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(54) **Title:** PRODUCTION OF SUCCINIC ACID FROM ORGANIC WASTE OR BIOGAS OR METHANE USING RECOMBINANT METHANOTROPHIC BACTERIUM

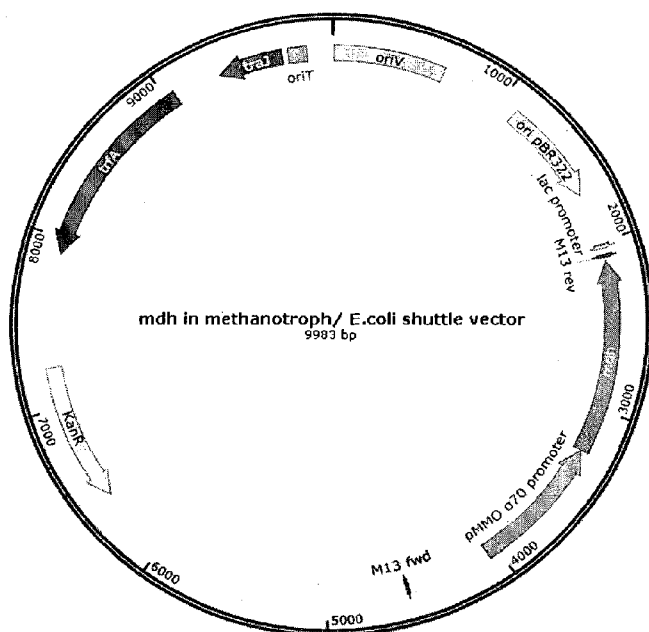


Figure 2

(57) **Abstract:** The present disclosure provides for production of succinic acid from organic waste or biogas or methane using recombinant methanotrophic bacterium. In one embodiment, the recombinant methanotrophic bacterium includes exogenous nucleic acid(s) or gene(s) encoding for specified enzymes. In a further embodiment, succinic acid producing capacity of the recombinant methanotrophic bacterium is increased above the basal level by overexpression or/and down-regulation of selected gene(s). In another embodiment, a process of producing succinic acid using the recombinant methanotrophic bacterium is disclosed. The present invention successfully solves the problems in converting organic waste to a useful chemical thereby providing an environment-friendly and commercially viable solution for waste management.



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PRODUCTION OF SUCCINIC ACID FROM ORGANIC WASTE OR BIOGAS OR METHANE USING RECOMBINANT METHANOTROPHIC BACTERIA

FIELD OF THE INVENTION

- 5 The present invention relates to the field of waste management, and more particularly relates to converting organic waste to succinic acid by employing recombinant methanotrophic bacteria.

BACKGROUND OF THE INVENTION

10 As global living standards and urban populations continue to rise, there's a concurrent increase in the amount of waste generated. Waste management has become the single largest expenditure for most municipalities. Ineffective management of waste is posing serious risk of rapid deterioration in levels of sanitation and general quality of urban life.

15 Disposal of wastes is commonly done by dumping (on land or into water bodies), incineration, and/or long term storage in a secured facility. All these methods have varying degrees of negative environmental impacts with adverse health risks if not properly executed. Apart from these methods, recycling, composting, recovery (including resource and energy), and biological reprocessing etc. are emerging as acceptable sustainable
20 modes of waste management. In recycling, materials generally undergo a chemical transformation and resultant products are recycled to be used for various purposes. For the purpose of resource recovery the organic waste is preferably anaerobically digested (also called Anaerobic Composting or Biomethanation) as compared to aerobic digestion to obtain compost which
25 can be used as an organic fertilizer on agricultural fields. Anaerobic digestion of organic waste results in energy in the form of biogas, and compost in the form of a liquid residual. The biogas consists of methane and carbon dioxide and can be used as fuel or, by using a generator, it can be converted to electricity on-site. This reduces greenhouse gas emissions

30 by using methane as an energy source which would otherwise be emitted from landfilling waste. Landfilling waste gas is similar in composition to biogas with lower amount of methane and differences in component gases. However, the conversion of biogas to electricity is not economically attractive and also results in significant loss of energy during conversion.

35 Methane, present in biogas or landfill gas, can also be converted to syngas and then to chemicals such as methanol. This gas to liquid conversion happens at high temperature and pressure necessitating huge capital investments.

Efficient utilization of biogas as well as methane has always been a
40 challenging task. Advances in biotechnology are enabling development of new and improved microorganisms for efficient conversion of biomass to useful products. However, the existing state of the art does not provide for a unified and efficient way of converting organic wastes and more specifically biogas or methane to target chemicals by employing recombinant
45 microorganisms.

SUMMARY OF THE INVENTION

The present disclosure provides recombinant methanotrophic bacterium for converting organic waste to succinic acid. It further provides methods for using the recombinant methanotrophic bacterium for converting biogas or
50 methane (produced by anaerobic digestion of the organic waste) to succinic acid. The present invention successfully solves the problems in converting organic waste to a useful chemical thereby providing an environment-friendly and commercially viable solution for waste management.

In one aspect of the present invention, a recombinant methanotrophic
55 bacterium for producing succinic acid from biogas or methane is provided. The said recombinant methanotrophic bacterium includes exogenous nucleic acid(s) or gene(s) encoding for a first group of enzymes consisting of malate dehydrogenase, pyruvate carboxylase, phosphoenol pyruvate

carboxylase, phosphofructokinase, citryl-CoA lyase, isocitrate lyase,
60 fumarate reductase, malate synthase, aspartate transaminase, succinyl
CoA synthetase, pyruvate kinase or any combination thereof.

In further aspect of the present invention, increasing production of succinic
acid in the recombinant methanotrophic bacterium from biogas or methane
is provided by overexpression or/and down-regulation of selected gene(s).

65 In another aspect of the present invention, a process of producing succinic
acid using the recombinant methanotrophic bacterium is provided. The
process comprises the steps of receiving biogas and/or methane as input,
culturing the bacterium in the input thereby converting the input into
succinic acid, and optionally purifying or separating the succinic acid
70 produced from the culture for obtaining the succinic acid.

In yet another aspect of the present invention, a process of producing
succinic acid using recombinant methanotrophic bacterium is provided. The
process comprises the steps of receiving organic waste as an input,
anaerobically digesting the organic waste to biogas, culturing the bacterium
75 in the biogas so generated thereby converting the biogas to the succinic
acid and optionally purifying the succinic acid produced from the culture for
obtaining the succinic acid. During the second step of culturing the
recombinant methanotrophic bacterium in the biogas following parameters
were maintained: temperature ranging from about 35 °C to about 50 °C,
80 preferably 45 °C, pH ranging from about 4 to about 7, preferably 5.8, and
dissolved oxygen concentration of <20%.

In a further aspect of the present invention, the process of producing
succinic acid using recombinant methanotrophic bacterium further
comprises cleaning the generated biogas to remove carbon dioxide and
85 other impurities present in the biogas so as to obtain methane. The
recombinant methanotrophic bacteria are cultured in the methane so
obtained.

Other features of the embodiments will be apparent from the accompanying drawings and from the detailed description that follows.

90

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Figure 1 illustrates some aspects of methanotroph metabolism and a pathway for conversion of methane to succinate in *M. capsulatus*, according to one embodiment.

95 **Figure 2** illustrates a plasmid map of malate dehydrogenase cloned in a bacterial shuttle vector, where malate dehydrogenase from *E.coli* (*mdh*) was PCR amplified and cloned under σ 70 promoter in a methanotroph/*E.coli* shuttle vector, according to one embodiment.

100 **Figure 3** is a graph illustrating the comparison between recombinant and wildtype *M.capsulatus* grown in culture tubes according to one embodiment. Genes overexpressed from pSB107 (SEQ ID 1), pSB108 (SEQ ID 2), pSB109 (SEQ ID 3) in *M.capsulatus* were tested for succinic acid production. Some recombinant strains had higher levels of succinic acid compared to control strain.

105 **Figure 4** is a graph depicting comparative growth profile of methanotroph strain on Biogas and Methane.

Figure 5 is a graph highlighting increase in succinic acid production as a function of time, wherein the conversion of biogas, generated from organic waste, to succinic acid is performed using recombinant methanotroph strain
110 with over-expressed malate dehydrogenase (SEQ ID 1; pSB107), according to one embodiment.

Figure 6 illustrates a plasmid map of a vector for cloning and expression of overexpression gene targets under the control of a σ 54 promoter, according to one embodiment.

115 **Figure 7** is a graph depicting effect of the overexpression of various gene combinations on succinic acid production in the recombinant *M. capsulatus*, according to one embodiment. The genes encode for Malate dehydrogenase, Phosphofructokinase, Pyruvate Kinase, Isocitrate Lyase, Citryl CoA Lyase (D,F,E subunits) Malate synthase A, Succinyl CoA
120 Synthetase (C, D subunits), Fumarate Reductase (A,B,C,D subunits).

DEPOSIT OF MICROORGANISM

The following microorganism has been deposited in accordance with the terms of the Budapest Treaty with the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India:

Identification ref.	Taxonomic designation	MTCC Accession number
STB18	<i>Methylococcus capsulatus</i>	MTCC 25005

125

The recombinant *Methylococcus capsulatus* capable of converting methane to succinic acid was deposited as MTCC Accession No.: MTCC 25005 on January 27 2015 with the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Sector 39-A, Chandigarh –
130 160036, INDIA. The MTCC issued an accession number in this matter on March 26 2015. STB18 refers to the recombinant *M.capsulatus* strain with the gene corresponding to SEQ ID 1 overexpressed from pSB107 plasmid. This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the
135 Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

DETAILED DESCRIPTION OF THE INVENTION

140 Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include the plural and plural terms shall include the singular. Generally, nomenclatures used in
145 connection with and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, genetics and protein/ nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art. Certain references and other documents cited herein are expressly incorporated herein by reference. In case of conflict,
150 the present specification, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

Before the present vectors, genomes, bacteria, microbes, compositions, methods, and other embodiments are disclosed and described, it is to be understood that the terminology used herein is for the purpose of describing
155 particular embodiments only and is not intended to be limiting. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

The term "comprising" as used herein is synonymous with "including" or
160 "containing," and is inclusive or open-ended and does not exclude additional, unrecited members, elements or method steps.

