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(54) Title: RECOMBINANT NITROGEN FIXING MICROORGANISM AND USES THEREOF

(57) Abstract: A recombinant microorganism is provided herein, in particular, a recombinant microorganism of the Azotobacteraceae family. The recombinant Azotobacter microorganism is capable of fixing atmospheric nitrogen continuously in the presence of oxygen and externally fixed nitrogen sources. The present invention further provides a process for production of the recombinant microorganism and a composition comprising the recombinant microorganism for use as biofertilizers and/or for use in the preparation of a fertilizer composition. The recombinant microorganism produced by this invention is an environmental friendly, highly beneficial microorganism.

TITLE

RECOMBINANT NITROGEN FIXING MICROORGANISM AND USES THEREOF.

FIELD OF INVENTION

The present invention relates to the field of microbiology and genetic engineering. The present invention relates to a recombinant microorganism, in particular it is related to recombinant *Azotobacter* capable of fixing atmospheric nitrogen in the presence of fixed nitrogen, oxygen or a combination thereof.

BACKGROUND OF THE INVENTION

Biological nitrogen fixation (BNF) occurs when atmospheric nitrogen is converted to ammonia by an enzyme called nitrogenase. Only some selected microorganisms are able to transform nitrogen from the abundant gaseous form to usable combined nitrogen compounds. Microorganisms that fix nitrogen are called diazotrophs. Enzymes responsible for nitrogenase action are very susceptible to destruction by oxygen. The *nif* genes are responsible for the coding of the nitrogenase proteins and other proteins related to and associated with the fixation of atmospheric nitrogen into a form of nitrogen available to plants. The *nif* genes have both positive and negative regulators. Besides the nitrogenase enzyme, the *nif* genes also encode a number of regulatory proteins involved in nitrogen fixation. The expression of *nif* genes is induced as a response to low concentrations of fixed nitrogen and low oxygen

concentrations (the low oxygen concentrations are actively maintained in the root environment). In most of the nitrogen fixing microorganisms, activation of transcription of the *nif* genes is done by the NifA protein. When there is not enough fixed nitrogen available for the use of the nitrogen fixing microorganisms, NifA expression is initiated from the native promoter of the *nifA* gene, while the NifA protein in turn leads to activation of the remaining *nif* genes transcription. If there is sufficient amount of reduced nitrogen or if oxygen is present, *nifA* gene promoter is not activated and no NifA protein expression takes place. On the other hand, another protein, NifL, is activated in presence of reduced nitrogen or oxygen, and this activated NifL inhibits NifA protein activity by interacting with it, resulting in the inhibition of the formation of nitrogenase and all other accessory proteins necessary for nitrogen fixation.

Nitrogen fixation in the free-living, aerobic, heterotrophic, diazotrophic gram negative soil bacterial genus, *Azotobacter* spp is regulated by the *nifLA* operon. Here also NifA protein activates the transcription of all the *nif* genes, while NifL protein antagonizes the transcriptional activator NifA in response to fixed nitrogen and molecular oxygen. The expression of the *nif* operons of *Azotobacter* is mediated by sigma-54 transcription factor, rather than the more common sigma-70 transcription factor. A typical characteristic of sigma 54 transcription factors is the requirement of an activator that must bind to DNA at a site about 100 or more bases upstream of the promoter. The *nifA* gene is present in *Azotobacter* in the *nifLA* operon and is located

distal to the promoter of *nifLA* operon. The *nifL* gene is also present in *Azotobacter* in the *nifLA* operon and is located proximal to the promoter of *nifLA* operon. Here also, NifL is the negative regulator, which is activated in presence of oxygen or ammonia. NifL inactivates NifA by interacting with it. In addition to NifA, the promoter of the native *nifLA* operon is also repressed by oxygen and ammonia. The *nifLA* operon serves as the master regulatory operon for the entire process of nitrogen fixation. Raina *et al.* [1993, *Mol. Gen. Genet.* 237: 400-406] has fully characterized the *nifL* gene of *Azotobacter vinelandii* and has elucidated its regulation. Unlike in the case of *Klebsiella pneumoniae*, the expression of the *nif LA* operon in *Azotobacter vinelandii* is not autogenously regulated. Bali *et al.* [1992, *App. Env. Microbiol.* 58: 1711-1718] inserted an antibiotic resistance cassette upstream of *nifA* of *Azotobacter vinelandii* and observed enhanced nitrogen fixation and excretion. Brewin *et al.* [1999, *J. Bacteriol.* 181: 7356-7362] studied ammonium excretion in *nifL* mutants of *Azotobacter vinelandii* obtained by insertion of antibiotic resistance cassette and concluded that ammonium is excreted from the cell passively. In *Azotobacter vinelandii*, current evidence suggests that NifL controls the activity of NifA by a relatively stable protein-protein interaction that is modulated by redox changes, ligand binding, and interactions with other signal transduction proteins and membrane components. *Azotobacter vinelandii* NifL contains a conserved histidine residue found in the transmitter domains of histidine kinases, suggesting that this NifL might employ a classical phosphoryl transfer mechanism to communicate

environmental signals to NifA. However, replacement of this conserved histidine by a number of other amino acids does not disable signal transduction. Furthermore, NifL is competent to inhibit NifA *in vitro* in the absence of ATP, and signal transduction requires stoichiometric protein-protein interactions between the two regulatory proteins (Martinez-Argudo *et al., J Bacteriol.*, 2004, 186(3): 601–610).

In both cereals and non-cereal crops, there is a need to supply extra fixed nitrogen by industrially-fixed nitrogen or biologically fixed nitrogen to supplement nitrogen availability in the soil. Nitrogen released to the available pool by mineralization is expected to depend on the amount of soil-nitrogen removed in the harvested produce, leaching of inorganic nitrogen (*e.g.* $NO3^-$ –N) to groundwater, the magnitude of denitrification of soil-nitrogen as N_2O or N_2, the extent and duration of immobilization of N and its rate of remobilization in the soil biomass (I.R. Kennedy *et al.*, 2004, Soil Biology & Biochemistry 36: 1229–1244). Inoculant biofertilizers, particularly nitrogen-fixing bacterial diazotrophs, can help ensure that the supply of nutrients contributing to optimized yield is maintained. However, in the presence of chemical nitrogenous fertilizers, there is no biological nitrogen fixation, because the ammonium generated by the chemical fertilizer switches off the *nifLA* operon and therefore, subsequently all the *nif* operons.

United States Patent 6548289 describes a method for increasing the rate of conversion of atmospheric nitrogen into ammonia in the genus *Rhizobium*, by increasing the intracellular level of the activator protein NifA, by introducing a

plasmid containing the *nifA* gene under an inducible or a constitutive promoter. The problem with this method is that the plasmid has an antibiotic resistance gene, which is undesirable from environmental considerations. Besides, the presence of the antibiotic in the medium or the soil would be essential for stable maintenance of the plasmid inside the bacteria. Thus, continuous selection pressure is required to maintain the stability of the plasmids in the bacteria.

United States Patent Application 20060270555 describes transformation of root nodule bacteria with a catalase gene leading to enhanced nitrogen-fixation ability, a preparation for leguminous crops containing the root nodule bacteria as an active ingredient, and a method of cultivating leguminous crops comprising contacting seeds of crops with the transformed bacteria.

Recombinant microorganisms showing uninterrupted biological nitrogen fixation has been investigated by other researchers by inserting an antibiotic resistance cassette into the *nifL* gene. However, such microorganisms harboring the antibiotic resistance genes are not acceptable for agricultural use due to environmental concerns. There is a dire need to reduce costs associated with chemical fertilizers and reduce the contribution of these chemical fertilizers to environmental pollution. Therefore, it is highly imperative that novel and inventive products and processes should be developed such that the agricultural costs are reduced with a beneficial effect on the environment.

SUMMARY OF THE INVENTION

One aspect of the present invention provides a recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

Another aspect of the present invention provides a process for producing a recombinant microorganism, wherein the process comprises (a) inserting a disrupted *nifL* gene and a *nifA* gene with the native promoter into the chromosome of a microorganism through homologous recombination with a recombinant DNA molecule comprising a disrupted chromosomal *nifL* gene, a *nifA* gene with native promoter and an antibiotic resistance marker gene to obtain a transformed microorganism, wherein the *nifL* gene is disrupted by insertional mutagenesis, deletion of genomic DNA, targeted gene disruption or introduction of a genomic or episomal vector, and (b) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the transformed microorganism through homologous recombination with a recombinant DNA molecule comprising the constitutive heterologous promoter to obtain a recombinant microorganism comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter, wherein the recombinant microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic

resistance marker gene.

Azotobacters are known to have multiple chromosomes [Punita et al J. Bacteriol 171(1989) 3133-3138]. This two-step procedure was essential to adopt so as to exert strong selection pressure for making sure that the mutation is transmitted to each and every copy of the chromosome, as otherwise reversion would take place after some time.

BRIEF DESCRIPTION OF ACCOMPANYING DRAWINGS

Figure 1 shows the following elements:

Figure 1A shows the partial restriction map of the 7.5 kb Bam HI fragment from *Azotobacter chroococcum* CBD15 which contains regions homologous to *nifL* and *nifA* of *Azotobacter vinelandii* UW, cloned in the Bam HI site of pUC7. This construct is denoted as pCL6. The restriction enzyme sites are denoted as E: EcoRI; B: BamHI; Nc: NcoI; Sa: SacII; S: SalI; K: KpnI. The restriction sub-fragments that hybridize with *nifL* and *nifA* of *Azotobacter vinelandii* UW are underlined.

Figure 1B shows the 4.8 kb EcoRI fragment from pCL6 containing the regions homologous to *nifL* and *nifA* of *Azotobacter vinelandii* UW cloned in pUC7. This construct is denoted as pCL6.2.

