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(54) Title: COMPOSITONS AND METHODS FOR THE PRODUCTION OF ISOPRENE

(57) Abstract: The present disclosure describes compositions and methods for production of isoprene from lignocellulosic plant bio-mass using a genetically engineered strain of a saprophytic bacteria.



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COMPOSITIONS AND METHODS FOR THE PRODUCTION OF ISOPRENE

RELATED APPLICATIONS

This application claims priority from U.S. Provisional Patent Application No.

5 61/507,532, filed July 13, 2011, incorporated by reference herein in its entirety.

FIELD

The present disclosure relates to methods for production of isoprene from lignocellulosic plant biomass using a genetically engineered strain of a saprophytic bacterium.

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BACKGROUND

Isoprene is an important chemical precursor for production of rubber and some plastics. According to some estimates, around 800,000 tons of isoprene were produced in 2008. Practically all isoprene used by chemical industry is derived from petroleum, however in nature this isoprene is produced by many plants. It is estimated that plants emit around 100 billion kg of isoprene a year into the atmosphere. Despite this there are few “green” alternatives to petrochemical production of isoprene. The most promising alternative technologies utilize a number of genetically-modified microorganisms to convert organic matter into isoprene. Currently, most of these organisms can use simple sugars as a substrate for isoprene biosynthesis. The amount of such sugars in nature is very limited. Besides, the same simple sugars are also valuable for use as animal feed or human food. At the same time, according to the Department of Energy (DOE), lignocellulosic biomass is the largest sustainable carbon resource on the earth. According to the same source a billion ton of cellulosic biomass is available in the US for conversion to biofuels each year. Currently there is a need for a method of producing isoprene from lignocellulosic biomass.

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SUMMARY

In some embodiments, the disclosure provides cells in culture that produce isoprene using either mono- and di- saccharides or complex carbohydrates including but not limited to cellulose, starch, hemicelluloses and chitin. In some embodiments, the cells have a heterologous nucleic acid encoding an isoprene synthase linked to a promoter.

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In some embodiments, the cells are cultured in a growth medium that includes mineral salts from sea water, an inorganic nitrogen source like ammonium chloride, peptides, proteins, vitamins and amino acids from yeast extract and tryptone, carbon sources like glucose, sucrose, lactose, starch or cellulose and hemicelluloses from corn cob.

- 5 In some embodiments, the cells contain a heterologous nucleic acid encoding an isoprene synthase and is operably linked to *tac* promoter. These cells start producing isoprene in the presence of isopropyl β -D-1-thiogalactopyranoside, abbreviated IPTG, in the growth medium.

- 10 In some embodiments, the cells contain a heterologous nucleic acid encoding an isoprene synthase that is operably linked to a *cel9A* promoter. These cells produce isoprene only in the presence of cellulose, hemicelluloses or pectins in the growth medium.

In some embodiments, the heterologous nucleic acid encoding an isoprene synthases designed to match codon usage of *S. degradans* and synthetically created.

15 BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1 is a schematic showing the MEP/DOXP pathway. The following abbreviations are used in Figure 1. Pur — pyruvate; G3P - glyceraldehyde 3-phosphate; DXS - 1-deoxy-D-xylulose 5-phosphate synthase; DXP - 1-deoxy-D-xylulose 5-phosphate; DXR - 1-deoxy-D-xylulose 5-phosphate reductase; MEP - 2-C-methylerythritol 4-phosphate; CMS - 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase; CDP-ME - 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; CMK - 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; CDP-MEP - 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; MCS - 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; MEcPP - 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate; HDS - (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate synthase; HMB-PP - (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; HDR - (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate reductase; IPP - isopentenyl pyrophosphate; DMAPP - dimethylallyl pyrophosphate.
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Figure 2 shows the oligonucleotide sequence of quaking aspen isoprene synthase gene optimized for expression in *S. degradans* (SEQ ID NO:1).