The term "polynucleotide", "nucleic acid molecule", "nucleic acid", or "nucleic acid sequence" refers to a polymeric form of nucleotides of at least 10 bases in length. The term includes DNA molecules and RNA molecules,
165 as well as analogs of DNA or RNA containing non-natural nucleotide analogs, non-native internucleoside bonds, or both. The nucleic acid can be in any topological conformation.

The term "protein" or "polypeptide" as used herein indicates a polymeric form of amino acids composed of two or more amino acidic monomers and/or analogs thereof. As used herein, the term "amino acid" or "amino acidic monomer" refers to any natural and/or synthetic amino acids.

The term "enzyme" as used herein refers to any substance that catalyzes or promotes one or more chemical or biochemical reactions, which usually includes enzymes totally or partially composed of a polypeptide, but can include enzymes composed of a different molecule including polynucleotides.

The term "Heterologous" or "exogenous" refers to molecules, specifically polynucleotides or polypeptides or enzymes that are not present naturally in the host or that is native to the host but at altered expression levels when compared to natural expression levels. These are expressed independently at levels of expression higher, equal or lower than the level of expression in a native organism.

As used herein, nucleic acid construct, nucleic acid (e.g., a polynucleotide), polypeptide, or host cell is referred to as "recombinant" when it is non-naturally occurring, artificial or engineered. In some embodiments, recombinant constructs contain two or more naturally-occurring sequences that are linked together in a way that does not occur naturally. A recombinant cell contains a recombinant polynucleotide. For clarity, reference to a cell of a particular strain refers to a parental cell of the strain as well as progeny and genetically modified derivatives of the same.

As used herein, "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked into a cell.

The terms "percent identity", "percent identical", "% identical" and "% identity" are used interchangeably herein to refer to the percent amino acid or polynucleotide sequence identity that is obtained by ClustalW2 analysis

(EMBL – EBI, Cambridge, UK), counting the number of identical matches in the alignment and dividing such number of identical matches by the length of the reference sequence, and using the following ClustalW2 parameters to achieve slow/accurate pairwise optimal alignments – DNA/Protein Gap
200 Open Penalty:15/10; DNA/Protein Gap Extension Penalty:6.66/0.1; Protein weight matrix: Gonnet series; DNA weight matrix: Identity; Toggle Slow/Fast pairwise alignments = SLOW or FULL Alignment; DNA/Protein Number of K-tuple matches:2/1; DNA/Protein number of best diagonals: 4/5; DNA/Protein Window size:4/5.

205 The term “methanotrophs” or “methanotrophic bacteria” herein refers to bacteria that utilize methane as a source of carbon and energy. These bacteria are widely present in nature and can be found in areas of high methane content such as oceans, mud, marshes, underground environments, soils, rice paddies and landfills. Some of these are obligate
210 and can only use methane as a source of carbon and energy. Some of these are facultative and are known to additionally use other substrates such as succinate, acetate, pyruvate etc.

The term “organic waste” herein refers to the components of waste that can be broken down into its base components in a reasonable amount of time
215 by microorganisms. Organic waste can be found in commonly occurring sources of waste such as municipal solid waste, green waste, food waste, paper waste, biodegradable waste, human waste, sewage, manure and slaughterhouse waste.

The term “Anaerobic digestion” herein refers to a set of processes wherein
220 several types of microorganisms break down biodegradable material in the absence of oxygen. The end products are a gas containing mostly methane and carbon dioxide, referred to as biogas, and a slurry or solid fraction, referred to as digestate. Different technologies are available for anaerobic digestion that vary in the process and process parameters affecting
225 digestion.

The term "biogas" herein refers to the major product resulting from anaerobic digestion of waste. Typical composition of biogas is methane (50-75%), carbon dioxide (25-50%), nitrogen (0-10%), hydrogen (0-1%), hydrogen Sulphide (0-3%), oxygen (0-2%) and water vapour (3-5%). The

230 biogas composition can vary depending on, among other factors, the type of waste, its organic matter load, feeding rate of digester and conditions of anaerobic digestion. Biogas is typically lighter than air and produces less calories by combustion compared to equal volume of natural gas. Biogas is typically used for heating, generating electricity or as cooking fuel.

235 As used herein the phrase "biogas cleaning" or "biogas upgrading" or "biogas scrubbing" refers to the process of removing the non-methane components of biogas. Depending on the use of the biogas, the extent of biogas cleaning can vary. Different methods of cleaning the various non-methane components of biogas are known and practiced. Hydrogen

240 Sulphide can be removed by among others biological fixation by using iron oxidizing bacteria, dosing with iron chloride, water scrubbing, absorption activated carbon or bubbling through sodium hydroxide. Water vapor present in biogas can be removed by among others passive cooling, refrigeration, absorption into a drying medium, or adsorption into silica gel.

245 Ammonia present in the biogas is usually in very low amounts and can be removed by water scrubbing. Oxygen and nitrogen are typically not present in large amounts in biogas and can be removed by adsorption with activated carbon, molecular sieves or membranes. "Biogas upgrading" more typically refers to the removal of carbon dioxide from the biogas to

250 increase the energy content of the gas. Some technologies for removing carbon dioxide are commercially available and some are at the pilot or demo scale. Pressure swing adsorption is a process wherein the carbon dioxide can be removed by adsorption onto materials like activated carbon or zeolites under elevated pressure. Another method is removal of carbon

255 dioxide by absorption. This is usually done by a counter current flow of biogas with a liquid in a column filled with plastic packaging. Absorption can

be done using water, organic solvents or amine solutions. Another classical method used is membrane separation using materials that are permeable to carbon dioxide, water and ammonia.

260 The terms "succinic acid" and "succinate" are used interchangeably in the context of the invention.

The present invention provides an environment-friendly and commercially viable way of handling waste by converting the organic waste into useful chemicals, namely succinic acid, by employing the recombinant
265 methanotrophic bacteria capable of converting methane or biogas into succinic acid.

In one of the embodiments, the present invention provides for a recombinant methanotrophic bacterium capable of producing succinic acid from biogas or methane. The recombinant methanotrophic bacterium
270 produces higher amounts of succinic acid as compared to wildtype methanotrophic bacterium when fed with biogas or methane. Further, most importantly the recombinant methanotrophic bacterium accumulates the succinic acid so produced. The methanotrophs in ordinary course do not accumulate succinate as it serves as a native intermediate for central
275 carbon metabolism.

Methanotrophs or methanotrophic bacteria are unique in their ability to utilize methane as a sole carbon and energy source. However, the methanotrophs are not well established industrial hosts. They are present in a wide variety of environments and play a critical role in the oxidation of
280 methane in the natural world (Hanson, R. S., & Hanson, T. E. (1996). Methanotrophic bacteria. *Microbiological Reviews*, 60(2), 439–471). The methanotrophs are classified into two major groups based on the pathways used for assimilation of formaldehyde, the major source of cell carbon, and other physiological and morphological features. Type I methanotrophs
285 employ the RuMP pathway for formaldehyde assimilation, whereas type II

methanotrophs employ the serine pathway for formaldehyde assimilation. The use of enzymes known as methane monooxygenases - MMOs (EC 1.14.13.25) to catalyze the oxidation of methane to methanol is a defining characteristic of methanotrophs. The oxidation of methane by aerobic methanotrophs is initiated by MMOs utilizing two reducing equivalents to split the O-O bonds of dioxygen. One of the oxygen atoms is reduced to form H₂O, and the other is incorporated into methane to form CH₃OH methanol. Two forms of MMOs have been found in methanotrophic bacteria, a soluble form (sMMO) and a membrane bound form, pMMO. Methanol is oxidized to formaldehyde by methanol dehydrogenase (MDH), an enzyme that's highly expressed in most methanotrophs. The further oxidation of formaldehyde to carbon dioxide via formate provides most of the reducing power required for the oxidation of methane. Multiple enzymes are known that catalyze the oxidation of formaldehyde to formate. The further oxidation of formate to carbon dioxide is catalyzed by an NAD-dependent formate dehydrogenase. Formaldehyde produced from the oxidation of methane and methanol by methanotrophic bacteria is assimilated to form intermediates of the central metabolic routes that are subsequently used for biosynthesis of cell material. The two known pathways used by methanotrophic bacteria for the synthesis of multicarbon compounds from formaldehyde are the serine pathway, in which 2 mol of formaldehyde and 1 mol of carbon dioxide are utilized to form a three-carbon intermediate, and the RuMP cycle for the assimilation of 3 mol of formaldehyde to form a three-carbon intermediate of central metabolism (Figure 1).

In one of the embodiments, the recombinant microorganism of the present invention is selected from a group of organisms comprising: *Methylococcus capsulatus*, *Methylobacterium extorquens*, *Methylobacterium organophilum*, *Methylocapsa acidiphila*, *Methylobacterium mesophilicum*, *Methylobacterium dichloromethanicum*, *Methylocella silvestris*, *Methylosinus trichosporium*, *Methylobacillus*

flagellatus KT, *Methylibium petroleiphilum* PM1, *Methylobacterium*
nodulans, *Methylobacterium populi*, *Methylobacterium chloromethanicum*,
Methylacidiphilum infernorum V4, *Methylophilus methylotrophus*,
 320 *Methylomonas methanica*, *Methylobacterium rhodesianum* MB 126,
Methylobacter tundripaludum, *Methylobacterium* sp. 4-46, *Methylovorus*
glucosetrophus SIP3-4, *Mycobacterium smegmatis*, *Methylobacterium*
rhodesianum, *Methylosinus sporium*, *Methylocella palustris*,
Methylobacterium fujisawaense, *Methylocystis parvus*, *Methylovulum*
 325 *miyakonense*, *Methylobacterium rhodinum*, *Methylocystis echinoides*,
Methylomonas rubra, *Methylococcus thermophilus*, *Methylobacterium*
aminovorans, *Methylobacterium thiocyanatum*, *Methylobacterium zatmanii*,
Acidithiobacillus ferrivorans, *Methylobacterium aquaticum*,
Methylobacterium suomiense, *Methylobacterium adhaesivum*,
 330 *Methylobacterium podarium*, *Methylobacter whittenburyi*, *Crenothrix*
polyspora, *Clonothrix fusca*, *Methylobacter bovis*, *Methylomonas*
aurantiaca, *Methylomonas fodinarum*, *Methylobacterium variabile*,
Methylocystis minimus, *Methylobacter vinelandii*, *Methylobacterium*
hispanicum, *Methylomicrobium japonense*, *Methylococcaceae bacterium*,
 335 *and Methylocystis methanolicus*.