Figure 1C shows the 2.0 kb EcoRI fragment from pHP45ΩKm containing the interposon ΩKm inserted in place of the Sal I sites of pCL6.2. This construct is denoted as pCL6.3.

Figure 1D shows the deletion of a 1.1 kb DNA fragment around the Sal I sites from pCL6.2 and insertion of 0.38 kb EcoRI-BamHI fragment containing the pBR322 constitutive promoter into the same site. This construct is denoted as pCL6.4.

Figure 1E shows the insertion of the construct pCL6.3 into *Azotobacter chroococcum* CBD15 by electroporation.

Figure 1F shows the integration of the ΩKm interposon into the *nifL* gene in the genome of *Azotobacter chroococcum* CBD15 *via* homologous recombination.

Figure 1G shows the introduction of the construct pCL6.4 harbouring the ΩKm interposon in the *nifL* gene, by electroporation into *Azotobacter chroococcum* CBD15.

Figure 1H shows the replacement of the 1.1 kb DNA fragment around the Sal I sites of the *nifL* gene comprising the ΩKm interposon in the genome of *Azotobacter chroococcum* CBD15 with EcoRI-BamHI fragment of the pCL6.4 construct containing the pBR322 constitutive promoter, via homologous recombination.

Figure 2 shows the enhanced production and excretion of ammonia by *Azotobacter chroococcum* HKD15 compared to *Azotobacter chroococcum* CBD15.

OBJECTS OF THE INVENTION

An object of the present invention is to provide a recombinant microorganism capable of converting atmospheric nitrogen into biological fixed nitrogen in the presence of fixed nitrogen and oxygen.

Another object of the present invention is to provide a recombinant microorganism with increased capacity to fix atmospheric nitrogen in the presence of fixed nitrogen, and oxygen, wherein the recombinant microorganism does not comprise any antibiotic resistance marker gene.

Another object of the present invention is to provide crop plant inoculants and biofertilizer composition comprising the recombinant microorganism of the present invention capable of continuous biological nitrogen fixation in presence of fixed nitrogen and oxygen thereby increasing the yield of the crop plants and reducing the degradation of soil.

Another object of the present invention is to provide a way of reducing the application of chemical nitrogenous fertilizers in agriculture and reducing environmental pollution.

DETAILED DESCRIPTION OF THE INVENTION

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

In accordance with the present invention, there may be employed conventional molecular biology, microbiology and recombinant DNA techniques within the skill of the art. Such techniques are fully explained in the literature (T. Maniatis, E. F. Fritsch and J. Sambrook; 1982, *Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory*; F. M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, 1987, *Current Protocols in Molecular Biology*, Greene Publishing Associates/Wiley Interscience, New York).

Definitions

For convenience, before further description of the present invention, certain terms employed in the specification, examples and appended claims are provided here. These definitions should be read in light of the remainder of the disclosure and understood as by a person of skill in the art. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art.

The articles "a," "an" and "the" are used to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article.

Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated element or step or group of element or steps but not the exclusion of any other element or step or group of element or steps. It is not intended to be construed as "consists of only."

The term "including" is used to mean "including but not limited to".

The term "nucleic acid" or "recombinant nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA).

The polynucleotides described in the present description include "genes" and nucleic acid molecules described include "vectors" or "plasmids." Accordingly, the term "gene", also called a "structural gene" refers to a polynucleotide that codes for a particular sequence of amino acids, which comprise all or part of one or more proteins or enzymes, and may include regulatory (non-transcribed) DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed.

The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein results from transcription and translation of the open reading frame sequence.

A "vector" is any means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include viruses, bacteriophage, pro-viruses, plasmids, phagemids, transposons, and artificial chromosomes such as YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes), and PLACs (plant artificial chromosomes), and the like, that are "episomes," that is, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a

naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that are not episomal in nature, or it can be an organism which comprises one or more of the above polynucleotide constructs such as an agrobacterium or a bacterium.

"Transformation" refers to the process by which a recombinant DNA molecule is introduced into a host cell. Transformation (or transduction, or transfection), can be achieved by any one of a number of means including electroporation, microinjection, biolistics (or particle bombardment-mediated delivery), or *Agrobacterium*-mediated transformation.

Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given amino acid sequence of the disclosure. The native DNA sequence encoding the biosynthetic enzymes described are referenced herein merely to illustrate an embodiment of the disclosure, and the disclosure includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the enzymes utilized in the methods of the disclosure. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. The disclosure includes such polypeptides with

alternate amino acid sequences, and the amino acid sequences encoded by the DNA sequences shown herein merely illustrate embodiments of the disclosure.

The disclosure provides nucleic acid molecules in the form of recombinant DNA expression vectors or plasmids. Generally, such vectors can either replicate in the cytoplasm of the host microorganism or integrate into the chromosomal DNA of the host microorganism. In either case, the vector can be a stable vector (i.e., the vector remains present over many cell divisions, even if only with selective pressure) or a transient vector (i.e., the vector is gradually lost by host microorganisms with increasing numbers of cell divisions).

The disclosure provides DNA molecules in isolated (i.e., not pure, but existing in a preparation in an abundance and/or concentration not found in nature) and purified (i.e., substantially free of contaminating materials or substantially free of materials with which the corresponding DNA would be found in nature) forms.

The term "promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. Promoters that cause a gene to be expressed in most cell types at most times or under most environmental conditions are commonly referred to as "constitutive promoters". Promoters that cause a gene to be expressed only in the presence of a particular compound or environmental condition are commonly referred to as "inducible promoters".

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For

example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence. Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions, and methods are clearly within the scope of the invention, as described herein.

An *Azotobacter* mutant showing constitutive nitrogen fixation would be useful as biofertilizers. Freeing all the genes involved in nitrogen fixation from any negative regulation should, therefore, lead to enhanced biological nitrogen fixation, even in the presence of ammonium or other fixed nitrogen, so that chemical fertilizers and bacterial fertilizers can be used together.

The present invention provides a recombinant microorganism capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen, wherein the chromosome of the microorganism comprises a disrupted *nifL* gene, a *nifA* gene operably linked to a constitutive heterologous promoter and does not comprise any antibiotic resistance marker gene, wherein the *nifL* gene is disrupted by insertional mutagenesis. The recombinant microorganism of the present invention also does not contain any other external genes like toxic genes that are harmful to the environment. The desired characteristics in the recombinant microorganism are obtained through manipulation of its chromosome and without the necessity of maintaining a plasmid.

The present invention also provides the polynucleotide of *nifL* gene having the nucleotide sequence as set forth in SEQ ID NO: 1 (GenBank: X70993.1) and encoding the amino acid sequence as set forth in SEQ ID NO: 2 and the nucleotide sequence as set forth in SEQ ID NO: 3.

The present invention also provides the polynucleotide of *nifA* gene having the nucleotide sequence as set forth in SEQ ID NO: 4 (GenBank: Y00554.1) and encoding the amino acid sequence as set forth in SEQ ID NO: 5.

The *nifLA* operon serves as the master regulatory operon for the entire process of nitrogen fixation in microorganisms. The native promoter for *nifA* gene is an inducible promoter which is regulated by the presence of fixed nitrogen and oxygen. The inventors found a surprising and unexpected result when they manipulated the chromosomal *nifLA* operon, a two-component regulatory system, by disrupting the negative regulatory component by partial deletion of *nifL*, and operably linking the positive component, *nifA*, under a constitutive heterologous promoter for continuous expression in a free-living, diazotrophic microorganism. It was unexpectedly found that the recombinant microorganism does not comprise any antibiotic resistance marker gene and there was successful, uninhibited continuous expression of the nitrogen-sensitive NifA transcription activator which increased the level of biological nitrogen fixation in the recombinant microorganism. The recombinant microorganism was also found to produce and excrete a high level of ammonia compared with the non-recombinant microorganism. The use of a constitutive

promoter renders the recombinant microorganism very efficient in fixing the atmospheric nitrogen. The disruption of *nifL* by partial deletion renders the reversion to an active negative regulatory component impossible, and thus makes the recombinant microorganism very stable. The recombinant microorganism such as recombinant *Azotobacter* having Accession number MTCC 5679 so obtained is an ideal biofertilizer component as it does not comprise any antibiotic resistance marker gene or any other external genes like *Bt* genes, or any toxic genes.

The present invention also provides a process for producing a recombinant microorganism capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen, wherein the process comprises (a) inserting a disrupted *nifL* gene and a *nifA* gene with the native promoter into the chromosome of a microorganism through homologous recombination with a recombinant DNA molecule comprising a disrupted chromosomal *nifL* gene, a *nifA* gene with native promoter and an antibiotic resistance marker gene to obtain a transformed microorganism, wherein the *nifL* gene is disrupted by insertional mutagenesis, deletion of genomic DNA, targeted gene disruption or introduction of a genomic or episomal vector and (b) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the transformed microorganism through homologous recombination with a recombinant DNA molecule comprising a constitutive heterologous promoter to obtain a recombinant microorganism comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter, wherein the recombinant

microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

The process of the present invention comprises a novel process for the transformation of the microorganism through homologous recombination, wherein the chromosome of the recombinant microorganism comprises a disrupted *nifL* gene, a *nifA* gene operably linked to a constitutive heterologous promoter and does not comprise any antibiotic resistance marker gene. Thus, the recombinant microorganism produced by the process of the present invention is capable of fixing atmospheric nitrogen in a continuous manner and is not under the control of external regulators. The principle underlying the present invention should be applicable to all nitrogen fixing microorganisms, particularly where a two-component regulation is employed via a positive and a negative regulatory element.

