GENE EXPRESSION CONTROL USING GUANIDINE BASED GENE INDUCTION

TECHNICAL FIELD OF THE INVENTION:

The present invention relates to the process for gene expression control in genetically engineered organisms using unique, natural inducer compounds. The present invention further relates to genetic modifications of microbes for development of efficient gene control mechanism by using natural and/ or non-natural inducer compounds such as carbon compounds and/ or nitrogen compounds like sugars, sugar analogues, nucleotides, etc. The invention also describes the gene expression control and tuning by application of unique, natural compound Guanidine, either alone or in combination with other inducer compounds, for efficient gene expression control.

BACKGROUND AND PRIOR ART OF THE INVENTION:

In current scientific era, gene expression strategies involves huge array of genetic regulatory tools that permit gene expression control at transcription and/ or translational levels. Recent years have seen improvements in the functionality of gene regulation tools, enhancing utility for dynamic, tuneable regulation, etc. tuneable regulation of gene expression can be achieved by inducing gene expression using efficient inducer compounds, such as lactose for Lac operon-based promoter usage, Arabinose for Ara operon-based promoter, or aminoacid auxotrophic promoters like promoter of Trp operon (Tryptophan metabolic pathway).

Messenger RNA (mRNA) has many regulatory regions along its sequence length, which will participate in enhancing or regulating protein expression. One of them is the 5′ untranslated region (5'UTR), which contains many regulatory elements such as upstream ORFs (uORFs), internal ribosome entry sites (IRESs), microRNA binding sites, and structural components involved in the regulation of mRNA stability, pre-mRNA splicing, and translation initiation.

Riboswitches are RNA segments found in the untranslated region of genes, which are involved in controlling gene expression by binding to small molecules, such as

metabolites, and changing the level of protein production from the messenger RNA (mRNA).

Inducer compounds play a central and classical role in the induction of gene expression systems by small molecules, such as lactose (*lac*), arabinose (*ara*), tetracycline (*tet*), etc. Inducible expression systems have been widely utilized for recombinant protein/ enzyme production, gene function studies and in synthetic biology for the control of gene networks and metabolic pathways to produce fine chemicals and biofuel.

Natural compounds which are nitrogen compounds also can be of good choice of inducible compounds, such as and not limited to amino acids, amino acid- derivatives, urea, guanidine, nucleotides, etc. Nitrogen rich Guanidyl moiety is a component of fundamental metabolites including the amino acid arginine, the energy carrier creatine, and the nucleobase guanine. Reports regarding the importance of free guanidine in biology are sparse and no biological receptors that specifically recognize this compound have been previously identified.

As per reports, Guanidine was first discovered as a thermal decomposition product of the nucleobase guanine. Industrial-scale production of guanidine has been exploited for the manufacture of explosives and other chemicals, but this compound is perhaps best known to the scientific community as a commonly-used reagent for protein denaturation.

Microbes capable of metabolizing guanidine have been previously studied, in part due to the interest in guanidine bioremediation. Guanidine also has been proposed to be generated by human cells as part of the guanidine cycle via the cleavage of canavanine. Though there are scattered findings on guanidine metabolism and pathways, guanidine has never been considered to be a major cellular metabolite. The lack of knowledge about the physiological role and source of free guanidine in biological systems is in bright contrast to the widespread occurrence of guanidine riboswitches. There is a plethora of guanidine-containing compounds ranging from arginine and creatinine to guanine and large secondary metabolites such as streptomycin. Although all these guanidine compounds comprise a potential source of guanidine, the known catabolic routes proceed mostly by hydrolytic attack of the guanidine carbon atom, giving raise to urea.

Based on literatures and reports, there are very few research works that are about the biological formation of guanidine and its hydrolysis in metabolism, only few biotic reactions have been characterized that produces or breaks down guanidine to date. The best studied enzyme that catalyzes the production of guanidine is the ethylene-forming enzyme (EFE). Here, guanidine is formed via the δ-hydroxylation of arginine and subsequent loss of guanidine. The recent discovery of a fourth class of guanidine riboswitches which is common in various bacterial clades and associated with genes encoding transporters like multidrug efflux pumps, urea carboxylases, purine biosynthesis, and amino acid metabolism enzymes.