Some species of methanotrophs including, but not limited to,
Methylococcus capsulatus, *Methylocella silvestris*, *Methylobacterium*
extorquens etc. are well-characterized and basic molecular biology tools for
 host manipulation have been developed (see
 340 www.methanotroph.org/genetics).

In an exemplary embodiment, the recombinant methanotrophic bacterium
 for producing succinic acid is created from *Methylococcus capsulatus*.

In another exemplary embodiment, the recombinant methanotrophic
 345 bacterium for producing succinic acid is created from *Methylococcus*
capsulatus (Bath).

In yet another embodiment, the recombinant methanotrophic bacterium for producing succinic acid is created from *Methylococcus trichosporium*.

In various embodiments of the recombinant methanotrophic bacterium, the present invention provides multiple ways of increasing the succinic acid production including:

- (a) overexpression of one or more genes encoding for certain key enzymes of TCA cycle;
- (b) overexpression of one or more genes encoding for certain key enzymes of central carbon metabolism;
- (c) overexpression of one or more genes encoding for enzymes of methane metabolism;
- (d) overexpression of one or more genes encoding for non-native enzymes;
- (e) overexpression of one or more genes encoding for transporters;
- (f) deletion or downregulation of genes encoding for enzymes of the central carbon metabolism/TCA cycle; and
- (g) deletion or downregulation of genes encoding for enzymes used by pathways that compete with succinate production.

It is to be noted that (a) to (g) could be used independently or in any combination thereof.

Expression of the heterologous genes may be accomplished by conventional molecular biology means (Green.M.R. & Sambrook.J , *Molecular Cloning- A laboratory Manual*, Fourth Edition). For example, the heterologous genes can be under the control of an inducible promoter or a constitutive promoter. The heterologous genes may either be integrated into a chromosome of the host microorganism, or exist as an extra-chromosomal genetic elements that can be stably passed on ("inherited") to

daughter cells. Such extra-chromosomal genetic elements (such as plasmids, BAC, YAC, etc.) may additionally contain selection markers that
375 ensure the presence of such genetic elements in daughter cells.

As used herein, the term "overexpress" is intended to encompass increasing the expression or activity of a gene or protein to a level greater than the cell normally produces. It is intended that the term encompass overexpression of endogenous, as well as heterologous gene or proteins.
380 Overexpression of genes or proteins can be done by conventional molecular biology methods. In some embodiments, the genes can be overexpressed by introducing additional copies of the genes on the chromosome or extra-chromosomally on plasmids, BACs or YACs. In certain embodiments, the expression can be increased by optimizing the
385 nucleotide sequence for expression in the specific host such as through codon optimization. In other embodiments, the gene expression can be increased by altering the promoter or ribosome binding site operably linked to the gene. In yet other embodiments the gene activity can be increased through mutations in the gene that enhance the enzymatic activity.

390 The term "down-regulated" or "deleted" used herein with reference to a gene or protein, indicates any modification in the genome and/or proteome of a microorganism that eliminates or reduces the biological activity of the gene, protein or enzyme either directly or indirectly. For example, deletion or downregulation of gene or protein can be performed by deleting or
395 mutating a native or heterologous polynucleotide encoding for the gene or protein in the microorganism, by deleting or mutating a native or heterologous polynucleotide encoding for an enzyme involved in the pathway for the synthesis of the gene or protein in the microorganism, by activating a further native or heterologous molecule that inhibits the
400 expression of the gene or protein in the microorganism. In particular, in some embodiments inactivation of a gene or protein such as an enzyme

can be performed by deleting from the genome of the recombinant microorganism one or more endogenous genes encoding for the enzyme.

For assembly of the constructs to enable overexpression or downregulation or deletion of specific gene, conventional molecular biology methods can be
405 used (Green, M.R. and Sambrook, J, 2001). *Molecular cloning: a laboratory manual*. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory; Ellis, T., Adie, T., & Baldwin, G. S. (2011); *DNA assembly for synthetic biology: from parts to pathways and beyond*. *Integrative Biology: Quantitative Biosciences from Nano to Macro*, 3(2), 109–18).

Assembly of DNA parts through restriction digestion and ligation is well-established and known to those skilled in the art. Other methods that offer standardized, scarless, sequence independent, multi piece DNA assembly such as SLIC (Sequence and Ligation Independent Cloning), Gibson
415 assembly, CPEC (Circular Polymerase Extension Cloning) or SLICE (Sequence and Ligation Cloning Extract) have more recently been established. In some embodiments, SLIC based assembly is used for generating DNA constructs or vectors for overexpression or downregulation or deletion (<https://j5.jbei.org/j5manual/pages/22.html>). In other
420 embodiments CPEC is used for assembly of DNA constructs for overexpression, deletion or down-regulation. In further embodiments, methods such as site-directed mutagenesis, transposon mutagenesis, crispr/cas assisted genome engineering and recombineering can be used directly for overexpression, down-regulation or deletion of specific gene or
425 protein.

In one embodiment of the recombinant methanotrophic bacterium, increase in succinate production is achieved by overexpressing the genes coding for enzymes of TCA cycle such as, but not limited to, fumarase (fumC/B/A), malate dehydrogenase (mdh), malate:quinone oxidoreductase (mqo),
430 isocitrate dehydrogenase (icd), 2-oxoglutarate dehydrogenase (sucA/B), 2-

oxoglutarate dehydrogenase (lpd), citryl-CoA lyase, isocitrate lyase, malate synthase, fumarate reductase and succinyl-CoA synthetase (suc C/D).

In another embodiment of the recombinant methanotrophic bacterium, increase in succinate production is achieved by overexpression of genes
 435 encoding for keys enzymes of central carbon metabolism such as, but not limited to, pyruvate dehydrogenase, pyruvate kinase, phosphoenolpyruvate carboxylase, hexulose 6-phosphate (hps) synthase, 6-phospho-3-hexuloisomerase, phosphor-fructo kinase, fructose-bisphosphate aldolase, transketolase, transaldolase, ribulose-5 phosphate epimerase, pyruvate
 440 carboxylase, aspartate transaminase and phosphoenolpyruvate carboxykinase.

In yet another embodiment of the recombinant methanotrophic bacterium, increase in succinate production is achieved by overexpression of genes encoding for enzymes of methane metabolism such as, but not limited to,
 445 methane monooxygenase and methanol dehydrogenase.

In yet another embodiment of the recombinant methanotrophic bacterium, increase in succinate production is achieved by overexpression of genes encoding for non-native enzymes such as, but not limited to, succinate transporters and dehydrogenases.

450 In a further embodiment of the recombinant methanotrophic bacterium, increase in succinate production is achieved by overexpression of gene encoding for transporters such as, but not limited to, *Schizosaccharomyces pombe* malate transporter gene SpMAE1 and *E.coli* C4 dicarboxylic/orotate symporter dctA.

455 **Table 1** enlists sources of the exogenic genes along with their Seq ID NOs. (encoding for the enzymes) selected for overexpression in the methanotrophic bacterium in various embodiments of the present invention.

SEQID	Gene	Host
SEQID 1	Malate dehydrogenase	<i>E.coli</i>
SEQ ID 2	Pyruvate carboxylase	<i>P.aeruginosa</i>
SEQ ID 3	Phosphoenol pyruvate carboxylase	<i>E.coli</i>
SEQ ID 4	Phosphofructokinase	<i>M. capsulatus</i> Bath
SEQ ID 5	Pyruvate Kinase	<i>E.coli</i>
SEQ ID 6	Isocitrate Lyase	<i>E.coli</i>
SEQ ID 7	Citryl CoA Lyase (D,F,E subunits)	<i>E.coli</i>
SEQ ID 8	Malate synthase A	<i>E.coli</i>
SEQ ID 9	Succinyl CoA Synthetase (C, D subunits)	<i>E.coli</i>
SEQ ID 10	Fumarate Reductase (A,B,C,D subunits)	<i>E.coli</i>
SEQ ID 11	Aspartate transaminase	<i>E.coli</i>

460

Table 1

The enzymes enlisted in **Table 1**, having gene sequence ID Nos: 1 to 11, code for amino acid sequences that are at least 80% identical to reference amino acid sequence set forth in SEQ ID Nos: 12 to 22.

Table 2 provides a list of genes encoding for the enzymes and its
465 respective amino acid sequence.

Enzyme	Gene SEQ ID	Amino Acid SEQ ID
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Malate dehydrogenase	SEQ ID 1	SEQ ID 12
Pyruvate carboxylase	SEQ ID 2	SEQ ID 13
Phosphoenol pyruvate carboxylase	SEQ ID 3	SEQ ID 14
Phosphofructokinase	SEQ ID 4	SEQ ID 15
Pyruvate Kinase	SEQ ID 5	SEQ ID 16
Isocitrate Lyase	SEQ ID 6	SEQ ID 17
Citryl CoA Lyase (D,F,E subunits)	SEQ ID 7	SEQ ID 18
Malate synthase A	SEQ ID 8	SEQ ID 19
Succinyl CoA Synthetase (C, D subunits)	SEQ ID 9	SEQ ID 20
Fumarate Reductase (A,B,C,D subunits)	SEQ ID 10	SEQ ID 21
Aspartate transaminase	SEQ ID 11	SEQ ID 22

Table 2

In another embodiment of the recombinant methanotrophic bacterium, increase in succinate production is achieved by deletion or downregulation of gene(s) encoding for enzyme(s) of the central carbon metabolism/TCA cycle or gene(s) encoding for enzymes used by pathways that compete with succinate production such as, but not limited to, glucose-6-phosphate dehydrogenase, succinate dehydrogenase, pyruvate decarboxylase, malate:quinone oxidoreductase, citrate synthase, isocitrate dehydrogenase, lactate dehydrogenase, acetyl CoA synthase, phosphotransacetylase, formaldehyde dehydrogenase and formate dehydrogenase.