In the present invention, deletion of a part of the *nifL* gene of *Azotobacter chroococcum* CBD15, a strain isolated from the soil at Indian Agricultural Research Institute, Delhi, India, was performed. A DNA fragment containing the constitutive tet promoter isolated from the plasmid pBR322 was inserted upstream of *nifA* of *Azotobacter chroococcum* CBD15 comprising the disrupted *nifL* gene. This newly created deletion mutant strain does not comprise antibiotic resistance marker gene and has been designated as *Azotobacter chroococcum* HKD15. The recombinant microorganism has been submitted at the MTCC, IMTECH, India. The Accession No. for the strain is MTCC 5679. The two-step strategy of first disrupting the *nifL*

gene by inserting an interposon and selecting for a *nif*-minus phenotype (absence of ability to fix atmospheric nitrogen) followed by replacing the interposon disrupted *nif L* gene with a partially deleted *nif L* gene and a heterologous constitutive promoter, to drive the positive regulatory element *nifA* resulting in a *nif*-plus phenotype (presence of ability to fix atmospheric nitrogen) is novel and not obvious.

In the present invention, the microbial DNA from *Azotobacter chroococcum* CBD15 is digested with restriction enzyme BamHI to obtain a genomic fragment comprising the *nifL* and the *nifA* genes. The nifA genes are identified and isolated on the basis of homology with the *nifA* and *nifL* genes already known in the art. A 7.5 kb genomic fragment comprising the *nifL* and the *nifA* genes is isolated on the basis of homology with the *nifA* and *nifL* genes of *Azotobacter vinelandii* UW (Figure 1A). This 7.5 Kb genomic fragment is cloned into pUC7 vector and subjected to further restriction to obtain a 4.8 kb genomic fragment comprising the *nifL* and the *nifA* genes. This 4.8 kb fragment is cloned into pUC7 vector and the construct was designated as pCL6.2 (Figure 1B). The pCL6.2 construct further comprises the 'second' antibiotic resistance marker gene (Ampicillin, Amp+) and is used for further transformation experiments.

Any vector that is stably replicated in any host cell for production of the constructs desired comprising the *nifL* and *nifA* genes of interest, but are unstable and do not replicate in the microorganisms which are to be transformed, may be used in this process.

In the first set of transformation experiments, the *nifL* gene is disrupted by insertional mutagenesis. An interposon of 2.0 kb having the 'first' antibiotic resistance marker gene (Kanamycin, Km+) is inserted in SalI restriction sites of the *nifL* gene of pCL6.2 construct, which is immediately upstream of the *nifA* gene and the construct is designated as pCL6.3(Figure 1C). This insertional mutagenesis renders the *nifL* gene non functional. The pCL6.3 recombinant plasmid is electroporated into the *Azotobacter* cells and homologous recombination is allowed to occur (Figure 1E), wherein the integration of the interposon into the chromosome of the cell takes place *via* homologous recombination. Transformed cells that have undergone successful homologous recombination *via* two-point crossing over are detected by their resistance to the 'first' antibiotic marker (Km+), sensitivity to the 'second' antibiotic marker (Amp-) and their inability to fix atmospheric nitrogen (Figure 1F).

In the second set of transformation experiments, 1.1 kb of DNA is deleted around the SalI sites from the pCL6.2 construct which renders partial deletion of the *nifL* gene and the 375 bp EcoRI-BamHI fragment containing the constitutive sigma-70 promoter/tet promoter of pBR322, as set forth in SEQ ID NO: 6, is inserted into SalI site to obtain the construct pCL6.4 (Figure 1D). Other constitutive promoters, either synthetic or natural, but, compatible with the microorganism, that may be used are synthetic promoters and natural promoters like P1, nptII, CAT promoter *etc.* The recombinant construct pCL6.4 containing the constitutive heterologous promoter is

further electroporated into the transformed *Azotobacter* cells obtained from the first set of transformation experiments. Successful homologous recombination *via* two-point crossing over between the flanking regions of the constitutive promoter and the chromosome of the first transformed *Azotobacter* cells result in deletion of the 'first' antibiotic resistance marker gene (Km), successful integration of the constitutive heterologous sigma-70/tet promoter operably linked to the *nifA* gene into the chromosome and the successful inhibition of the production of the Nif L protein of the microorganism (Figure 1H). The recombinant *Azotobacter* obtained by this second transformation step comprises a non-functional *nifL* gene, an over-expressing nifA gene and is devoid of any antibiotic resistance (Km-). The recombinant *Azotobacter* obtained by this process are tested for successful transformation based on their sensitivity to the 'first' antibiotic (Km-) and their ability to fix atmospheric nitrogen. The partial deletion of the *nifL* gene and the insertion of the constitutive heterologous promoter upstream of the *nifA* gene are further confirmed by conventional methods available in the art. This recombinant *Azotobacter* has been designated as *Azotobacter chroococcum* HKD15 and has been submitted at the MTCC, IMTECH, India on 14th December 2011. The Accession No. for the strain is MTCC 5679.

The biological nitrogen fixation in the presence of fixed nitrogen by the recombinant microorganism, *Azotobacter chroococcum* HKD15 having Accession number MTCC 5679 is improved over that of the non-recombinant, native *Azotobacter chroococcum*

CBD15. A 3-fold increase in nitrogen fixation was observed in the *Azotobacter chroococcum* HKD15 having Accession number MTCC 5679 compared to that of the non-recombinant native *Azotobacter chroococcum* CBD15 strain (Table 1). The *Azotobacter chroococcum* HKD15 having Accession number MTCC 5679 also show enhanced expression and excretion of ammonia wherein the ammonia excretion is increased by 9-fold compared to that of the non-recombinant native *Azotobacter chroococcum* CBD15 strain. Further, it was observed that expression is unaffected by the presence of fixed nitrogen in the form of urea or ammonium salts (Figure 2). The biofertilizer efficacy of the recombinant microorganism, *Azotobacter chroococcum* HKD15 having Accession number MTCC 5679 was higher than the *Azotobacter chroococcum* CBD15, as observed when the crop plants were inoculated with the recombinant *Azotobacter chroococcum* HKD15 having Accession number MTCC 5679, compared to non-recombinant native *Azotobacter chroococcum* CBD15 strain (Table 2).

In one embodiment of the present invention, there is provided a recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment of the present invention, there is provided a recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal

nifA gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene, wherein the microorganism is selected from a group consisting of Green sulfur bacteria, Firmibacteria, Thallobacteria, Heliobacteria, Cyanobacteria, Campylobacter, Proteobacteria, Archaeobacteria and Propionispira.

In another embodiment of the present invention, there is provided a recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene, wherein the microorganism is *Azotobacter spp.*

In another embodiment of the present invention, there is provided a recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene, wherein the *Azotobacter* is selected from a group consisting of *Azotobacter chroococcum, Azotobacter vinelandii, Azotobacter armenaicus, Azotobacter beijerinckii, Azotobacter nigricans and Azotobacter paspali.*

In a particular embodiment of the present invention, there is provided a recombinant *Azotobacter* having Accession number MTCC 5679 wherein, the *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment of the present invention, there is provided a recombinant *Azotobacter* having Accession number MTCC 5679 wherein, the *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen, wherein the *Azotobacter* comprises a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter and does not comprise antibiotic resistance marker gene.

In another embodiment of the present invention, there is provided a recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene, wherein the *nifL* gene is disrupted by insertional mutagenesis, deletion of genomic DNA, targeted gene disruption or introduction of a genomic or episomal vector.

In one embodiment of the present invention, there is provided a recombinant DNA molecule comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter.

In another embodiment of the present invention, there is provided a recombinant vector comprising the recombinant DNA molecule comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter.

In one embodiment of the invention, there is provided a process for producing a recombinant microorganism, wherein said process comprises (a) inserting a disrupted *nifL* gene and a *nifA* gene into the chromosome of a microorganism through homologous recombination with a recombinant DNA molecules comprising a disrupted chromosomal *nifL* gene, a *nifA* gene with native promoter and an antibiotic resistance marker gene to obtain a transformed microorganism, wherein the *nifL* gene is disrupted by insertional mutagenesis, deletion of genomic DNA, targeted gene disruption or introduction of a genomic or episomal vector and (b) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the transformed microorganism through homologous recombination with a recombinant DNA molecule comprising a constitutive heterologous promoter to obtain a recombinant microorganism comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter, wherein the recombinant microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In one embodiment of the invention, there is provided a process for producing a recombinant microorganism, wherein said process comprises (a) inserting a

disrupted *nifL* gene and a *nifA* gene into the chromosome of a microorganism through homologous recombination with a recombinant DNA molecule comprising a disrupted chromosomal *nifL* gene, a *nifA* gene with native promoter and an antibiotic resistance marker gene to obtain a transformed microorganism, wherein the *nifL* gene is disrupted by insertional mutagenesis carried out by interposon insertion and (b) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the transformed microorganism through homologous recombination with a recombinant DNA molecule comprising a constitutive heterologous promoter to obtain a recombinant microorganism comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter, wherein the recombinant microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment of the invention there is provided a recombinant microorganism produced by the process comprising (a) inserting a disrupted *nifL* gene and a *nifA* gene into the chromosome of a microorganism through homologous recombination with a recombinant DNA molecules comprising a disrupted chromosomal nifL gene, a *nifA* gene with native promoter and an antibiotic resistance marker gene to obtain a transformed microorganism, wherein the *nifL* gene is disrupted by insertional mutagenesis, deletion of genomic DNA, targeted gene disruption or introduction of a genomic or episomal vector and (b) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the