US8440810B2 discloses method of inhibiting gene expression by bringing into contact a compound and a cell, wherein the cell comprises a gene encoding an RNA comprising a guanine-responsive riboswitch and the compound inhibits expression of the gene by binding to the guanine-responsive riboswitch. The compound is not guanine, hypoxanthine, or xanthine. Overall, there are lacuna in scientific researches on efficient production and application of Guanidine. Only few enzymes are discovered to be sensitive to Guanidine. Therefore, there is a need in the art to provide for efficient gene expression control mechanisms using natural inducer compounds.

Utilising the available knowledge on Guanidine metabolism and applications, the present inventors have come up with a Guanidine based gene induction process for controlling the gene expression of rate-limiting enzymes and utilised it in production of various agri and non-agri beneficial products, sugars, polymers, Nitrogen compounds, proteins, enzymes, amino acids, therapeutics, dairy products, aqua products, etc.

SUMMARY OF THE INVENTION:

The present invention provides genetic modification of microbes for development of efficient gene control mechanism by using natural and/ or non-natural inducer compounds such as carbon compounds and/ or nitrogen compounds like sugars, sugar analogues, nucleotides, etc.

In a main aspect, the present invention provides a process for gene expression control in genetically engineered organisms using natural inducer compounds selected from the group comprising but not limited to Xanthine, Guanine, Deoxy guanosine, Guanidine, etc.

In another aspect, the process for controlled inducible gene expression uses inducer compounds selected from the group comprising but not limited to, Nitrogen compound Guanidine and Guanidine derivatives.

In another aspect, the present invention provides a method of gene induction and production of Guanidine as a product for application of Guanidine-based gene control in the formation of different products.

In a further aspect, the microbial strain for genetic modification is selected from but not limited to Bacteria Fungi, Yeast, Algae etc., for overproduction of Guanidine, by overexpression of enzymes such as and not limited to Ethylene forming enzyme (EFE) and related rate-limiting enzymes, such as Arginase, glutamine synthase, carbamoyl phosphate synthase etc.

In yet another aspect, the present invention provides the use of Guanidine based gene induction for controlled gene expression of rate-limiting enzymes involved in pathway of products such as amino acids, protein, fatty acids, carbohydrate production.

In another aspect, the present invention provides the inclusion of Guanidine sensitive gene expression elements such as and not limited to promoter, operator, Translation regulators like UTR (UnTranslated regions), RBS (Ribosome binding site) etc., for target gene expression.

The gene expression elements under control of Guanidine based induction may be selected from the group comprising of Guanidine-sensitive enzymes such as, and not limited to Urea carboxylase, Urease, Allophane hydrolase etc.

In certain aspect, the invention details that the promoter with Riboswitch UTR element inclusion, can include constitutive promoters such as and not limited to gap (glyceraldehyde 3-phosphate dehydrogenase), pyk (pyruvate kinase), pfk (phspho fructo kinase), recombinant promoters like trc, tac which are not sensitive to IPTG or lactose; or inducible promoters such as and not limited to lac (lactose sensitive), trp (tryptophan sensitive), aox (alcohol oxidase), ara (arabinose sensitive), etc.

In yet another aspect, the invention mentions that the inducible promoter with guanidine-specific riboswitch can be controlled by both the inducer compounds specific for the promoter and by Guanidine specific for the UTR element. For example, if the promoter element includes Lac promoter and Guanidine specific UTR sequence, gene expression can be activaed ny either addition of lactose/ lactose analog like TPTG or by addition of Guanidine alone.

One major aspect of ythe invention provides the list of strains which can be modified for production of Guanidine can be from the group of microbes including and not limited to E.coli, Bacillus, Methylobacterium, Corynebacterium, Cyanobacteria, Lactobacillus, Saccharomyces, Pichia, Kluyveromyces, etc.

In another aspect, the present invention provides the application of Guanidine based gene expression control for either in-vitro or in-vivo production by genetically engineered microbes, for production of compounds such as and not limited to agri and non-agri products, sugars, polymers, nitrogen compounds, organic acids, protein, carbohydrates, fatty acids, aqua products, therapeutics, biofuels, etc.