The present invention also provides for a method of creating the recombinant methanotrophic bacterium that converts methane to succinic acid at an efficient rate while accumulating the same. In one embodiment, the method of creating the recombinant *Methylococcus capsulatus* strain includes:

(a) the genes/operons encoding for enzymes, used in the succinate pathway, Malate dehydrogenase (*mdh*), Fumarase (*fumA/fumB/fumC*), Fumarate reductase (*frdA/frdB/frdC/frdD*) were amplified and cloned into a shuttle vector; and

(b) the vector is transformed into *M. capsulatus* and positive transformants of *M. capsulatus* containing the genes/operons encoding for enzymes Malate dehydrogenase (*mdh*), Fumarase (*fumA/fumB/fumC*), Fumarate reductase (*frdA/frdB/frdC/frdD*) were verified by PCR.

Thereafter, the recombinant *M. capsulatus* is grown using standard methods in the presence of methane or biogas. The recombinant methanotrophs so created when fed with methane not only produces greater amount of succinic acid as compared to natural occurring *M. capsulatus* but also accumulates the succinic acid so produced.

In one exemplary embodiment, Malate dehydrogenase (*mdh E.coli*; SEQ ID 1), pyruvate carboxylase (*pyc P.aeruginosa*, SEQ ID 2), phospho enol pyruvate carboxylase (*pepc E.coli*, SEQ ID 3), genes were amplified individually from genomic DNA using primers flanked with *SacI* and *SphI/PciI* restriction enzymes. The amplified gene/operon was restriction digested with *SacI* and *SphI/PciI* and cloned into the same sites in the broad host range vector pMHA201 to create pSB107, pSB108, and pSB109. pMHA201 (Alit, H., & Murrell, J. C., *Development and validation of promoter-probe vectors for the study of methane monooxygenase gene expression in Methylococcus capsulatus Bath*, Microbiology, 155(3), pp 761–771 (2009)) is a plasmid with a broad range Origin of replication

(OriV), Kanamycin resistance gene, Ampicillin resistance gene and OriT for conjugative transfer. Plasmid pSB107, pSB108 and pSB109 were sequence verified.

Methanotroph strains were cultivated in nitrate mineral salt (NMS) medium
510 (<http://www.methanotroph.org/wiki/culturing-tips/>). NMS agar plates were prepared with 1.5 % (w/v) Bacto agar. Antibiotics were added as required: Kanamycin (30 µg/ml) and Gentamicin (5µg/ml). Methanotrophs were typically grown in 250 ml conical flasks with 24/29 joint containing 50 ml NMS medium. Flasks were sealed with suba-seals and gassed with 50 ml
515 (i.e. ~20 %) methane/carbon dioxide (95/5, v/v mix). Methanotrophs grown on NMS agar plates were incubated in gas-tight container under a methane/air/carbon dioxide atmosphere (50/45/5, by vol.) at the appropriate temperature. The gas was replenished every 2 days until colonies formed, usually within 5–10 days. *M.capsulatus* Bath.(From Prof.Colin Murrell,
520 University of Norwich) derived strains were incubated at 45°C. Conjugation of pSB107, pSB108 and pSB109 into *M.capsulatus* was done based on the protocol described by Martin, H., & Murrell, J. C. (1995), *Methane monooxygenase mutants of Methylosinus trichosporium constructed by marker-exchange mutagenesis*, FEMS Microbiology Letters, 127(3), 243–
525 248. 30 mL of *M.capsulatus* culture was spun down and resuspended in 5mL of NMS media. 2 mL of *E.coli* S.17 lambda pir bearing the plasmid to be conjugated was spun down, washed with 1 mL of NMS and resuspended again in 1 mL of NMS. The two cultures were mixed and filtered onto a 0.2µ nitrocellulose membrane. The membrane was placed on
530 an NMS agar plate containing 0.02 % (w/v) proteose peptone and incubated for a duration of 24 hours at 37°C in a gas-tight container under a methane/air/carbon dioxide atmosphere (50/45/5, by vol.). Following incubation, the cells were washed with 1 ml NMS and collected by centrifugation (7,000 x g for 5 minutes). Aliquots (50-100 µl) of the cells
535 were spread onto NMS plates containing 30µg/ml kanamycin for plasmid selection and incubated at 45 °C in a gas-tight container under a

methane/air/carbon dioxide atmosphere (50/45/5, by vol.). Colonies typically formed on the plates after 8-12 days. Colonies were re-streaked onto NMS agar plates with 30ug/ml of kanamycin to confirm the true
540 recombinant strains.

Recombinant methanotrophic strains with SEQ ID 1 had >10% improved levels of succinic acid compared to control. Recombinant methanotrophic strains with SEQ ID 2 had >50% improved levels of succinic acid when compared to control.

545 Recombinant strains that had combinations of genes overexpressed had further improvements in levels of succinic acid compared to recombinant strains with single gene overexpression. Recombinant strain with SEQ ID 1 and 6 had >40% improved levels of succinic acid compared to recombinant strains with a single gene overexpression.

550 The present invention also provides a process for organic waste management by using the recombinant methanotrophic bacterium capable of producing succinic acid from biogas or methane. The process broadly involves (a) converting the waste to biogas by anaerobic digestion, (b) converting biogas to succinic acid by using the recombinant methanotrophic
555 bacterium, or alternatively scrubbing the biogas so produced to have methane and then converting it to succinic acid by using the recombinant methanotrophic bacterium.

In one embodiment, biogas is used as an input for producing succinic acid by employing the recombinant methanotrophic bacterium. The process
560 steps include:

A. Anaerobically digesting organic waste to break it down to biogas via three distinct stages of hydrolysis, acetogenesis, and methanogenesis. In the first stage, a group of microorganisms comprising fermentative bacteria, secreting enzymes (lipases, proteases, cellulases, amylases, etc.), hydrolyses polymeric
565

materials to monomers such as sugars and amino acids. In the next stage, products of the first stage are subsequently converted by a second group of bacteria comprising acetogenic bacteria to simple organic acids, carbon dioxide and hydrogen. In the final stage, a third group of bacteria comprising methanogens converts carbon dioxide, hydrogen and acetate to methane. Various aspects of the process of breaking down of solid waste have been well-researched, stream lined, and solutions at various scales have been developed. The most valuable component of biogas is methane (CH_4) which constitutes around 50-60%, the remaining portion comprises carbon dioxide (CO_2) and small percentages of other gases. The overall process of anaerobic digestion and output varies depending on the size of plant, type of waste, process conditions for fermentation, type of fermentation process etc.

B. Cleaning up the biogas to remove carbon dioxide and other impurities present in the gas. The cleaning further includes two steps- (i) cleaning of hydrogen sulphide (H_2S), NH_3 , water vapour and other impurities, and (ii) removal of carbon dioxide. Methods employed for biogas purification include, but not limited to, chemical absorption, high pressure water scrubbing, pressure swing adsorption, cryogenic separation, and membrane separation. The steps employed are well-researched and optimised to achieve efficient purification. The main output from this process is the methane gas.

C. Third step of the process plays most significant part where the methane gas is converted to succinic acid by using the recombinant methanotrophic bacterium capable of metabolising methane/biogas to produce succinic acid. The process of converting methane to succinic acid includes:

- 595 (1) Conversion of methane to methanol involves oxidation of methane to methanol by the methane monooxygenase enzyme (EC number EC 1.14.13.25). As mentioned above the methane monooxygenases (MMOs) are unique enzymes that can catalyze the oxidation of methane in the presence of oxygen;
- 600 (2) Conversion of methanol to formaldehyde involves oxidation of methanol to formaldehyde by methanol dehydrogenase (EC 1.1.1.244). Gram negative methanotrophs have a periplasmic methanotroph that is cytochrome c dependent. Gram positive methanotrophs have a NAD dependent enzyme that catalyzes
- 605 this step;
- (3) Conversion of formaldehyde to pyruvate via central carbon metabolism involving assimilation of formaldehyde into central carbon metabolism of the methanotrophs and conversion to pyruvate via the steps of the RuMP pathway or serine pathway.
- 610 Formaldehyde is a key intermediate that gets assimilated into the central carbon metabolism;
- (4) Conversion of pyruvate into acetyl CoA by components of pyruvate dehydrogenase complex (EC 1.2.4.1); and
- (5) Conversion of acetyl CoA into succinate via enzymes of the
- 615 tricarboxylic acid cycle.

The pathway for succinate production in some instances encompasses carbon dioxide fixation. One mole of carbon dioxide is incorporated into phosphoenol pyruvate to make oxaloacetate catalyzed by phosphoenol pyruvate carboxylase. In addition, malic enzyme and pyruvate carboxylase

620 incorporate one mole of carbon dioxide into pyruvate to form malate and oxaloacetate, respectively. Oxaloacetate or malate is then converted to succinic acid by enzymes of the TCA cycle.

In an alternate embodiment, biogas is directly used as input without cleaning it up to remove carbon dioxide and other impurities, hence omitting
625 the step B provided for the above described embodiment, for producing succinic acid.

In another alternate embodiment, the biogas used as the input has varying ratios of methane to carbon dioxide such as, but not limited to, from 95% methane:5% CO₂ to 50% methane:50% CO₂. Depending on the type of
630 substrates used, anaerobic digestion, biogas cleaning *etc.* the ratio of methane to carbon dioxide in the input may vary. In some embodiments the ratio of methane to carbon dioxide is 95%:5%. In other embodiments it can be 50% methane:50% carbon dioxide. In another alternate embodiment, the biogas used as the input has varying ratios of methane to carbon
635 dioxide such as, but not limited to, from 95% methane:5% CO₂ to 50% methane:50% CO₂.