transformed microorganism through homologous recombination with a recombinant DNA molecule comprising a constitutive heterologous promoter to obtain a recombinant microorganism comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter, wherein the recombinant microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment of the invention there is provided a recombinant *Azotobacter spp* produced by the process comprising (a) inserting a disrupted *nifL* gene and a *nifA* gene into the chromosome of *Azotobacter* through homologous recombination with a recombinant DNA molecules comprising a disrupted chromosomal *nifL* gene, a *nifA* gene with native promoter and an antibiotic resistance marker gene to obtain a transformed *Azotobacter*, wherein the *nifL* gene is disrupted by insertional mutagenesis, deletion of genomic DNA, targeted gene disruption or introduction of a genomic or episomal vector and (b) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the transformed *Azotobacter* through homologous recombination with a recombinant DNA molecule comprising a constitutive heterologous promoter to obtain a recombinant *Azotobacter* comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter, wherein the recombinant *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In a particular embodiment of the present invention, there is provided a process for producing a recombinant *Azotobacter*, wherein said process comprises (a) providing a recombinant DNA construct comprising a disrupted *nifL* gene and a *nifA* gene with native promoter, wherein the *nifL* gene is disrupted by insertional mutagenesis, deletion of genomic DNA, targeted gene disruption or introduction of a genomic or episomal vector (b) inserting a disrupted *nifL* gene and a *nifA* gene with native promoter into the chromosome of a microorganism through homologous recombination by transforming an *Azotobacter* cell with the recombinant DNA construct of step a (c) selecting the transformed *Azotobacter*, wherein the transformed *Azotobacter* is unable to fix atmospheric nitrogen, (d) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the in the transformed *Azotobacter* through homologous recombination with a recombinant DNA molecule comprising a constitutive heterologous promoter to obtain a recombinant *Azotobacter* comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter and (e) selecting the recombinant *Azotobacter* from step (d) on nutrient medium lacking nitrogen source, wherein the recombinant *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In a particular embodiment of the present invention, there is provided a process for producing a recombinant *Azotobacter*, wherein said process comprises (a) providing a recombinant DNA construct comprising a disrupted *nifL* gene and a *nifA* gene with

native promoter, wherein the *nifL* gene is disrupted by insertional mutagenesis carried out by interposon insertion (b) inserting a disrupted *nifL* gene and a *nifA* gene with native promoter into the chromosome of a microorganism through homologous recombination by transforming an *Azotobacter* cell with the recombinant DNA construct of step a (c) selecting the transformed *Azotobacter*, wherein the transformed *Azotobacter* is unable to fix atmospheric nitrogen, (d) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the transformed *Azotobacter* through homologous recombination with a recombinant DNA molecule comprising a constitutive heterologous promoter to obtain a recombinant *Azotobacter* comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter and (e) selecting the recombinant *Azotobacter* from step (d) on nutrient medium lacking nitrogen source, wherein the recombinant *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In yet another embodiment of the invention, there is provided a process for producing a recombinant *Azotobacter chroococcum* HKD15 having Accession number MTCC 5679, wherein said process comprises (a) providing a recombinant DNA construct comprising a disrupted *nifL* gene and a *nifA* gene with native promoter, wherein the *nifL* gene is disrupted by insertional mutagenesis carried out by interposon insertion (b) inserting a disrupted *nifL* gene and a *nifA* gene with native promoter into the chromosome of a microorganism through homologous

recombination by transforming an *Azotobacter* cell with the recombinant DNA construct of step a (c) selecting the transformed *Azotobacter*, wherein the transformed *Azotobacter* is unable to fix atmospheric nitrogen, (d) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the transformed *Azotobacter* through homologous recombination with a recombinant DNA molecule comprising a constitutive heterologous promoter to obtain a recombinant *Azotobacter* comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter and (e) selecting the recombinant *Azotobacter* from step (d) on nutrient medium lacking nitrogen source, wherein the recombinant *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment there is provided a recombinant *Azotobacter* produced by the process comprising (a) providing a recombinant DNA construct comprising a disrupted *nifL* gene and a *nifA* gene with native promoter, wherein the *nifL* gene is disrupted by insertional mutagenesis, deletion of genomic DNA, targeted gene disruption or introduction of a genomic or episomal vector (b) inserting a disrupted *nifL* gene and a *nifA* gene with native promoter into the chromosome of a microorganism through homologous recombination by transforming an *Azotobacter* cell with the recombinant DNA construct of step (a), (c) selecting the transformed *Azotobacter*, wherein the transformed *Azotobacter* is unable to fix atmospheric nitrogen, (d) inserting a heterologous constitutive promoter to the upstream of the

nifA gene of the transformed *Azotobacter* through homologous recombination with a recombinant DNA molecule comprising a constitutive heterologous promoter to obtain a recombinant *Azotobacter* comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter and (e) selecting the recombinant *Azotobacter* from step (d) on nutrient medium lacking nitrogen source, wherein the recombinant *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment there is provided a recombinant *Azotobacter* produced by the process comprising (a) providing a recombinant DNA construct comprising a disrupted *nifL* gene and a *nifA* gene with native promoter, wherein the *nifL* gene is disrupted by insertional mutagenesis carried out by interposon insertion (b) inserting a disrupted *nifL* gene and a *nifA* gene with native promoter into the chromosome of a microorganism through homologous recombination by transforming an *Azotobacter* cell with the recombinant DNA construct of step (a), (c) selecting the transformed *Azotobacter*, wherein the transformed *Azotobacter* is unable to fix atmospheric nitrogen, (d) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the transformed *Azotobacter* through homologous recombination with a recombinant DNA molecule comprising a constitutive heterologous promoter to obtain a recombinant *Azotobacter* comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter and (e) selecting the

recombinant *Azotobacter* from step (d) on nutrient medium lacking nitrogen source, wherein the recombinant *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In an advantageous embodiment of the present invention there is provided a composition comprising the recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment of the present invention there is provided a composition comprising a recombinant *Azotobacter* produced by the process comprising (a) providing a recombinant DNA construct comprising a disrupted *nifL* gene and a *nifA* gene with native promoter, wherein the *nifL* gene is disrupted by an interposon insertion (b) inserting a disrupted *nifL* gene and a *nifA* gene with native promoter into the chromosome of a microorganism through homologous recombination by transforming an *Azotobacter* cell with the recombinant DNA construct of step (a), (c) selecting the transformed *Azotobacter*, wherein the transformed *Azotobacter* is unable to fix atmospheric nitrogen, (d) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the transformed *Azotobacter* through homologous recombination with a recombinant DNA molecule comprising a constitutive

heterologous promoter to obtain a recombinant *Azotobacter* comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter and (e) selecting the recombinant *Azotobacter* from step (d) on nutrient medium lacking nitrogen source, wherein the recombinant *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment of the present invention there is provided a composition comprising (a) a recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene and (b) a carrier.

In another embodiment of the present invention there is provided a composition comprising (a) a recombinant *Azotobacter* comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene and (b) a carrier.

In another embodiment of the present invention there is provided a composition comprising (a) a recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous

constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene and (b) an agriculturally acceptable carrier.

In a preferred embodiment of the present invention there is provided a biofertilizer composition comprising a recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment of the present invention there is provided a biofertilizer composition comprising a recombinant *Azotobacter* comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment of the present invention there is provided a biofertilizer composition comprising a recombinant *Azotobacter* having Accession number MTCC 5679 comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment of the present invention there is provided a composition comprising (a) a recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene and (b) one or more constituents selected from a group comprising sources of potassium, phosphorus, carbon, nitrogen, iron, sulphur, calcium, magnesium, zinc, trace elements, growth factors, pesticides, anti-termites, bacteria and/or algae.

In an embodiment of the present invention, there is provided use of a recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter for the preparation of a fertilizer composition for enhancing the fertility of the soil, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment of the present invention there is provided use of the recombinant *Azotobacter* comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter for the preparation of a fertilizer composition for enhancing the fertility of the soil, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

In another embodiment of the present invention there is provided use of the recombinant *Azotobacter* having Accession number MTCC 5679 comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter for the preparation of a fertilizer composition for enhancing the fertility of the soil, wherein the *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions, and methods are clearly within the scope of the invention, as described herein. Although the subject matter has been described in considerable detail with reference to certain preferred embodiments thereof, other embodiments are possible. As such, the spirit and scope of the appended claims should not be limited to the description of the preferred embodiment contained therein.

EXAMPLES

The disclosure will now be illustrated with working examples, which is intended to illustrate the working of disclosure and not intended to take restrictively to imply any limitations on the scope of the present disclosure.

The inventors initially carried out restriction deletion of a small part of the *nifL* gene of *Azotobacter vinelandii* UW, a strain obtained from the University of Wisconsin. A

restriction fragment containing a constitutive sigma 70 promoter of 375 bases isolated from the plasmid pBR322 was inserted upstream of *nifA* of *Azotobacter vinelandii* UW. The expression of *nifA,* yield and excretion of ammonium was enhanced several fold, and this expression was unaffected by the presence of urea or ammonium salts.

Example 1

Isolation of *nifL* and *nifA* genes of *Azotobacter chroococcum* CBD15

Azotobacter chroococcum CBD15, a strain isolated from the soil at Indian Agricultural Research Institute, Delhi, India, was used in the present study.

A genomic fragment of 7.5 kb size from the *Azotobacter chroococcum* CBD15 that contains the *nifL* gene comprising the nucleotide sequence as set forth in SEQ ID NO: 3 and the *nifA* genes was isolated using conventional methods and on the basis of homology with the known sequences of the *nifL* and the *nifA* genes of *Azotobacter vinelandii* UW. This fragment was purified and ligated into the cloning vector pUC7 at the BamHI restriction site to obtain a first recombinant vector. This recombinant vector comprising the 7.5 kb fragment was designated as pCL6 (Figure 1A) and was introduced into competent *E.coli* cells. The transformed cells were screened using radiolabelled probes and colony hybridization technique.

The plasmid from these transformed cells harboring the 7.5 kb insert was further subjected to restriction digestion with various restriction enzymes. A fragment of 4.8

kb containing the *nifL* and *nifA* genes was obtained by restriction digestion with EcoRI restriction enzyme. The fragment was purified and subsequently cloned into the vector pUC7 to obtain a second recombinant vector. This recombinant vector comprising the 4.8 kb insert was designated as pCL6.2 (Figure 1B) and used for further experiments.