DESCRIPTION OF FIGURES:

Figure 1: Depicts the biochemical reaction involved in Guanidine production along with Ethylene formation

Figure 2: depicts the Gene construct assembly for Guanidine based gene expression control

Figure 3: depicts the visual assessment of Bacterial culture expressing Green fluorescent protein (GFP) under the control of Guanidine induced promoter.

DETAILED DESCRIPTION OF THE INVENTION:

The invention will now be described in detail in connection with certain preferred and optional embodiments, so that various aspects thereof may be more fully understood and appreciated.

In an embodiment, the present invention provides genetic modifications of microbes for development of efficient gene control mechanism by using natural and/ or non-natural

inducer compounds such as carbon compounds and/ or nitrogen compounds like sugars, sugar analogues, nucleotides, etc.

The microbial strain for genetic modification include but not limited to Pseudomonas, Rhizobium sp, or Cyanobacteria, Green algae etc., capable of Guanidine production as part of ethylene formation pathway.

In a preferred embodiment, the present invention provides a process for gene expression control in genetically engineered organisms using natural inducer compounds selected from the group comprising Xanthine, Guanine, Deoxy guanosine, Guanidine etc.

In an embodiment, the present process for controlled inducible gene expression uses compounds selected from but not limited to Nitrogen compound Guanidine and Guanidine derivatives.

In yet another embodiment, the present invention provides a method of gene induction and production of Guanidine as a product for application of Guanidine-based gene control in the formation of different products.

In another embodiment, the present invention provides for the development of microbial strain for overproduction of Guanidine, by overexpressing Ethylene forming enzyme (EFE) and related rate-limiting enzymes. This production strain can be cultured at higher level for Guanidine production and use for induction in various applications.

In yet another embodiment, the present invention provides the use of Guanidine based gene induction for controlled gene expression of rate-limiting enzymes involved in pathway of products such as amino acids, protein, fatty acids, carbohydrate production.

In a further embodiment, the present invention provides the inclusion of Guanidine sensitive gene expression elements such as and not limited to promoter, operator, Translation regulators like UTR (UnTranslated regions), RBS (Ribosome binding site), Riboswitch, etc. for target gene expression.

In another embodiment, the gene expression elements under control of Guanidine based induction may be selected from the group comprising of Guanidine-sensitive enzymes

such as, and not limited to Urea carboxylase, Urease, Allophane hydrolase etc., which are all the enzymes involved related to Arginine degradation.

In certain embodiment, the invention details that the promoter with Riboswitch UTR element inclusion, can include constitutive promoters such as and not limited to gap (glyceraldehyde 3-phosphate dehydrogenase), pyk (pyruvate kinase), pfk (phspho fructo kinase), recombinant promoters like trc, tac which are not sensitive to IPTG or lactose; or inducible promoters such as and not limited to lac (lactose sensitive), trp (tryptophan sensitive), aox (alcohol oxidase), ara (arabinose sensitive), etc.

In yet another embodiment, the invention mentions that the inducible promoter with guanidine-specific riboswitch can be controlled by both the inducer compounds specific for the promoter and by Guanidine specific for the UTR element. For example, if the promoter element includes Lac promoter and Guanidine specific UTR sequence, gene expression can be activaed ny either addition of lactose/ lactose analog like TPTG or by addition of Guanidine alone.

One major embodiment of ythe invention provides the list of strains which can be modified for production of Guanidine can be from the group of microbes including and not limited to E.coli, Bacillus, Methylobacterium, Corynebacterium, Cyanobacteria, Lactobacillus, Saccharomyces, Pichia, Kluyveromyces, etc.

In yet another embodiment, the present process for Guanidine based gene expression control is useful for in-vitro or in-vivo production by genetically engineered microbes, for production of agri and non-agri products, nitrogen compounds, organic acids, protein, carbohydrates, fatty acids etc.

Advantages of the invention:

The process of Guanidine based gene expression control has the following advantages

1. Guanidine is a unique, natural Nitrogen compound produced by few of the microbes, which is similar to urea in function.
2. Only few upstream controlling elements such as promoters, Riboswitches, operator, UTR regions are sensitive to Guanidine presence, thus making Guanidine, a specific inducer compound.