In yet another embodiment, purified methane gas is used as an input for producing succinic acid by employing the recombinant methanotrophic bacterium. The process of converting methane to succinic acid includes:

- 640 (1) Conversion of methane to methanol involves oxidation of methane to methanol by the methane monooxygenase enzyme (EC number EC 1.14.13.25). As mentioned above the methane monooxygenases (MMOs) are unique enzymes that can catalyze the oxidation of methane in the presence of oxygen;
- 645 (2) Conversion of methanol to formaldehyde involves oxidation of methanol to formaldehyde by methanol dehydrogenase (EC 1.1.1.244). Gram negative methanotrophs have a periplasmic methanotroph that is cytochrome c dependent. Gram positive methanotrophs have a NAD dependent enzyme that catalyzes this step;
- 650 (3) Conversion of formaldehyde to pyruvate via central carbon metabolism involving assimilation of formaldehyde into central carbon metabolism of

the methanotrophs and conversion to pyruvate via the steps of the RuMP pathway or serine pathway. Formaldehyde is a key intermediate that gets assimilated into the central carbon metabolism;

655 (4) Conversion of pyruvate into acetyl CoA by components of pyruvate dehydrogenase complex (EC 1.2.4.1); and

(5) Conversion of acetyl CoA into succinate via enzymes of the tricarboxylic acid cycle.

In another embodiment, efficiency of succinate production and
660 accumulation is increased by overexpressing enzymes involved in carbon dioxide fixation such as, but not limited to, pyruvate carboxylase, phosphoenol pyruvate carboxylase, malic enzyme, and phosphoenolpyruvate carboxykinase etc.

The conditions of fermentation of the biogas or methane to succinic acid
665 also directly affects the production of succinic acid. Some of the key parameters affecting the fermentation of biogas are conditions such as pH, temperature, dissolved oxygen concentration in the media, composition of the media etc. Some of the conditions are optimized for the recombinant methanotroph for optimal production of succinic acid. In one embodiment,
670 preferred temperature for fermentation is the optimal temperature for growth of *M.capsulatus*, i.e. 45°C. In other embodiments, the temperature for fermentation may vary from 35°C to 50°C. In another embodiment, the pH during fermentation is maintained at the pH which is optimal for the strain, i.e. pH 5.8. In other embodiments, the pH during fermentation is
675 maintained at pH lower than 6.8. In a further embodiment, the pH is maintained from about 3 to about 7. In yet another embodiment, the pH is maintained from about 4 to about 6. Another parameter that has a critical effect on the succinic acid production is the dissolved oxygen concentration in the media. In some instances this is maintained at 20% of maximum DO.
680 In another embodiment, the DO is maintained at <20% DO.

Succinic acid has been traditionally used as following: a surfactant- an additive, as a detergent and foaming agent; an ion chelator- for preventing the corrosion and spot corrosion of metal in the electroplating industry; an acidulant- a pH regulator and flavoring agent in the food industry; 685 pharmaceutical products- including the production of antibiotics, amino acids, and vitamins (*Zeikus et al.* 1999).

Recently, succinic acid is getting a lot of attention for production of biodegradable polymers. Succinic acid and its derivative diamines and diols can be used as monomer units of a variety of plastics, such as polyesters, 690 polyamides, and polyester amides (*Bechthold et al.* 2008). Among them, poly(butylene succinate) (PBS) and its copolymers are a family of biodegradable polyesters synthesized from succinic acid. The PBS owing to its excellent thermal processability, balanced mechanical properties, and good biodegradability can be used as a suitable substitute for conventional 695 plastics. The PBS can be used to prepare supermarket bags, packaging film, mulch film, and other disposable articles.

Succinic acid produced from organic waste can be more cost-effective than the fossil-based processes. The present invention is further making the situation better by providing the recombinant methanotrophic bacterium 700 capable of converting methane or biogas to succinic acid at an efficient rate.

The present invention provides a cradle to cradle solution for managing organic waste. The organic waste is anaerobically digested to produce biogas and compost. The resultant biogas is further efficiently converted to 705 succinic acid by employing the recombinant methanotrophic bacterium. At present, succinic acid can be made commercially by hydrogenation of fossil-derived maleic acid (anhydride). However, non-renewability and the rising price of the fossil resources have limited the use of succinic acid. Hence, the present invention not only produces succinic acid from waste at 710 a cost effective rate but also reduces the greenhouse emission by

effectively utilising methane forming a major part of biogas. The target chemical succinic acid is an excellent building block for manufacturing variety of commercially viable products including, but not limited to, biodegradable polymers. The biodegradable polyester synthesised from succinic acid is PBS which owing to its properties of excellent thermal processability, balanced mechanical properties, and good biodegradability make it an appropriate substitute for conventional plastics. The PBS can be used for industrial packaging, wrapping, milk sachets, foodservice, personal care, pharmaceuticals, surgical implants, medical devices, recreation, etc.

These products can be reused to make the input methane or biogas stream once the lifecycle of these products is completed. The increase in production and re-use of biodegradable plastic will effectively solve disposability problem associated with the use of conventional plastics, since the waste biodegradable plastics do not create environmental hazard and can be converted to target chemicals which may be used again to produce useful products. This can effectively contribute to the ongoing efforts of ensuring sustainability in environment.

EXAMPLES

The present invention is explained further in the following specific examples which are only by way of illustration and are not to be construed as limiting the scope of the invention.

Example 1: Cloning succinate pathway genes into a shuttle vector

Malate dehydrogenase (*mdh E.coli*; SEQ ID 1), pyruvate carboxylase (*pyc P.aeruginosa* (MTCC 424), SEQ ID 2), phospho enol pyruvate carboxylase (*pepc E.coli*, SEQ ID 3), genes were amplified individually from genomic DNA using primers flanked with *SacI* and *SphI/PciI* restriction enzymes. The amplified gene/operon was restriction digested and cloned into the same sites in the broad host range vector pMHA201 to create pSB107 (Figure 2), pSB108, and pSB109. pMHA201 (Alit, H., & Murrell, J. C,

Development and validation of promoter-probe vectors for the study of methane monooxygenase gene expression in Methylococcus capsulatus Bath, Microbiology, 155(3), pp 761–771. (2009)) is a plasmid with a broad range Origin of replication (OriV), Kanamycin resistance gene, Ampicillin resistance gene and OriT for conjugative transfer. Plasmid pSB107, 745 pSB108 and pSB109 were sequence verified.

Example 2: Transformation of succinate pathway genes into methanotrophs

Methanotrophs were cultivated in nitrate mineral salt (NMS) medium. NMS 750 medium was prepared following the protocol outlined at <http://www.methanotroph.org/wiki/culturing-tips/> and included here. NMS agar plates were prepared with 1.5 % (w/v) Bacto agar. Antibiotics were added as required: kanamycin (30 ug/ml) and Gentamicin (5ug/ml).

Methanotrophs were typically grown in 250 ml conical flasks with 24/29 joint 755 containing 50 ml NMS medium. Flasks were sealed with suba-seals (Sigma Aldrich, Cat Num: Z279773-10EA) and gassed with 50 ml (i.e. ~20 %) methane/carbon dioxide (95/5, v/v mix). *M.capsulatus Bath* (from Prof. Colin Murrell, University of Norwich) derived strains were incubated at 45°C with shaking at 200 rpm. *M.trichosporium* (from Prof. Colin Murrell, 760 University of Norwich) derived strains were incubated at 30°C. A typical methanotrophic culture took about 4–6 days to reach stationary phase. Methanotrophs grown on NMS agar plates were incubated in gas-tight container under a methane/air/carbon dioxide atmosphere (50/45/5, by vol.) at the appropriate temperature. The gas was replenished every 2 days until 765 colonies formed, usually within 5–10 days depending on the strain.

Conjugation of pSB107, pSB108, pSB109 individually into *M.capsulatus* was done based on the protocol described by individually into *M.capsulatus* was done based on the protocol described by Martin, H., & Murrell, J. C. (1995), *Methane monooxygenase mutants of Methylosinus trichosporium*

770 constructed by marker-exchange mutagenesis, FEMS Microbiology Letters, 127(3), 243–248. 30 mL of methanotroph culture was spun down and resuspended in 5mL of NMS. 2 mL of *E.coli* S.17 lambda pir (Strand, T. A., Lale, R., Degnes, K. F., Lando, M., & Valla, S., *A New and Improved Host-Independent Plasmid System for RK2-Based Conjugal Transfer. PLoS ONE*, 9(3), (2014)) bearing the plasmid to be conjugated was spun down, 775 washed with 1 mL of NMS and resuspended again in 1 mL of NMS. The two cultures were mixed and filtered onto a 0.2µ nitrocellulose membrane. The membrane was placed on an NMS agar plate containing 0.02 % (w/v) proteose peptone and incubated for a duration of 24 hours at 37 °C.

780 Following incubation, the cells were washed with 1 ml NMS and collected by centrifugation (7,000 x g for 5 minutes). Aliquots (50-100 µl) of the cells were spread onto NMS plates containing 30µg/ml kanamycin for plasmid selection and incubated at 45 °C in a gas-tight container under a methane/air/carbon dioxide atmosphere (50/45/5, by vol.). Colonies 785 typically formed on the plates after 8-12 days. Colonies were re-streaked onto NMS agar plates with 30ug/ml of kanamycin to confirm the true transformants.

Example 3: Growth and assay of methane to succinate activity

Positive transformants of *M.capsulatus* containing the malate 790 dehydrogenase (*mdh*; SEQ ID 1; pSB107), pyruvate carboxylase (*pyc*; SEQ ID 2, pSB108), phospho enol pyruvate carboxylase (*ppc*; SEQ ID 3, pSB109) genes were verified by PCR. These were inoculated into 5ml of liquid NMS media taken in 30ml culture tubes and sealed with suba seals. 15ml of Methane mixture (95% CH₄; 5% CO₂) was introduced into the 795 culture tube using a syringe. The tubes were incubated at 45 °C at 250rpm agitation. Once the culture OD reached 1, the cultures was centrifuged and the supernatant samples were taken and assayed for succinic acid. The organic acid concentrations were measured using Succinic Acid assay kit

(Megazyme International; K-SUCC) according to manufacturer's protocol
800 (Figure 3).