Other known DNA sequences for *nifL* gene having GenBank Accession Numbers X70993.1 (SEQ ID NO: 1), SEQ ID NO: 3, NC_012560.1, X64832.1, 3. AF450501.1 can also be used for genetic manipulation and production of recombinant microorganism capable of fixing nitrogen in presence of fixed nitrogen, oxygen or a combination thereof.

Other known DNA sequences for *nifA* gene having GenBank Accession Numbers Y00554.1 (SEQ ID NO: 4), J03411.1 and M26751.1 can also be used for genetic manipulation and production of recombinant microorganism capable of fixing nitrogen in presence of fixed nitrogen, oxygen or a combination thereof.

Example 2

Site-directed insertional mutagenesis of *nifL*

Insertion of the interposon ΩKm into *nifL* of *Azotobacter chroococcum* CBD15

The 2.0kb EcoRI fragment from pHP45ΩKm (R. Fellay, J. Frey, H. Krisch, 1987; *Gene;* **52:** 147) containing the interposon ΩKm was inserted into the pCL6.2 at the SalI sites of the *nifL* gene, which was immediately upstream of *nifA*. This construct

is denoted as pCL6.3 and comprises the 'first' antibiotic resistance marker gene (Km+) (Figure 1C). The ampicillin resistance marker gene of the plasmid is denoted as the 'second' antibiotic resistance marker gene (Amp+).

Electroporation of *Azotobacter chroococcum* CBD15 with pCL6.3 for insertion of the interposon ΩKm at the *nifL* gene in its genome

Azotobacter chroococcum CBD15 was electroporated with the recombinant plasmid construct pCL6.3 to obtain the first set of transformed cells. The transformed cells showing kanamycin resistance (Km+) were selected. The cells were assayed for absence of ampicillin resistance (Amp-) and absence of nitrogen fixation. This ensured that pCL6.3 was not stable in *Azotobacter chroococcum* CBD15 and the kanamycin resistance of the cells was the result of homologous recombination *via* two point cross-over between the flanking regions of the interposon ΩKm in pCL6.3 and the chromosome of *Azotobacter chroococcum* CBD15 (Figures 1E and 1F).

Example 3

Partial deletion of *nifL* gene and cloning of the pBR322 constitutive promoter

The 375 bp EcoRI-BamHI fragment from pBR322 comprising the constitutive promoter (nucleotides 1 to 377), as set forth in SEQ ID NO: 6, was used.

A 1.1 kb DNA fragment around the Sal I sites of pCL6.2 was deleted and the EcoRI–BamHI fragment of 375bp containing the pBR322 constitutive promoter (SEQ ID NO: 6) was inserted at the site of deletion of 1.1 kb DNA fragment around the SalI

sites in pCL6.2. This construct containing the constitutive promoter was denoted as pCL6.4 (Figure 1 D).

Example 4

Integration of partially deleted *nifL* and the constitutive promoter upstream of *nifA* into the genome of the microorganism

The first set of transformed *Azotobacter chroococcum* CBD15 cells comprising the ΩKm interposon and the 'first' antibiotic resistance marker gene (Km+) were electroporated with the recombinant plasmid construct pCL6.4 (Figure 1G). Those cells that were successfully transformed by the result of homologous recombination *via* two point cross-over between the flanking regions of the pBR322 constitutive promoter in pCL6.4 and the chromosome of the first set of transformed cells of *Azotobacter chroococcum* CBD15 (Km+) were assayed for absence of 'first' antibiotic resistance marker gene and their ability to fix nitrogen (Figure 1H). The transformed cells that have undergone successful homologous recombination are sensitive to the 'first' antibiotic resistance marker gene, kanamycin. The recombinant microorganism has been designated as *Azotobacter chroococcum* HKD15. The recombinant microorganism has been submitted at the MTCC, IMTECH, India on 14th December 2011. The Accession No. for the strain is MTCC 5679.

The deletion of a part of the *nifL* gene and the insertion of the pBR322 constitutive promoter in the chromosome of *Azotobacter chroococcum* HKD15 has been confirmed by Southern blotting and PCR analysis.

Example 5

Characterization of the recombinant *Azotobacter chroococcum* HKD15

Nitrogen fixation activity of *Azotobacter chroococcum* HKD15 in the presence of fixed nitrogen

It is difficult to assay reduction of nitrogen. It is, however, known that the enzyme nitrogenase is also capable of reducing acetylene. Hence, the reduction of acetylene to ethylene is accepted as a measure of nitrogenase. The bacterial culture in a screw-capped tube is fed with specific amount of gaseous acetylene and incubated at 30°C. After 24 hours, the amount of ethylene produced along with the amount of acetylene left over are monitored by gas chromatography and compared with the initial amount of acetylene.

Acetylene reduction was enhanced 3-fold by the constitutive promoter in *Azotobacter chroococcum* HKD 15 compared to the native strain *Azotobacter chroococcum* CBD 15 (see Table 1).

Table 1: Effect of fixed nitrogen source (ammonium acetate) on acetylene reduction by *Azotobacter chroococcum* CBD 15 and *Azotobacter chroococcum* HKD 15

Sl No.	Strain	Nitrogen status	Percent acetylene reduced
1.	CBD 15	N minus	14.2 ± 2.4
2.	CBD 15	N plus (10 mM)	1.9 ± 0.7
3.	CBD 15	N plus (20 mM)	1.7 ± 0.5
4.	HKD 15	N minus	42.9 ± 2.5
5.	HKD 15	N plus (10 mM)	41.1 ± 1.9
6.	HKD 15	N plus (20 mM)	39.6 ± 1.6

Ammonia production and excretion by *Azotobacter chroococcum* HKD15

Samples of cultures growing at 30°C were withdrawn periodically, centrifuged and ammonium in the supernatant solution was estimated according to the indophenol method [Bergersen F. J, 1980, Methods for evaluating biological nitrogen fixation. John Wiley and Sons]. This consisted of the addition of 0.5 ml of phenol-sodium nitroprusside solution (phenol, 50 g/liter; sodium nitroprusside, 0.25 g/liter), 0.5 ml of sodium hypochlorite solution (0.1 M), and 2 ml of distilled water. The mixture was incubated for 30 min at room temperature. The optical density of the solution was then read at A_{625} and the ammonium concentration was estimated from a standard curve obtained with ammonium solutions at various concentrations assayed with the same reagent solutions.

It was found that the recombinant microorganism *Azotobacter chroococcum* CBD 15 showed a 9-fold increase in ammonia excretion compared with *Azotobacter*

chroococcum HKD 15. Figure 2 shows the ammonia excretion by the recombinant and the non-recombinant *Azotobacter* microorganism.

Effect of *Azotobacter chroococcum* HKD15 as plant inoculants

Wheat seeds are soaked in a suspension of the recombinant or the non-recombinant microorganism for three hours and air dried at around 25°C for 12-24 hours. The treated seeds are then sown in pots of 30 cm diameter. Three plants are kept per pot. The seeds were sown in mid-November and the crop was harvested in mid-April.

The yield of the crop in the absence of chemical fertilizer and the presence of the recombinant microorganism HKD15 was found to be higher than the yield of the crop in the presence of *Azotobacter chroococcum* CBD15, as shown in Table 2. The recombinant *Azotobacter chroococcum* HKD15 was found to be an efficacious crop inoculant and a biofertilizer.

Table 2: Effect of inoculation of wheat seed with *Azotobacter chroococcum* on yield of wheat crop in pot experiments

Sl. No.	Treatment	Crop yield per 100 plants
1.	Absence of urea, Absence of inoculation with *Azotobacter spp.*	155 gm
2.	Absence of urea Inoculation with *Azotobacter chroococcum* CBD15	167 gm
3.	Absence of urea	246 gm

	Inoculation with *Azotobacter chroococcum* HKD15	

SEQ ID NO: 1 shows nucleotide sequence of the *nifL* gene from *Azotobacter vinelandii* (1927 bp) (GENBANK: X70993.1)

TGCCAGCGCT CAAAATTTGC ACAGGCGTAT CGCGGGAGCC
CCTCTAAAAT TGACCTGGAT CAACAAATAG CTTCGGCACG
CCAGCCGCCT ATCCACCCGG CGGCCCCGGT TTTGTAAGGT
TTGTGACAGC TCGTTACTGA GCCTGCCGCC CGGCTGTGCG
CTTTCGCACA GCTAGAGGGC GACCACCCCG AAAATCCATG
TTTCGAGGTT TTTCCGAGCA ATTCGGCGCA CCCGGGCGAT
TAAGGTGCGG CACAGGATTT GCTAATCTTC TCTCAGGCCC
AACACGCCCC TCCGGCGGAC GCAGCCGCGC TCGCCGGTTT
TCTTGGATAG ACGAGGCACA GCATGACCCC GGCCAACCCG
ACCCTGAGCA ACGAGCCGCA AGCGCCTCAC GCCGAGAGCG
ACGAGCTGCT TCCCGAGATC TTTCGCCAGA CGGTGGAGCA
TGCGCCCATC GCCATTTCCA TCACCGACCT CAAGGCCAAC
ATTCTTTACG CCAATCGCGC TTTCCGCACC ATCACCGGCT
ACGGCAGCGA GGAAGTGCTC GGCAAGAACG AATCGATCCT
CTCCAACGGC ACCACGCCGC GCCTGGTCTA CCAGGCCCTG
TGGGGCCGGC TGGCGCAGAA GAAGCCCTGG TCCGGCGTGC
TGGTCAACCG CCGCAAGGAC AAGACCCTGT ACCTGGCCGA
ACTGACCGTG GCGCCGGTGC TCAACGAGGC CGGCGAGACC
ATCTACTACC TGGGCATGCA CCGCGACACC AGCGAATTGC
ACGAACTGGA ACAACGCGTC AACAACCAGC GCCTGATGAT
CGAGGCGGTG GTCAACGCCG CCCCGGCGGC GATGGTGGTG
CTCGACCGCC AGCACCGGGT GATGCTCTCC AACCCGAGCT
TCTGCCGCCT GGCCCGCGAC CTGGTCGAGG ATGGCAGCAG
CGAGAGCCTG GTGGCGCTGC TGCGGGAAAA CCTCGCCGCC

