

**“GENE EXPRESSION CONTROL USING GUANIDINE BASED
GENE INDUCTION”**

TECHNICAL FIELD OF THE INVENTION:

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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/
10 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
15 control.

BACKGROUND AND PRIOR ART OF THE INVENTION:

In current scientific era, gene expression strategies involves huge array of
20 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,
25 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
30 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.

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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).

10 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

15 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

20 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.

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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

30 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
5 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
10 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.

15 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,
20 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.

25 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
30 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.

5 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
10 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/
15 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
20 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,
25 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.

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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 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.

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DESCRIPTION OF FIGURES:

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

5 **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.

10 **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.

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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.

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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.

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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.

5 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.

10 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.

15 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.

20 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.

25 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, Allophanase hydrolase etc., which are all the enzymes involved related to Arginine degradation.

30 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:

5 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,
10 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.

15 **EXAMPLES:**

Example 1: Over expression of Ethylene forming enzyme (EFE) and related rate-limiting enzyme genes such as Arginase, glutamine synthase, carbamoyl phosphate synthase, and other genes of Urea synthesis cycle, etc to increase
20 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.
- 25 • 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
30 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.

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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:

10 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

5 Software Name: WIPO Sequence

Software Version: 2.3.0

Production Date: 2024-09-13

General Information:

10 Current application / IP Office: IN

Current application / Application number: 202341016651

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Current application / Applicant file reference: GNANLex_16651

Earliest priority application / IP Office: IN

15 Earliest priority application / Application number: 202341016651

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

20 BASED GENE INDUCTION (en)

Sequence Total Quantity: 4

Sequences:

Sequence Number (ID): 1

25 Length: 1053

Molecule Type: DNA

Features Location/Qualifiers:

- source, 1..1053

> mol_type, other DNA

30 > organism, EFE coding sequence

Residues:

atgaccaacc tacagacttt cgagttgcct accgaggtaa ccggctgcgc cgccgatatc 60
tcattgggaa gggcgctgat ccaagcctgg caaaaagatg gcattttca gatcaagacc 120
gatagtgagc aggatcgcaa aacgcaggaa gcaatggctg ctagcaagca gttttgcaag 180
gaaccgctga ctttaagag tagctgcgtt agcgatctga cctacagcgg ctatgttgcg 240
5 tcaggcgagg aagtcacagc tggtaaacct gatttcctg aaatcttcac tgtctgcaag 300
gacttgctgg taggcgatca gcgtgtaaaa gccggctggc ctgccatgg tccggtgcca 360
tgcccaaata acacctatca gaaaagcatg aagaccttca tggaagagct gggtttagcg 420
ggcgaacggt tgctcaaaact gacagcgctc ggctttgaac taccatcaa cacgttcacc 480
gacttaactc gcgatggtg gcaccacatg cgtgtattac gttcccgcc ccaaacatcc 540
10 acgctgtccc gtggaattgg tgcgcacact gactatgggt tgttgtaat tgccgctcag 600
gacgatgttg gtggcttata tattcgccct ccagtcgagg gagagaagcg taatcgtaac 660
tggttgctcg gtgagagctc agcaggcatg ttgagcacg atgaaccttg gaccttcgtg 720
acgccccccc caggcgctg gacagtttc ccaggtgata tctgcagtt catgaccggc 780
ggccagctgc ttccactcc gcacaaggtt aagctcaata cccgcgaacg ttgcgctgc 840
15 gcttatttc atgagcctaa tttgaagca tccgcctatc cgttggtcga gcccagcgcc 900
aatgagcgta ttcattatgg tgagcactt accaacaatg ttatgcgttg ctatccagat 960
cggatcacca ccagaggat caacaaggag aatcgctgg cgcacttga ggacttgaag 1020
aagtattcgg acaccgcgc gacaggctca tga 1053

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

Sequence Number (ID): 3

Length: 278

Molecule Type: DNA

Features Location/Qualifiers:

- source, 1..278

- 5 > mol_type, other DNA
> organism, Tuf Promoter (promoter of Translation elongation factor Tu) with Guanidine specific 5'UTR

Residues:

tgctcgatct cggcgagcgc cgcggcggcg ccccgagggc gcagcggctc tacgagcagg 60
10 tcggcgaggc cgcgtcctga cccctcattc ggcgcgatcc gccctgccgg caaacctgcc 120
gcttgatcat cgtcgacaaa gcctctaatac ggtccgcaaa atagaataaa tactccaccg 180
ggagttaaat cgtatgaacg attgtttgca tttagtagg tctgagaaga aatgtagata 240
gtcgttcttt ttttagctga aggagagtga aacccatg 278

- 15 Sequence Number (ID): 4

Length: 922

Molecule Type: DNA

Features Location/Qualifiers:

- source, 1..922

- 20 > mol_type, other DNA
> organism, Coding sequence of GFP (Green fluorescent protein)

Residues:

tacacacgaa taaaagataa caaagatgag taaaggagaa gaactttca ctggagtgt 60
cccaattctt gttgaattag atggcgatgt taatgggcaa aaattctctg tcagtggaga 120
25 gggtgaaggt gatgcaacat acggaaaact tacccttaaa ttatttgca ctactgggaa 180
gctacctgtt ccatggccaa cactgtcac tactttctct tatggtgttc aatgctttc 240
aagataccca gatcatatga aacagcatga cttttcaag agtgccatgc ccgaaggta 300
tgtacaggaa agaactatat ttacaaaga tgacgggaac tacaagacac gtgctgaagt 360
caagtttgaa ggtgataccc ttgtaatag aatcgagtta aaaggattg attttaaaga 420
30 agatggaaac attcttgac acaaaatgga atacaactat aactcacata atgtatacat 480
catggcagac aaaccaaaga atggaatcaa agttaacttc aaaattagac acaacattaa 540
agatggaagc gttcaattag cagaccatta tcaacaaat actccaattg gcgatggccc 600

tgctcttta ccagacaacc attacctgtc cacacaatct gccctttcca aagatccaa 660
cgaaaagaga gatcacatga tccttcttga gtttgtaaca gctgctggga ttacacatgg 720
catggatgaa ctatacaaat aaatgtccag acttccaatt gacactaaag tgtccgaaca 780
attactaaat tctcagggtt cctgggttaa ttcaggctga gactttattt atatatttat 840
5 agattcatta aaattttatg aataatttat tgatgttatt aataggggct attttcttat 900
taaataaggct actggagtgt at 922

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We claim,

1. A method of gene modifications for efficient gene expression and control using natural and/ or non-natural inducer compounds.
- 5 2. The method as claimed in claim 1, where in the inducer compounds are selected from but not limited to carbon and/ or nitrogen compounds like sugars, sugar analogues, nucleotides, amino acids, peptides and the like
3. The method as claimed in claim 1, wherein the gene modifications for efficient gene expression includes modifications in the promoter
10 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
15 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
20 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 .
7. The method as claimed in claim 5, where in the inducer compound Guanidine is generated form strain modified with overexpression of
25 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 system was assessed by expression of marker proteins such as
30 and not limited to Green fluorescence protein (GFP), Red fluorescence protein (RFP), Lysozyme, maltose binding protein (MBP) and the like.

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.
- 5 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. 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
- 10 acids, protein, carbohydrates, fatty acids, therapeutics, aqua products, dairy products, etc.

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