Some of the recombinant strains had higher levels of succinic acid compared to the control strain. Recombinant strain with SEQ ID 1 was >15% improved when compared to the native strain. Recombinant strain with SEQ ID 2 was >50% improved when compared to the native strain.

805

Example 4: Growth of methanotroph strains on biogas generated from organic waste

Methanotroph strain *Methylococcus capsulatus* was grown on methane and biogas in parallel to test the effect of biogas constituents on growth. Biogas
810 used for this analysis was from an anaerobic digester that processes kitchen waste. Food waste was anaerobically digested using BioOrja biomethanation reactor (GPS Renewables, Bangalore). Bioorja generates 70kg of LPG equivalent from 1 ton of food waste. The composition of the biogas was largely 60-65% CH₄; 35-30% CO₂; Traces – H₂S; Traces- NH₃.
815 For methane, a commercial mixture of 95% CH₄; 5% CO₂ was used. Nitrate mineral salts medium was used for strain growth. Methanotroph strain, *M.capsulatus*, was inoculated into 5ml of NMS media taken in 30ml culture tubes. The tubes were sealed with suba seals. 15ml of methane or biogas was fed into the tubes using a syringe. The tubes were incubated at 45 °C
820 at 250rpm agitation. Samples were taken from the tubes every 24hrs and cell growth was measured by monitoring OD at 600nm. When growth was compared for *M.capsulatus* between biogas and methane, the growth profile of the strain on biogas was similar to the growth profile on commercial methane mixture (Figure 4).

825 **Example 5: Growth and assay of biogas to succinic acid fermentation**

Recombinant *Methylococcus capsulatus* with malate dehydrogenase gene cloned in a broad host range vector (SEQ ID 1, pSB107) was grown in

biogas and tested for conversion of biogas to succinic acid. Biogas used for this analysis was obtained from kitchen waste digested using the BioOrja reactor. The composition of the biogas was largely 60-65% CH₄; 35-30% CO₂; Traces – H₂S; Traces- NH₃. Recombinant strain and control were inoculated in 5ml of NMS media containing 30ug/ml of Kanamycin taken in 30ml culture tubes. The culture tubes were sealed with suba seals. 15ml of biogas was fed into the culture tubes using a syringe. The cells were growth in conditions optimal for growth: 45°C and 200rpm. 0.5ml samples were taken at every 24hrs and measured for OD and succinic acid levels. The samples were centrifuged and the supernatant was assayed for succinic acid using the Succinic acid assay kit (Megazyme International; K-SUCC) according to manufacturer's protocol. **Figure 5** shows the results for production of succinic acid from biogas. Succinic acid levels in the strain increase with time.

These studies were done with biogas without upgrading the biogas to remove carbon dioxide. Alternately, the biogas can be cleaned up to remove the carbon dioxide by having a basic purification unit in place. Water scrubbing is a basic method used to remove the carbon dioxide. Pressurized biogas is fed to the bottom of a packed column where water is fed on the top and the absorption process is operated counter-currently. The cleaned up gas with >90% of methane can be used for growth of strains and succinic acid production.

Examples 6: Effect of gene(s) over expression on methane to succinic acid production

To improve the levels of succinate production via gene overexpression, specific gene(s) were targeted for overexpression in *Methylococcus capsulatus*. **Table 3** enlists genes cloned into vector pSB107 and tested for overexpression.

Sl.	Gene	Host Organism	Size of cloned	SEQ ID
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No.			fragment	
1	6-Phosphofructokinase	<i>M. capsulatus</i> Bath	1,263 bp	SEQ ID 4
2	Pyruvate Kinase	<i>E.coli</i>	1,467 bp	SEQ ID 5
3	Isocitrate Lyase	<i>E.coli</i>	1,305 bp	SEQ ID 6
4	Citryl CoA Lyase	<i>E.coli</i>	2,745 bp	SEQ ID 7
5	Malate synthase A	<i>E.coli</i>	1,602 bp	SEQ ID 8
6	Succinyl CoA Synthetase	<i>E.coli</i>	2,036 bp	SEQ ID 9
7	Fumarate Reductase	<i>E.coli</i>	3,312 bp	SEQ ID 10
8	Aspartate transaminase	<i>E.coli</i>	1,191 bp	SEQ ID 11

Table 3

In order to clone the genes into pSB107, $\sigma 54$ promoter was cloned into pSB107 using Sequence and Ligation Independent Cloning (SLIC). Above genes were amplified with a 20bp overlap to the above base vector and introduced under the $\sigma 54$ promoter using SLIC (**Figure 6**). SLIC was done in a 10 μ l reaction according to the following set up: 50-100ng of vector; 200-400ng of insert; 1X Buffer 2.1 (NEB); 0.3ul of T4 DNA Polymerase. All components except the enzyme was added and kept on ice for 5 minutes. The enzyme was added to the mixture, mixed well and incubated on ice for 10 minutes. 4 μ l of the reaction mixture was transformed into *E.coli* and selected on LB/Kan plates to select for true transformants. True transformants were confirmed by PCR.

Vectors were purified from *E.coli* and conjugated into *M.capsulatus* based on the protocol described by Martin & Murrell 1995 and elaborated above. True conjugants were selected on NMS agar plates with 30 μ g/ml of Kanamycin.

To test the effect of overexpression on succinic acid production, recombinant strains and control were inoculated in 5ml of NMS media containing 30µg/ml of Kanamycin taken in 30ml culture tubes. The culture tubes were sealed with suba seals. 15ml of methane was fed into the culture tubes using a syringe. The cells were growth in conditions optimal for growth: 45°C and 200rpm. Samples were taken at 72hours and measured for OD and succinic acid levels. The samples were centrifuged and the supernatant was assayed for succinic acid using the Succinic acid assay kit (Megazyme International; K-SUCC) according to manufacturer's protocol.

Specific combinations of genes had a positive effect on succinic acid production when compared to the recombinant strain with a single gene overexpression. The comparative analysis/assessment of various combinations of genes is depicted in **Figure 7**. It is evident that some of the combinations of genes are showing better results in terms of fold improvement in succinic acid production, such as SEQ ID 1,6 > SEQ ID 1,7> SEQ ID 1,5> SEQ ID 1,10 and so on.

While the invention has been described in detail with reference to preferred embodiments thereof, it will be apparent to one skilled in the art that various changes can be made, and equivalents employed, without departing from the scope of the invention.

Claims:

1. A recombinant methanotrophic bacterium for producing succinic acid from organic waste or biogas or methane, comprising:

900 one or more exogenous nucleic acids or genes encoding for a first group of enzymes consisting at least one of

 malate dehydrogenase, pyruvate carboxylase, phosphoenol pyruvate carboxylase, phosphofructokinase, citryl-CoA lyase, isocitrate lyase, fumarate reductase, malate synthase, aspartate

905 transaminase, succinyl CoA synthetase, pyruvate kinase.
2. The recombinant methanotrophic bacterium as claimed in claim 1, wherein the recombinant methanotrophic bacterium produces higher amounts of succinic acid as compared to wildtype methanotrophic
- 910 bacterium and accumulates the succinic acid so produced when cultured in the presence of methane or biogas.
3. The recombinant methanotrophic bacterium as claimed in claim 1, wherein the one or more exogenous nucleic acids or genes encoding
- 915 the first group of enzymes are selected from a group of gene sequences set forth as SEQ ID NOs. 1 to 11.
4. The recombinant methanotrophic bacterium as claimed in claim 1, wherein the bacterium is selected from a group consisting of
- 920 *Methylococcus capsulatus*, *Methylobacterium extorquens*, *Methylomicrobium album*, *Methylocapsa acidiphila*, *Methylobacterium organophilum*, *Methylobacterium mesophilicum*, *Methylobacterium dichloromethanicum*, *Methylocella silvestris*, *Methylosinus trichosporium*, *Methylobacillus flagellatus* KT,
- 925 *Methylibium petroleiphilum* PM1, *Methylobacterium nodulans*, *Methylobacterium populi*, *Methylobacterium chloromethanicum*, *Methylacidiphilum infernorum* V4, *Methylophilus methylotrophus*,

930 *Methylomonas methanica*, *Methylobacterium rhodesianum* MB 126,
Methylobacter tundripaludum, *Methylobacterium* sp. 4-46,
Methylovorus glucosetrophus SIP3-4, *Mycobacterium smegmatis*,
Methylobacterium rhodesianum, *Methylosinus sporium*,
Methylocella palustris, *Methylobacterium fujisawaense*,
Methylocystis parvus, *Methylovulum miyakonense*,
 935 *Methylobacterium rhodinum*, *Methylocystis echinoides*,
Methylomonas rubra, *Methylococcus thermophilus*,
Methylobacterium aminovorans, *Methylobacterium thiocyanatum*,
Methylobacterium zatmanii, *Acidithiobacillus ferrivorans*,
Methylobacterium aquaticum, *Methylobacterium suomiense*,
Methylobacterium adhaesivum, *Methylobacterium podarium*,
 940 *Methylobacter whittenburyi*, *Crenothrix polyspora*, *Clonothrix fusca*,
Methylobacter bovis, *Methylomonas aurantiaca*, *Methylomonas*
fodinarum, *Methylobacterium variabile*, *Methylocystis minimus*,
Methylobacter vinelandii, *Methylobacterium hispanicum*,
Methylomicrobium japanense, *Methylococcaceae bacterium*, and
 945 *Methylocystis methanolicus*.

5. The recombinant methanotrophic bacterium as claimed in claim 1,
 wherein the bacterium is *Methylococcus capsulatus*.
- 950 6. The recombinant methanotrophic bacterium as claimed in claim 1,
 wherein the bacterium is *Methylococcus capsulatus* (Bath)
7. The recombinant methanotrophic as claimed in claim 1, wherein the
 bacterium is *Methylococcus trichosporium*.
- 955 8. The recombinant methanotrophic bacterium as claimed in claims 1 to
 7, further comprises one or more genes coding for enzymes that are

overexpressed or downregulated or deleted or a combination thereof for increasing the production of succinic acid.