CCCTTCGAGA CGCTGGAAAA CCAGGGCAGC GCCTTCTCCG
GCAAGGAGAT CTCCTTCGAC CTGGGCGGCC GCTCGCCGCG
CTGGCTGTCC TGCCACGGCC GGGCCATCCA CATCGAGAAC
GAGCAGGCCC ACGTGTTCTT CGCGCCCACC GAGGAACGCT
ACCTGCTGCT GACCATCAAC GACATCTCCG AGCTGCGCCA
GAAGCAGCAG GATTCGCGGC TCAACGCGCT GAAGGCGCTG
ATGGCCGAGG AAGAGCTGCT GCAAGGCATG CGCGAGACCT
TCAACGCCGC CATCCATCGC CTGCAGGGCC CGGTCAACCT
GATCAGCGCG GCGATGCGCA TGCTCGAACG GCGCCTCGGC
GACAAGGCCG GCAACGACCC GGTGCTGAGC GCCATGCGCG
AAGCCAGCAC GGCCGGAATG GAGGCACTGG AGAACCTCAG
TGGCTCCATT CCGGTGCGCA TGGCCGAGTC CAAGATGCCG
GTCAACCTCA ACCAGTTGAT CCGCGAGGTG ATCACCCTGT
GCACCGACCA GTTGCTGGCC CAGGGCATCG TCGTCGACTG
GCAGCCGGCG CTGCGCCTGC CCTGGGTGAT GGGCGGGGAA
AGCAGCCTGC GCAGCATGAT CAAGCACCTG GTCGACAACG
CCATCGAGTC CATGAGCCAG AACCAGGTCA GCCGCCGCGA
GCTGTTCATC AGCACCCGCG TGGAGAACCA CCTGGTGCGC
ATGGAGATCA CCGACAGCGG CCCGGGCATT CCGCCCGACC
TGGTGCTGAA GGTGTTCGAG CCGTTCTTCA GCACCAAGCC
GCCACACCGC GTCGGGCGCG GCATGGGCCT GCCGGTGGTG
CAGGAGATCG TCGCCAAGCA CGCCGGCATG GTGCACGTAG
ACACCGACTA TCGCGAAGGC TGCCGGATCG TCGTCGAGCT
GCCCTTCTCG GCCTCCACCT GAACAGCGAC AGGGAATGCC CATGAAT

SEQ ID NO: 2 shows amino acid sequence encoded by the SEQ ID NO: 1

MTPANPTLSNEPQAPHAESDELLPEIFRQTVEHAPIAISITDLKANILYANRAF
RTITGYGSEEVLGKNESILSNGTTPRLVYQALWGRLAQKKPWSGVLVNRRK
DKTLYLAELTVAPVLNEAGETIYYLGMHRDTSELHELEQRVNNQRLMIEAV

VNAAPAAMVVLDRQHRVMLSNPSFCRLARDLVEDGSSESLVALLRENLAAP
FETLENQGSAFSGKEISFDLGGRSPRWLSCHGRAIHIENEQAHVFFAPTEERY
LLLTINDISELRQKQQDSRLNALKALMAEEELLQGMRETFNAAIHRLQGPVN
LISAAMRMLERRLGDKAGNDPVLSAMREASTAGMEALENLSGSIPVRMAES
KMPVNLNQLIREVITLCTDQLLAQGIVVDWQPALRLPWVMGGESSLRSMIK
HLVDNAIESMSQNQVSRRELFISTRVENHLVRMEITDSGPGIPPDLVLKVFEP
FFSTKPPHRVGRGMGLPVVQEIVAKHAGMVHVDTDYREGCRIVVELPFSAS
T

SEQ ID NO: 3 shows Partial nucleotide sequence of *nifL* gene of *Azotobacter chroococcum* CBD15 (1.4kb)

ATCGCGCTTTCCGCACCATCACCGGCTACGGCAGCGAGGAAGTGCTCGG
CAAGAACGAATCGATCCTCTCCAACGGCACCACGCCGCGCCTGGTCTACC
AGGCCCTGTGGGGCTGGCTGGCGCAGAAGAAGCCCTGGTCCGGCGTGCT
GGTCAACCGCCGCAAGGACAAGACCCTGTACCTGGCCGAACTGACCGTG
GCGCCGGTGCTCAACGAGGCCGGCGAGACCATCTACTACCTGGGCATGC
ACCGCGACACCAGCGAATTGCACGAACTGGAACAACGCGTCAACAACCA
GCGCCTGATGATCGAGGCGGTGGTCAGCGCCGCCCCGGCGGCGATGGTG
GTGCTCGACCGCCAGCACCGGGTGATGCTCTCCAACCCGAGCTTCTGCCG
CCTGGCCCGCGACCTGGTCGAGGATGGCAGCAGCGAGAGCCTGGTGGCG
CTGCTGCGGGAAAACCTCGCCGCCCCCTTCGAGACGCTGGAAAACCAGG
GCAGCGCCTTCTCCGGCAAGGAGATCTCCTTCGACCTGGGCGGCCGCTCG
CCGCGCTGGCTGTCCTGCCACGGCCGGGCCATCCACATCGAGAACGAGC
AGGCCCACGTGTTCTTCGCGCCCACCGAGGAACGCTACCCTGCTGCTGAC
CATCAACGACATCTCCGAGCTGCGCCAGAAGCAGCAGGATTCGCGGCTC
AACGCGCTGAAGGCGCTGATGGCCGAGGAAGAGCTGCTGGAAGGCATGC
GCGAGACCTTCAACGCCGCCATCCATCGCCTGCAGGGCCCGGCCAACCT
GATCAGCGCGGCGATGCGCATGCTCGAACGGCGCCTCGGCGGCAAGGCC
GGCAACGACCCGGTGCTGAGCGCCATGCGCGAAGCCAGCACGGCCGGAA

TGGAGGCACTGGAGAACCTCAGTGGCTCCATTCCGGTGCGCATGGCCGA
GTCCAAGATGCCGGTCAACCTCAACCAGTTGATCCGCGAGGTGATCACCC
TGTGCACCGACCAGTTGCTGGCCCAGGGCATCGTCGTCGACTGGCAGCCG
GCGCTGCGCCTGCCCTGGGTGATGGGCGGGGAAAGCAGCCTGCGCAGCA
TGATCAAGCACCTGGTCGACAACGCCATCGAGTCCATGAGCCAGAACCA
GGTCAGCCGCCGCGAGCTGTTCATCAGCACCCGCGTGGAGAACCACCTG
GTGCGCATGGAGATCACCGACAGCGGCCCGGGCATTCCGCCCGACCTGG
TGCTGAAGGTGTTCGAGCCGTTCTTCAGCACCAAGCCGCCACACCGCGTC
GGGCGCGGCACGGGCCTGCCGGTGGTGCAGGAGATCGTCGCCAAGCACG
CCGGCATGGTGCACGTAGACACCGACTATCGCGAAGGCTGCCGGATCGT
CGTCGAGCTGCCCTTCTCGGCCTCACCTCTAGAGTCGA

SEQ ID NO: 4 shows nucleotide sequence of *nifA* gene of *Azotobacter vinelandii* (1569 bp) (GenBank: Y00554.1)

ATGAATGCA ACCATCCCTC AGCGCTCGGC CAAACAGAAC
CCGGTCGAAC TCTATGACCT GCAATTGCAG GCCCTGGCGA
GCATCGCCCG CACGCTCAGC CGCGAACAAC AGATCGACGA
ACTGCTCGAA CAGGTCCTGG CCGTACTGCA CAATGACCTC
GGCCTGCTGC ATGGCCTGGT GACCATTTCC GACCCGGAAC
ACGGCGCCCT GCAGATCGGC GCCATCCACA CCGACTCGGA
AGCGGTGGCC CAGGCCTGCG AAGGCGTGCG CTACAGAAGC
GGCGAAGGCG TGATCGGCAA CGTGCTCAAG CACGGCAACA
GCGTGGTGCT CGGGCGCATC TCCGCCGACC CGCGCTTTCT
CGACCGCCTG GCGCTGTACG ACCTGGAAAT GCCGTTCATC
GCCGTGCCGA TCAAGAACCC CGAGGGCAAC ACCATCGGCG
TGCTGGCGGC CCAGCCGGAC TGCCGCGCCG ACGAGCACAT
GCCCGCGCGC ACGCGCTTCC TGGAGATCGT CGCCAACCTG
CTGGCGCAGA CCGTGCGCCT GGTGGTGAAC ATCGAGGACG
GCCGCGAGGC GGCCGACGAG CGCGACGAAC TGCGTCGCGA