Figure 3 shows the oligonucleotide sequence of kudzu isoprene synthase gene optimized for expression in *S. degradans* (SEQ ID NO:2).

Figure 4 shows the oligonucleotide sequence of peanut isoprene synthase gene optimized for expression in *S. degradans* (SEQ IS NO:3).

5 Figure 5 shows a schematic of plasmid pMMB503EH.

Figure 6 shows a schematic of plasmid pZym-IPTG.

Figure 7 is a line graph showing isoprene concentration in the overhead space of the fermenter over time.

10 Figure 8 shows a sequence of the *cel9A* promoter area used in the cellulose-inducible construct (SEQ ID NO:4).

Figure 9 shows sequences of primers used to amplify *cel9A* promoter area (SEQ ID NOs:5 (PromD) and 6 (PromR)). The *MluI* and *EcoRI* restriction endonuclease sites are shown in the lower case.

Figure 10 shows a schematic of plasmid pZym-Cel.

15 DETAILED DESCRIPTION

The present disclosure describes compositions and methods taking advantage of the natural ability of saprophytic bacteria *Saccharophagus degradans* (*S. degradans*) 2-40 to metabolize practically all complex polysaccharides found in lignocellulosic biomass. By altering the isoprenoid biosynthesis pathway in this organism, an isoprene-producing strain of *S. degradans* utilizing plant-derived biomass as a major carbon source was created as disclosed below.

20 There are two major isoprenoid biosynthesis pathways utilized by living organisms: *a*) the mevalonate pathway or HMG-CoA reductase pathway and *b*) the non-mevalonate pathway or 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP pathway) (Figure 1). Typically the mevalonate pathway present in all higher eukaryotes and many bacteria while plants and some protozoa utilize the non-mevalonate pathway.

The genome of *Saccharophagus degradans* has been sequenced and annotated. The genome annotation clearly shows that this microorganism exclusively utilizes the non-mevalonate pathway for terpenoid biosynthesis. As was mentioned earlier, the same pathway is utilized by plants for synthesis of isoprene. The only key element missing in
5 *S. degradans* for synthesis of this hydrocarbon is isoprene synthase (IspS). This enzyme converts dimethylallyl diphosphate into isoprene and diphosphate. Cells of many higher plants (*tracheophytes*) contain isoprene synthases. Amino acid sequences for quaking aspen, kudzu and peanut isoprene synthases were obtained from Gene Bank. Based on these sequences, oligonucleotide sequences for all three isoprene synthases were
10 designed. The oligonucleotide sequences were designed to match the codon usage table of 10 highest expressed genes in *S. degradans*. (Figures 2 - 4) The oligonucleotide sequences encoding the aforementioned isoprene synthases were synthesized with *EcoRI* endonuclease site followed by the ribosome-binding site upstream from the start-codon of the IspS-encoding sequences and with *BamHI* endonuclease site following the stop-
15 codon of the gene. The synthetic isoprene synthase genes were lacking the signal-peptide-encoding region present in the wild-type versions of the genes.

In certain embodiments, any heterologous nucleic acid encoding an isoprene synthase can be introduced into any saprophytic bacteria, including *S. degradans*. Moreover, any variant of a heterologous nucleic acid encoding an isoprene synthase can be introduced
20 into any saprophytic bacteria including *S. degradans*. Isoprene synthase amino acid sequences include *Arachis hypogaea* (peanut) isoprene synthase (GenBank accession number: EZ721087.1); *Populus tremuloides* (aspen) isoprene synthase (GenBank accession number: AAQ16588); *Pueraria lobata* (Kudzu vine) (*Pueraria montana* var. *lobata*) isoprene synthase (Uniprot accession number: UPI00003B9580).

25 Any variant of these amino acid sequences may be introduced into a saprophytic bacterium, including *S. degradans*. These variants may have between 70-99% sequence identity with any wild type isoprene synthase, as long as they have the ability to synthesize isoprene.