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9. The recombinant methanotrophic bacterium as claimed in claims 1 and 8, wherein the gene(s) encoding the enzymes responsible for increased production of succinic acid by overexpression are selected from a second group consisting of *Schizosaccharomyces pombe* malate transporter gene SpMAE1, *E.coli* C4 dicarboxylic/orotate symporter, fumarase, isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, methane monooxygenase, methanol dehydrogenase, hexulose 6-phosphate (hps) synthase, 6-phospho-3-hexuloisomerase, fructose-bisphosphate aldolase, transketolase, transaldolase, ribulose-5 phosphate epimerase or combinations thereof.

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10. The recombinant methanotrophic bacterium as claimed in claims 1 and 8, wherein the gene(s) encoding the enzymes responsible for increased production of succinic acid by down-regulation or deletion are selected from a third group of enzymes consisting of glucose-6-phosphate dehydrogenase, succinate dehydrogenase, pyruvate decarboxylase, lactate dehydrogenase, malate:quinone oxidoreductase, citrate synthase, isocitrate dehydrogenase, succinate dehydrogenase, formaldehyde dehydrogenase, acetyl CoA synthase, phospho transacetylase and formate dehydrogenase.

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11. The recombinant methanotrophic bacterium as claimed in claim 1, wherein the one or more exogenous nucleic acids or genes encoding for the first group of enzymes are from one or more micro-organisms selected from a group consisting of *E. coli*, *P. eruginosa*, *Methylosinus trichosporium*, *Methylococcus capsulatus* and *Schizosaccharomyces pombe*.

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- 990 12. The recombinant methanotrophic bacterium as claimed in claim 1, wherein the one or more exogenous nucleic acids or genes encoding for a first group of enzymes set forth as SEQ ID Nos: 1 -11 have corresponding amino acid sequences set forth as SEQ ID Nos: 12 to 22.
- 995 13. The recombinant methanotrophic bacterium as claimed in claim 12, wherein the amino acid sequence is at least 80% identical to a reference amino acid sequence set forth as SEQ ID NOs: 12 to 22.
- 1000 14. A process for producing succinic acid from a carbon source using recombinant methanotrophic bacterium as claimed in claim 1, said process comprising acts of:
- receiving at least one carbon source selected from a group consisting of biogas or methane as input;
 - 1005 culturing the bacterium in the input, thereby converting the input into succinic acid; and
 - optionally, purifying or separating the succinic acid produced from the culture for obtaining the succinic acid.
- 1010 15. The process as claimed in claim 14, wherein the input carbon source has a ratio of methane to carbon dioxide ranging from about 95 :5 to about 50 : 50.
- 1015 16. The process as claimed in claim 14, wherein the temperature maintained throughout the act of converting the input to succinic acid is in the range of about 35 to 50°C.

1020 17. The process as claimed in claim 14, wherein the temperature maintained throughout the act of converting the input to succinic acid is 45 °C.

1025 18. The process as claimed in claim 14, wherein the pH maintained during the act of converting the input to succinic acid is in the range of about 3 to about 7.

19. The process as claimed in claim 14, wherein the pH maintained during the act of converting the input to succinic acid is in the range of about 4 to about 6.

1030 20. A process for producing succinic acid from organic waste using recombinant methanotrophic bacterium as claimed in claim 1, said process comprising the acts of f:

Receiving organic waste as input;

anaerobically digesting the organic waste to biogas;

1035 culturing the bacterium in the biogas so generated thereby converting the biogas to the succinic acid while maintaining:

temperature in the range of about 35 °C to about 50 °C, preferably 45 °C,

1040 pH in the range of about 4 to about 7, preferably 5.8, and dissolved oxygen concentration of <20%; and

optionally, purifying the succinic acid produced from the culture for obtaining the succinic acid

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1050 21. The process as claimed in claim 20, wherein the biogas produced by anaerobically digesting the organic waste is optionally cleaned to remove carbon dioxide and other impurities present in the biogas to obtain methane for producing succinic acid from methane thereafter

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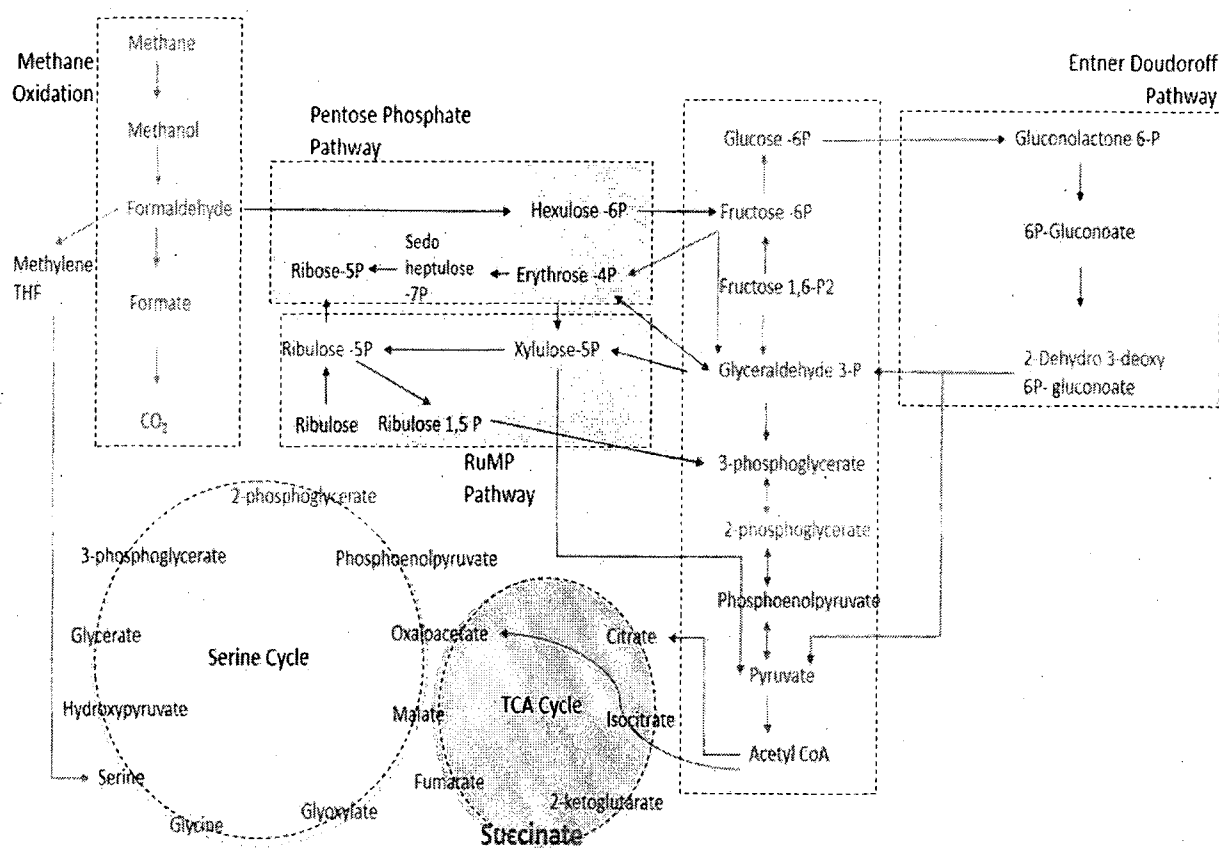


Figure 1

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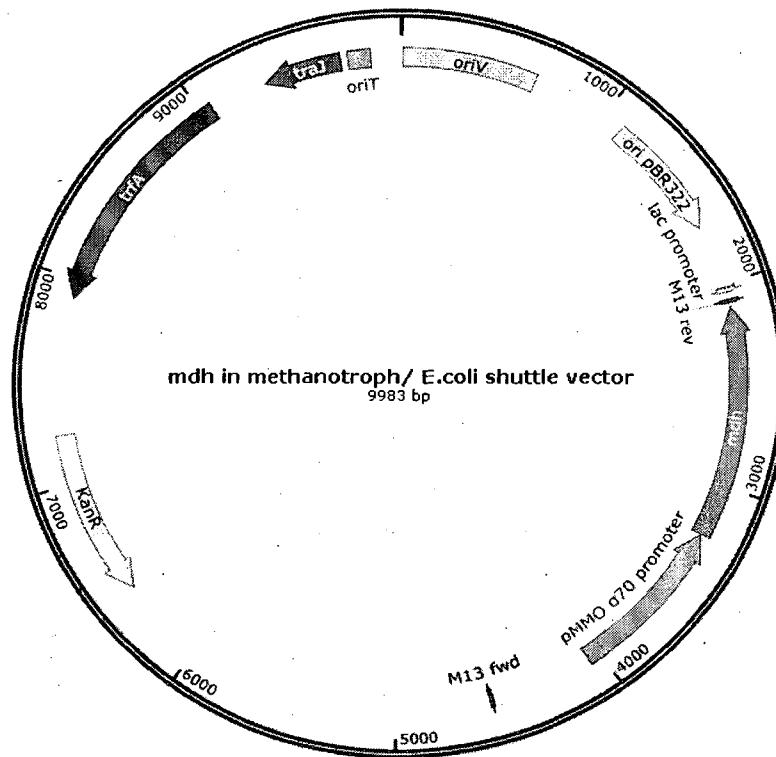
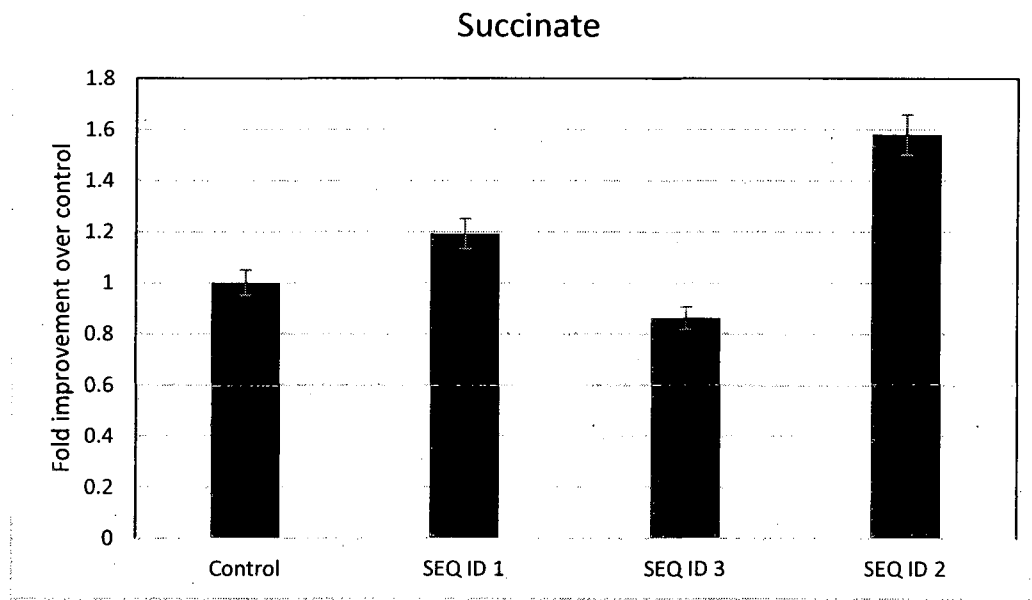