GGTGCGCGGC AAGTACGGCT TCGAGAACAT GGTGGTGGGC
CACACCCCCA CCATGCGCCG GGTGTTCGAT CAGATCCGCC
GGGTCGCCAA GTGGAACAGC ACCGTACTGG TCCTCGGCGA
GTCCGGTACC GGCAAGGAAC TGATCGCCAG CGCCATCCAC
TACAAGTCGC CGCGCGCGCA CCGCCCCTTC GTCCGCCTGA
ACTGCGCCGC GCTGCCGGAA ACCCTGCTCG AGTCCGAACT
CTTCGGCCAC GAGAAGGGCG CCTTCACCGG CGCGGTGAAG
CAGCGCAAGG GGCGTTTCGA GCAGGCCGAC GGCGGCACCC
TGTTCCTCGA CGAGATCGGC GAGATCTCGC CGATGTTCCA
GGCCAAGCTG CTGCGCGTGC TGCAGGAAGG CGAGTTCGAG
CGGGTCGGCG GCAACCAGAC GGTGCGGGTC AACGTGCGCA
TCGTCGCCGC CACCAACCGC GACCTGGAAA GCGAGGTGGA
AAAGGGCAAG TTCCGCGAGG ACCTCTACTACCGCCTGAAC
GTCATGGCCA TCCGCATTCC GCCGCTGCGC GAGCGTACCG
CCGACATTCCCGAACTGGCG GAATTCCTGC TCGGCAAGAT
CGGCCGCCAG CAGGGCCGCC CGCTGACCGT CACCGACAGC
GCCATCCGCC TGCTGATGAG CCACCGCTGG CCGGGCAACG
TGCGCGAACT GGAGAACTGC CTGGAGCGCT CGGCGATCAT
GAGCGAGGAC GGCACCATCA CCCGCGACGT GGTCTCGCTG
ACCGGGGTCGACAACGAGAG CCCGCCGCTC GCCGCGCCGC
TGCCCGAGGT CAACCTGGCC GACGAGACCC TGGACGACCG
CGAACGGGTG
ATCGCCGCCCTCGAACAGGCCGGCTGGGTGCAGGCCAAGGCCGCGCGGC
TGCTGGGCATGACGCCGCGGCAGATCGCCTACCGCATCCAGACCCTCAA
CATCCACATGCGCAAGATCTGA

SEQ ID NO: 5 shows amino acid sequence of the product encoded by SEQ ID NO: 4

MNATIPQRSAKQNPVELYDLQLQALASIARTLSREQQIDELLEQVLAVLHND
LGLLHGLVTISDPEHGALQIGAIHTDSEAVAQACEGVRYRSGEGVIGNVLKH

GNSVVLGRISADPRFLDRLALYDLEMPFIAVPIKNPEGNTIGVLAAQPDCRAD
EHMPARTRFLEIVANLLAQTVRLVVNIEDGREAADERDELRREVRGKYGFE
NMVVGHTPTMRRVFDQIRRVAKWNSTVLVLGESGTGKELIASAIHYKSPRA
HRPFVRLNCAALPETLLESELFGHEKGAFTGAVKQRKGRFEQADGGTLFLDE
IGEISPMFQAKLLRVLQEGEFERVGGNQTVRVNVRIVAATNRDLESEVEKGK
FREDLYYRLNVMAIRIPPLRERTADIPELAEFLLGKIGRQQGRPLTVTDSAIRL
LMSHRWPGNVRELENCLERSAIMSEDGTITRDVVSLTGVDNESPPLAAPLPE
VNLADETLDDRERVIAALEQAGWVQAKAARLLGMTPRQIAYRIQTLNIHMR
KI

SEQ ID NO: 6 shows nucleotide sequence of the fragment of DNA from the plasmid pBR322 inserted upstream of the *nif A* gene of *Azotobacter*

GAATTCTCAT GTTTGACAGC TTATCATCGA TTAGCTTTAA
TGCGGTAGTT TATCACAGTT AAATTGCTAA CGCAGTCAGG
CACCGTGTAT GAAATCTAAC AATGCGCTCA TCGTCATCCT
CGGCACCGTC ACCCTGGATG CTGTAGGCAT AGGCTTGGTT
ATGCCGGTAC TGCCGGGCCT CTTGCGGGAT ATCGTCCATT
CCGACAGCAT CGCCAGTCAC TATGGCGTGC TGCTAGCGCT
ATATGCGTTG ATGCAATTTC TATGCGCACC CGTTCTCGGA
GCACTGTCCG ACCGCTTTGG CCGCCGCCCA GTDCCTGCTC
GCTTCGCTAC TTGGAGCCAC TATCACTACG CGATCATGGC
GACCACACCC GTCCTGTGGA TCC

CLAIMS

1. A recombinant microorganism comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter, wherein the microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

2. The recombinant microorganism as claimed in claim 1, wherein the microorganism is selected from a group consisting of Green sulfur bacteria, Firmibacteria, Thallobacteria, Heliobacteria, Cyanobacteria, Campylobacter, Proteobacteria, Archaeobacteria and Propionispira.

3. The recombinant microorganism as claimed in claim 1, wherein the microorganism is *Azotobacter* spp.

4. The recombinant microorganism as claimed in claim 3, wherein the *Azotobacter* is selected from a group consisting of *Azotobacter chroococcum, Azotobacter vinelandii, Azotobacter armenaicus, Azotobacter beijerinckii, Azotobacter nigricans and Azotobacter paspali.*

5. A recombinant *Azotobacter* having Accession number MTCC 5679, wherein the *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

6. The recombinant microorganism as claimed in claim 1, wherein the *nifL* gene is disrupted by insertional mutagenesis, deletion of genomic DNA, targeted gene disruption or introduction of a genomic or episomal vector.

7. A recombinant DNA molecule comprising a disrupted chromosomal *nifL* gene and a chromosomal *nifA* gene operably linked to a heterologous constitutive promoter.

8. A recombinant vector comprising the recombinant DNA molecule as claimed in claim 7.

9. A process for producing a recombinant microorganism, wherein said process comprises

(a) inserting a disrupted *nifL* gene and a *nifA* gene into the chromosome of a microorganism through homologous recombination with a recombinant DNA molecules comprising a disrupted chromosomal *nifL* gene, a *nifA* gene with native promoter and an antibiotic resistance marker gene to obtain a transformed microorganism, wherein the *nifL* gene is disrupted by insertional mutagenesis, deletion of genomic DNA, targeted gene disruption or introduction of a genomic or episomal vector.

(b) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the transformed microorganism through homologous

recombination with a recombinant DNA molecule comprising a constitutive heterologous promoter to obtain a recombinant microorganism comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter,

wherein the recombinant microorganism is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

10. The process as claimed in claim 9, wherein the insertional mutagenesis is carried out by interposon insertion.

11. A recombinant microorganism produced by the process as claimed in claim 9.

12. The recombinant microorganism as claimed in claim 11, wherein the microorganism is *Azotobacter spp.*

13. A process for producing a recombinant *Azotobacter* capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen, wherein said process comprises

(a) providing a recombinant DNA construct comprising a disrupted *nifL* gene and a *nifA* gene with native promoter, wherein the *nifL* gene is disrupted by an interposon insertion

(b) inserting a disrupted *nifL* gene and a *nifA* gene with native promoter into the chromosome of a microorganism through homologous recombination by transforming an *Azotobacter* cell with the recombinant DNA construct of step (a)

(c) selecting the transformed *Azotobacter*, wherein the transformed *Azotobacter* is unable to fix atmospheric nitrogen,

(d) inserting a heterologous constitutive promoter to the upstream of the *nifA* gene of the transformed *Azotobacter* through homologous recombination with a recombinant DNA molecule comprising a constitutive heterologous promoter to obtain a recombinant *Azotobacter* comprising a disrupted *nifL* gene and a *nifA* gene operably linked to the heterologous constitutive promoter and

(e) selecting the recombinant *Azotobacter* from step (d) on nutrient medium lacking nitrogen source,

wherein the recombinant *Azotobacter* is capable of fixing atmospheric nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

14. A recombinant *Azotobacter* obtained by the process as claimed in claim 13, wherein the recombinant *Azotobacter* is capable of fixing atmospheric

nitrogen in presence of fixed nitrogen and oxygen and does not comprise antibiotic resistance marker gene.

15. A composition comprising the recombinant microorganism as claimed in claim 1 or *Azotobacter* as claimed in claim 14.

16. The composition as claimed in claim 15, wherein the composition optionally comprises a carrier.

17. The composition as claimed in claim 16, wherein the carrier is an agriculturally acceptable carrier.

18. The composition as claimed in claim 15, wherein the composition is a biofertilizer.

19. The composition as claimed in any of the preceding claims optionally comprises one or more constituents selected from a group comprising sources of potassium, phosphorus, carbon, nitrogen, iron, sulphur, calcium, magnesium, zinc, trace elements, growth factors, pesticides, anti-termites, bacteria, algae.

20. Use of the recombinant microorganism as claimed in claim 1 or *Azotobacter* as claimed in claim 14 or the composition as claimed in any of the preceding claims for the preparation of a fertilizer composition for enhancing the fertility of the soil.