The heterologous nucleic acid encoding an isoprene synthase can include any promoter
30 that allows for the expression of isoprene synthase. This includes promoters from *S. degradans* genes. These genes include any of the ones described in U.S. Patent Publication No. 2008-0293115, incorporated herein by reference in its entirety.

Promoters from other organisms can also be used. These promoters include the *tac* promoter and the LacUV5 promoter. The LacUV5 promoter can be used in a triple configuration.

5 DNA sequences encoding isoprene synthase may be cloned into any suitable vectors for expression in intact saprophytic bacteria or in cell-free translation systems by methods well-known in the art.

Expression and cloning vectors will likely contain a selectable marker, a gene encoding a protein necessary for survival or growth of a host cell transformed with the vector. The presence of this gene ensures growth of only those host cells that express the inserts.

10 Typical selection genes encode proteins that 1) confer resistance to antibiotics or other toxic substances, *e.g.*, ampicillin, neomycin, methotrexate, *etc.*; 2) complement auxotrophic deficiencies, or 3) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for Bacilli. Markers may be an inducible or non-inducible gene and will generally allow for positive selection. Non-

15 limiting examples of markers include the ampicillin resistance marker (*i.e.*, β -lactamase), tetracycline resistance marker, neomycin/kanamycin resistance marker (*i.e.*, neomycin phosphotransferase), dihydrofolate reductase, glutamine synthetase, and the like. The choice of the proper selectable marker will depend on the saprophytic bacteria, and appropriate markers for different saprophytic bacteria as understood by those of skill

20 in the art.

Vectors can contain one or more replication and inheritance systems for cloning or expression, one or more markers for selection in the saprophytic bacteria, *e.g.*, antibiotic resistance, and one or more expression cassettes. The inserted coding sequences can be synthesized by standard methods, isolated from natural sources, or prepared as hybrids.

25 Ligation of the coding sequences to transcriptional regulatory elements (*e.g.*, promoters, enhancers, and/or insulators) and/or to other amino acid encoding sequences can be carried out using established methods.

Expression vectors for saprophytic bacteria ordinarily include an origin of replication (where extrachromosomal amplification is desired, as in cloning, the origin will be a

30 bacterial origin), a promoter located upstream from the isoprene synthase coding sequences, together with a ribosome binding site (the ribosome binding or Shine-

- Dalgarno sequence is only needed for prokaryotic expression), RNA splice site (if the isoprene synthase DNA contains genomic DNA containing one or more introns), a polyadenylation site, and a transcriptional termination sequence. As noted, the skilled artisan will appreciate that certain of these sequences are not required for expression in certain saprophytic bacteria. An expression vector for use with microbes need only contain an origin of replication recognized by the intended saprophytic bacteria, a promoter which will function in the host and a phenotypic selection gene, for example a gene encoding proteins conferring antibiotic resistance or supplying an auxotrophic requirement.
- Saprophytic bacteria can be transformed, transfected, or infected as appropriate by any suitable method including electroporation, calcium chloride-, lithium chloride-, lithium acetate/polyethylene glycol-, calcium phosphate-, DEAE-dextran-, liposome-mediated DNA uptake, spheroplasting, injection, microinjection, microprojectile bombardment, phage infection, viral infection, or other established methods. Vectors may integrate into the saprophytic bacteria genome or remain separate from the host cell genome. Alternatively, vectors containing the nucleic acids of interest can be transcribed *in vitro*, and the resulting RNA introduced into the saprophytic bacteria by well-known methods, *e.g.*, by injection. The cells into which have been introduced nucleic acids described above are meant to also include the progeny of such cells. Methods of transfection include nucleofection, electroporation, sonoporation, heat shock, magnetofection and proprietary transfection reagents such as Lipofectamine, Dojindo, GenePORTER, Hilymax, Fugene, jetPEI, Effectene or DreamFect.