3. As Guanidine is sensed by very specific Riboswitches, Guanidine as an inducer will have very specific gene expression control.

EXAMPLES:

Example 1: Over expression of Ethylene forming enzyme (EFE) and related rate-limiting enzymegenes such as Arginase, glutamine synthase, carbamoyl phosphate synthase, and other genes of Urea synthesis cycle, etc to increase production of Guanidine:-

- Genetic modifications were performed on genes coding for Ethylene forming enzyme in bacterial strain. This resulted in increased enzyme activity of these enzymes and thereby increased production of Guanidine.
- Selected Microbes produce Guanidine by pathway involving Arginase, glutamine synthase, carbamoyl phosphate synthase, and other genes of Urea synthesis cycle, etc.(as depicted in Figure 2).
- To identify such modifications, site-directed mutagenesis was performed in and around the active sites of the genes that code for Ethylene forming enzyme (EFE) and other rate-limiting enzymes. Modified enzymes were analysed by Bioinformatic softwares prior to expression studies.
- Gene coding for EFE- Ethylene forming enzyme (represented by SEQ ID NO: 1) was expressed under control of methanol induction promoter (Mxa) in Methylobacterium sp.

Example 2: Expression of GFP (Green fluorescent protein) as an example of controlled gene expression using Guanidine as inducer compound.

- Guanidine based gene control was assessed using Green fluorescent protein (GFP) as visual marker protein
- Gene coding for GFP (represented by SEQ ID NO: 4) was cloned under promoter with Guanidine specific 5'UTR and transformed into *E.coli* cells. Transformant *E.coli* cells (GFP-S1 and S2) were cultured and induced for expression using Guanidine as inducer. Cultures expressing GFP were compared against *E.coli* host cells under Ultra violet- UV light transillumination.

Result: Recombinant *E.coli* cells induced with Guanidine showed expression of GFP protein, which was visually observed under UV irradiation (as depicted in Figure 3)

Example 3: Guanidine based induction of protein expression - Tuning of gene expression by varying the inducer compound Guanidine – Expression of Lysozyme as marker protein

Gene coding for Lysozyme was cloned downstream of the promoter with Guanidine-specific 5'UTR region (represented by SEQ ID NO: 2), in *E.coli*.
SEQ ID 3 depicts the Promoter sequence and 5'UTR sequence together (as example, Tuf promoter sequence+UTR sequence).

Expression of lysozyme was assessed in different sets with addition of Guanidine in varying concentrations. The effect of lysozyme expression was observed by the cell density (absorbance at 600nm) measurements.

Table 1

Guanidine concentration	**A600 at 0hr**	**A600 at 24hrs**
Control (0 mM)	2.13	2.75
100 mM	2.11	2.09
200 mM	2.04	1.92
300 mM	2.12	1.77

Result:

As the concentration of Guanidine varied from 100 to 200 mM concentration, the cell density decreased showing the linear tuning of lysozyme expressing in correlation with Guanidine concentration

SEQUENCE LISTING

Sequence Listing Information:
DTD Version: V1_3
File Name: Sequence listing.xml
Software Name: WIPO Sequence
Software Version: 2.3.0

Production Date: 2024-09-13

General Information:

Current application / IP Office: IN

Current application / Application number: 202341016651

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Current application / Applicant file reference: GNANLex_16651

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Earliest priority application / Filing date: 2023-09-13

Applicant name: Fertis India Pvt. Ltd.

Applicant name / Language: en

Invention title: GENE EXPRESSION CONTROL USING GUANIDINE BASED GENE INDUCTION (en)

Sequence Total Quantity: 4

Sequences:

Sequence Number (ID): 1

Length: 1053

Molecule Type: DNA

Features Location/Qualifiers:

- source, 1..1053

> mol_type, other DNA

> organism, EFE coding sequence

Residues:

atgaccaacc tacagacttt cgagttgcct accgaggtaa ccggctgcgc cgccgatatc 60

tcattgggaa gggcgctgat ccaagcctgg caaaaagatg gcatttttca gatcaagacc 120

gatagtgagc aggatcgcaa aacgcaggaa gcaatggctg ctagcaagca gttttgcaag 180

gaaccgctga cttttaagag tagctgcgtt agcgatctga cctacagcgg ctatgttgcg 240

tcaggcgagg aagtcacagc tggtaaacct gatttccctg aaatcttcac tgtctgcaag 300

gacttgtcgg taggcgatca gcgtgtaaaa gccggctggc cttgccatgg tccggtgcca 360

tggccaaata acacctatca gaaaagcatg aagaccttca tggaagagct gggtttagcg 420

ggcgaacggt tgctcaaact gacagcgctc ggctttgaac tacccatcaa cacgttcacc 480

gacttaactc gcgatggttg gcaccacatg cgtgtattac gcttcccgcc ccaaacatcc 540

acgctgtccc gtggaattgg tgcgcacact gactatgggt tgttggtaat tgccgctcag 600
gacgatgttg gtggcttata tattcgccct ccagtcgagg gagagaagcg taatcgtaac 660
tggttgcctg gtgagagctc agcaggcatg tttgagcacg atgaaccttg gaccttcgtg 720
acgcccaccc caggcgtgtg gacagttttc ccaggtgata tcttgcagtt catgaccggc 780
ggccagctgc tttccactcc gcacaaggtt aagctcaata cccgcgaacg tttcgcctgc 840
gcttattttc atgagcctaa ttttgaagca tccgcctatc cgttgttcga gcccagcgcc 900
aatgagcgta ttcattatgg tgagcacttt accaacatgt ttatgcgttg ctatccagat 960
cggatcacca cccagaggat caacaaggag aatcgcctgg cgcacttgga ggacttgaag 1020
aagtattcgg acacccgcgc gacaggctca tga 1053

Sequence Number (ID): 2
Length: 121
Molecule Type: DNA
Features Location/Qualifiers:
- source, 1..121
> mol_type, other DNA
> organism, Guanidine specific 5'UTR region
Residues:
aaaatagaat aaatactcca ccgggagtta aatcgtatga acgattgttt gcatttcagt 60
aggtctgaga agaaatgtag atagtcgttc ttttttagc tgaaggagag tgaaacccat 120
g 121

Sequence Number (ID): 3
Length: 278
Molecule Type: DNA
Features Location/Qualifiers:
- source, 1..278
> mol_type, other DNA
> organism, Tuf Promoter (promoter of Translation elongation factor Tu) with Guanidine specific 5'UTR
Residues:
tgctcgatct cggcgagcgc cgcggcggcg ccccggaggc gcagcggctc tacgagcagg 60
tcggcgaggc cgcgtcctga cccctcattc ggcgcgatcc gccctgccgg caaacctgcc 120
gcttgatcat cgtcgcacaa gcctctaatc ggtccgcaaa atagaataaa tactccaccg 180

ggagttaaat cgtatgaacg attgtttgca tttcagtagg tctgagaaga aatgtagata 240
gtcgttcttt ttttagctga aggagagtga aacccatg 278

Sequence Number (ID): 4
Length: 922
Molecule Type: DNA
Features Location/Qualifiers:
- source, 1..922
> mol_type, other DNA
> organism, Coding sequence of GFP (Green fluorescent protein)
Residues:
tacacacgaa taaaagataa caaagatgag taaaggagaa gaacttttca ctggagttgt 60
cccaattctt gttgaattag atggcgatgt taatgggcaa aaattctctg tcagtggaga 120
gggtgaaggt gatgcaacat acggaaaact tacccttaaa tttatttgca ctactgggaa 180
gctacctgtt ccatggccaa cacttgtcac tactttctct tatggtgttc aatgcttttc 240
aagataccca gatcatatga aacagcatga ctttttcaag agtgccatgc ccgaaggtta 300
tgtacaggaa agaactatat tttacaaaga tgacgggaac tacaagacac gtgctgaagt 360
caagtttgaa ggtgataccc ttgttaatag aatcgagtta aaaggtattg attttaaaga 420
agatggaaac attcttggac acaaaatgga atacaactat aactcacata atgtatacat 480
catggcagac aaaccaaaga atggaatcaa agttaacttc aaaattagac acaacattaa 540
agatggaagc gttcaattag cagaccatta tcaacaaaat actccaattg gcgatggccc 600
tgtcctttta ccagacaacc attacctgtc cacacaatct gccctttcca aagatcccaa 660
cgaaaagaga gatcacatga tccttcttga gtttgtaaca gctgctggga ttacacatgg 720
catggatgaa ctatacaaat aaatgtccag acttccaatt gacactaaag tgtccgaaca 780
attactaaat tctcagggtt cctggttaaa ttcaggctga gactttattt atatatttat 840
agattcatta aaattttatg aataatttat tgatgttatt aataggggct attttcttat 900
taaataggct actggagtgt at 922