Figure 2

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**Figure 3**

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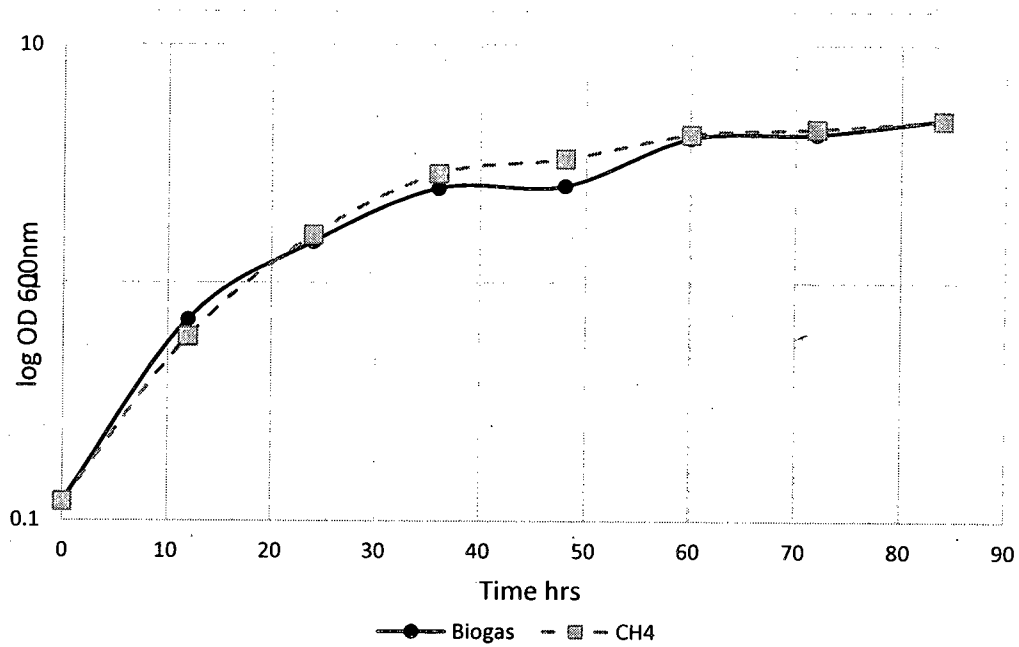


Figure 4

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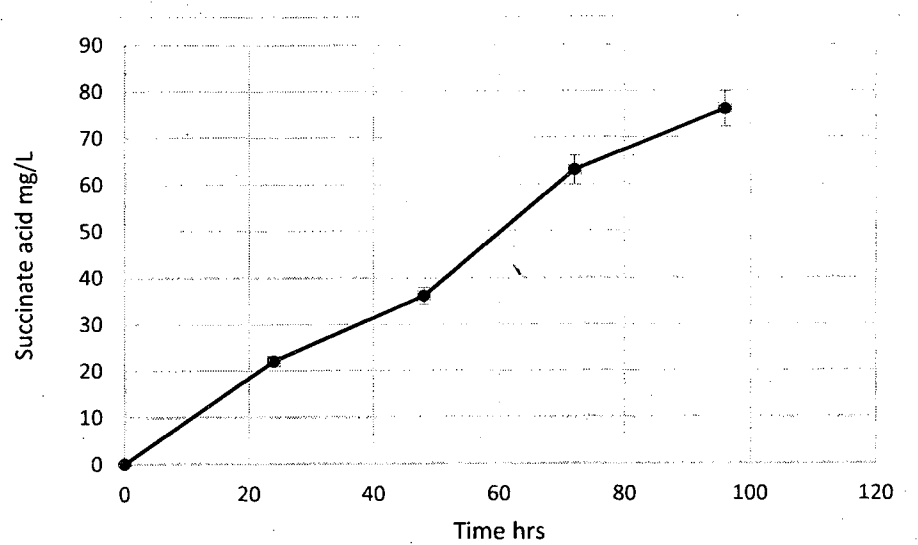
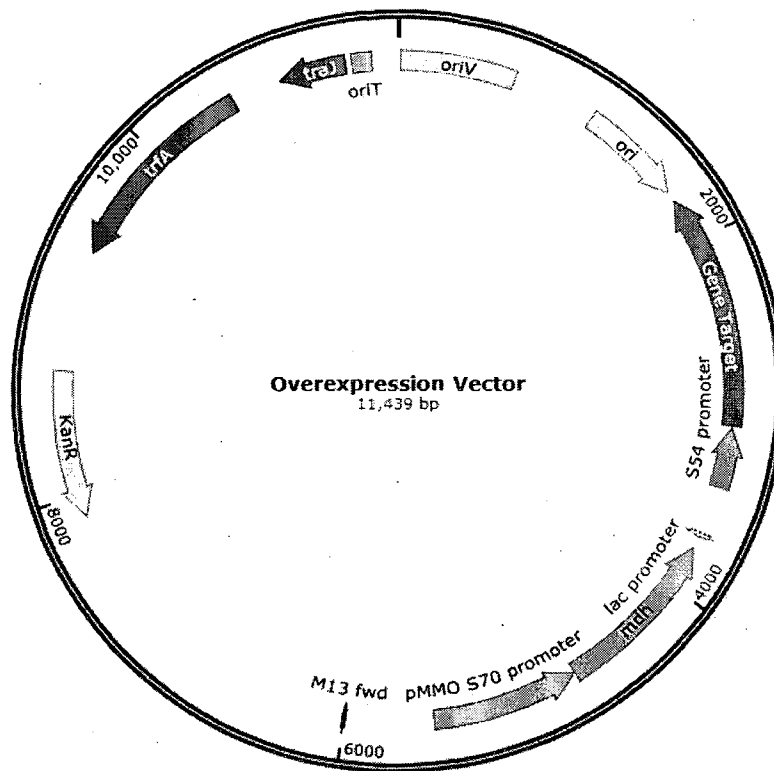


Figure 5

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**Figure 6**

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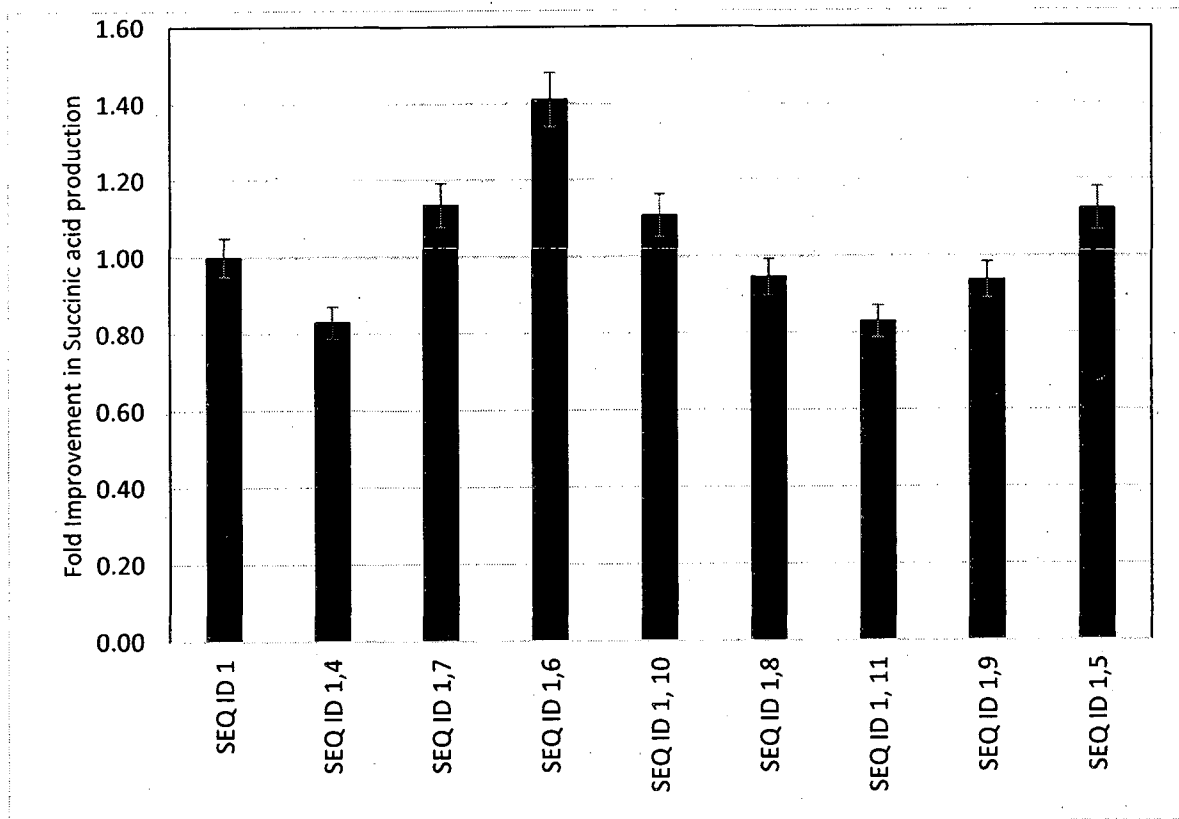


FIGURE 7