FIGURE 1

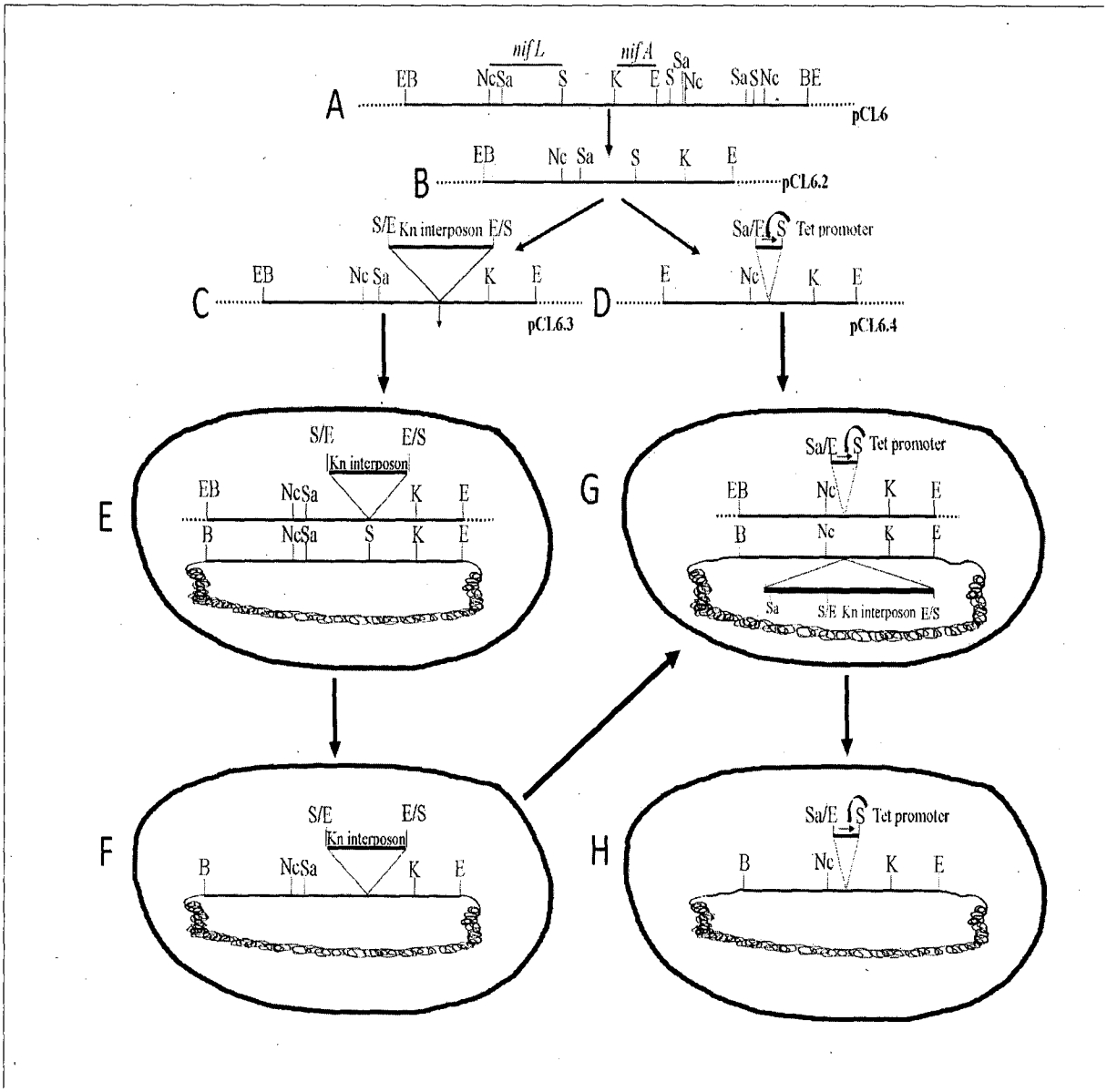
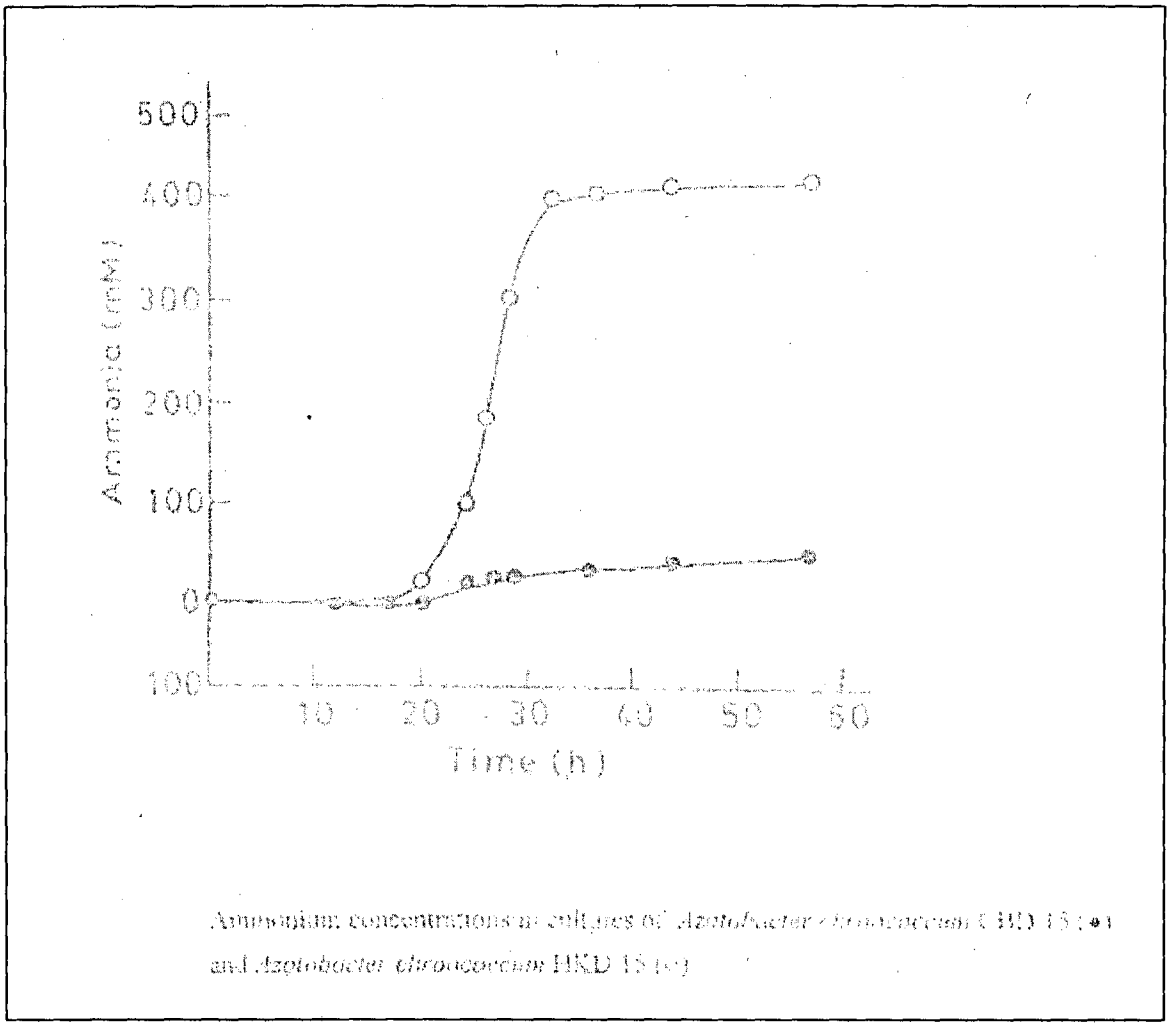

nifL
nifA
Sa
A
EB
NcSa
S
K
E
S
Nc
SaSNc
BE
pCL6
B
EB
Nc Sa
S
K
E
pCL6.2
S/E Kn interposon E/S
Sa/E S Tet promoter
C
EB
Nc Sa
K
E
pCL6.3
D
E
Nc
K
E
pCL6.4
E
S/E
E/S
Kn interposon
EB
NcSa
K
E
B
NcSa
S
K
E
G
Sa/E S Tet promoter
EB
Nc
K
E
B
Nc
K
E
Sa
S/E Kn interposon E/S
F
S/E
E/S
Kn interposon
B
NcSa
K
E
H
Sa/E S Tet promoter
B
Nc
K
E

FIGURE 2



Ammonium concentrations in cultures of *Azotobacter chroococcum* HKD 15 (●) and *Azotobacter chroococcum* HKD 15 (○)

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2013/000123

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/90 C07K14/195 C05F11/08
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C07K C05F

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2 259 302 A (BALI ANIL KUMAR [IN]; BLANCO GONZALO [ES]; KENNEDY CHRISTINA KAY [GB]) 10 March 1993 (1993-03-10) the whole document	1-20
X	EP 0 339 830 A2 (BIOTEKNIKA INTERNATIONAL [US]) 2 November 1989 (1989-11-02) the whole document	1-20

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

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 2013	18/07/2013

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

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	F. REYES-RAMIREZ ET AL: "Mutant Forms of the Azotobacter vinelandii Transcriptional Activator NifA Resistant to Inhibition by the NifL Regulatory Protein", JOURNAL OF BACTERIOLOGY, vol. 184, no. 24, 15 December 2002 (2002-12-15), pages 6777-6785, XP055070122, ISSN: 0021-9193, DOI: 10.1128/JB.184.24.6777-6785.2002 the whole document ----- MCMANUS P S ET AL: "Antibiotic use in plant agriculture", ANNUAL REVIEW OF PHYTOPATHOLOGY, ANNUAL REVIEWS INC, US, vol. 40, 1 January 2002 (2002-01-01), pages 443-465, XP002442836, ISSN: 0066-4286, DOI: 10.1146/ANNUREV.PHYTO.40.120301.093927 abstract ----- PALMEROS B ET AL: "A family of removable cassettes designed to obtain antibiotic-resistance-free genomic modifications of Escherichia coli and other bacteria", GENE, ELSEVIER, AMSTERDAM, NL, vol. 247, no. 1-2, 1 April 2000 (2000-04-01), pages 255-264, XP004196572, ISSN: 0378-1119, DOI: 10.1016/S0378-1119(00)00075-5 the whole document ----- CHEREPANOV P P ET AL: "Gene disruption in Escherichia coli: Tc<R> and Km<R> cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant", GENE, ELSEVIER, AMSTERDAM, NL, vol. 158, no. 1, 1 January 1995 (1995-01-01), pages 9-14, XP004206666, ISSN: 0378-1119, DOI: 10.1016/0378-1119(95)00193-A the whole document ----- -/--	1-20 1-20 1-20 1-20

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2013/000123

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COLNAGHI RITA ET AL: "Strategies for increased ammonium production in free-living or plant associated nitrogen fixing bacteria", PLANT AND SOIL, vol. 194, no. 1-2, July 1997 (1997-07), pages 145-154, XP002700267, ISSN: 0032-079X the whole document ----- MALDONADO RAFAEL ET AL: "Gene dosage analysis in Azotobacter vinelandii", GENETICS, vol. 132, no. 4, 1992, pages 869-878, XP002700268, ISSN: 0016-6731 abstract -----	1-20 1-20

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IN2013/000123

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		JP H02131581 A	21-05-1990