I/We claim, **CLAIMS**

1. A method of gene modifications for efficient gene expression and control using natural and/ or non-natural inducer compounds.
2. A method of gene modifications in promoter elements for Guanidine-based gene expression in promoting gene expression, gene silencing, gene repression, growth promotion, cell lysis, etc., under in-vitro and/ or in vivo conditions
3. The method as claimed in claim 1, wherein the gene modifications for efficient gene expression includes modifications in the promoter elements such as and not limited to promoter, operator, Riboswitch elements and Translation regulators like UTR (UnTranslated regions), RBS (Ribosome binding site) and the like.
4. The method as claimed in claim 3, wherein the promoter modification is particularly with respect to incorporation of Riboswitch element such as 5'UTR incorporation with Promoter sequence.
5. The method as claimed in claim 4, wherein the modified promoter with 5'UTR is used for protein expression using Guanidine as the Inducer compound.
6. The method as claimed in claim 4, wherein the gene expression elements under control of Guanidine based induction are selected from the group comprising of Guanidine-sensitive enzymes such as, and not limited to Urea carboxylase, Urease, Allophane hydrolase and the like.

The method as claimed in claim 4, wherein the said promoter element is selected from the guanidine sensing promoters of enzymes such as and not limited to urea carboxylase, Allophane hydrolase, Urease, guanidine breakdown enzyme such as Guanidinase, etc.

7. The method as claimed in claim 5, where in the inducer compound Guanidine is generated from strain modified with overexpression of enzymes such as and not limited to Ethylene forming enzyme (EFE) and related rate-limiting enzymes, such as Arginase, glutamine synthase, carbamoyl phosphate synthase and the like.
8. The method as claimed in claim 5, wherein the Guanidine specific promoter elements for expression of proteins or enzymes or system assessed by expression of marker proteins such as and not limited to Green fluorescence protein (GFP), Red fluorescence protein (RFP), Lysozyme, maltose binding protein (MBP) and the like.

The method as claimed in claim 5, wherein the guanidine based expression system is for expression of proteins, regulatory proteins, DNA binding proteins, fluorescent

proteins, enzymes, RNAs such as antisense RNA, siRNA, lytic enzymes, hormones, etc.

9. The method as claimed in claim 1 to 8, wherein the Guanidine-specific induced expression can be applied for gene expression and product formation in various microbes including but not limited to bacteria, algae, fungi, yeast, and the like.
10. The method as claimed in claim 9, where in the gene expression of product formation are for *in vitro* and/ or *in vivo* applications.
11. The method as claimed in claim 4, wherein the said promoter with Riboswitch UTR element inclusion, can include constitutive promoters (such as and not limited to gap, pyk, pfk, trc, tac, etc.) or inducible promoters (such as and not limited to lac, trp, aox, ara, etc.)
12. The method as claimed in claim 11, wherein the inducible promoter with guanidine-specific riboswitch can be controlled by both the inducer compounds specific for the promoter and by Guanidine specific for the UTR element.
13. The method as claimed in claim 7, where in the strain modified for production of Guanidine can be from the group of microbes including and not limited to E.coli, Bacillus, Methylobacterium, Corynebacterium, Cyanobacteria, Lactobacillus, Saccharomyces, Pichia, Kluyveromyces, etc.
14. Guanidine based gene expression control for use in in-vitro or in-vivo applications including but not limited to production of agri and non-agri products, sugars, polymers, organic acids, nitrogen compounds, amino acids, protein, carbohydrates, fatty acids, therapeutics, aqua products, dairy products, etc.
15. A method as claimed in claim 14, where the application of Guanidine for in vivo applications includes formulation with excipients such as stickers, binding compounds, etc.