- Saprophytic bacteria carrying an expression vector (*i.e.*, transformants or clones) are selected using markers depending on the mode of the vector construction. The marker may be on the same or a different DNA molecule, preferably the same DNA molecule. In prokaryotic hosts, the transformant may be selected, *e.g.*, by resistance to ampicillin, tetracycline or other antibiotics. Production of a particular product based on temperature sensitivity may also serve as an appropriate marker.

Definitions

- As used herein, the terms “transformed”, “stably transformed” or “transgenic” with reference to a cell means the cell has a non-native (heterologous) nucleic acid sequence

integrated into its genome or as an episomal plasmid that is maintained through multiple generations.

As used herein, the term “expression” refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both
5 transcription and translation.

The term “introduced” in the context of inserting a nucleic acid sequence into a cell, means “transfection”, or “transformation” or “transduction” and includes reference to the incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell where the nucleic acid sequence may be incorporated into the genome of the cell (for example,
10 chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (for example, transfected mRNA).

“A”, “an” and “the” include plural references unless the context clearly dictates otherwise.

Numeric ranges are inclusive of the numbers defining the range.

15 The term “variant” refers to a protein or polypeptide in which one or more amino acid substitutions, deletions, and/or insertions are present as compared to the amino acid sequence of an protein or peptide and includes naturally occurring allelic variants or alternative splice variants of an protein or peptide. The term “variant” includes the replacement of one or more amino acids in a peptide sequence with a similar or
20 homologous amino acid(s) or a dissimilar amino acid(s). There are many scales on which amino acids can be ranked as similar or homologous. (Gunnar von Heijne, Sequence Analysis in Molecular Biology, p. 123-39 (Academic Press, New York, N.Y. 1987.) Preferred variants include alanine substitutions at one or more of amino acid positions. Other preferred substitutions include conservative substitutions that have little
25 or no effect on the overall net charge, polarity, or hydrophobicity of the protein. Conservative substitutions are set forth in the table below. According to some embodiments, the SP1NT1 and TMPRSS4 polypeptides have at least 80%, 85%, 88%, 95%, 96%, 97%, 98% or 99% sequence identity with the amino acid sequences of the preferred embodiments.

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Conservative Amino Acid Substitutions

Basic:	arginine lysine histidine
Acidic:	glutamic acid aspartic acid
Uncharged Polar:	glutamine asparagines serine threonine tyrosine
Non-Polar:	phenylalanine tryptophan cysteine glycine alanine valine praline methionine leucine isoleucine

The table below sets out another scheme of amino acid substitution:

Original Residue	Substitutions
Ala	Gly;Ser
Arg	Lys
Asn	Gln;His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala;Pro

His	Asn;Gln
Ile	Leu;Val
Leu	Ile;Val
Lys	Arg;Gln;Glu
Met	Leu;Tyr;Ile
Phe	Met;Leu;Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp;Phe
Val	Ile;Leu

Other variants can consist of less conservative amino acid substitutions, such as selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions that in general are expected to have a more significant effect on function are those in which (a) glycine and/or proline is substituted by another amino acid or is deleted or inserted; (b) a hydrophilic residue, *e.g.*, seryl or threonyl, is substituted for (or by) a hydrophobic residue, *e.g.*, leucyl, isoleucyl, phenylalanyl, valyl, or alanyl; (c) a cysteine residue is substituted for (or by) any other residue; (d) a residue having an electropositive side chain, *e.g.*, lysyl, arginyl, or histidyl, is substituted for (or by) a residue having an electronegative charge, *e.g.*, glutamyl or aspartyl; or (e) a residue having a bulky side chain, *e.g.*, phenylalanine, is substituted for (or by) one not having such a side chain, *e.g.*, glycine. Other variants include those designed to either generate a novel glycosylation and/or phosphorylation site(s), or those designed to delete an existing glycosylation and/or phosphorylation site(s). Variants include at least one amino acid substitution at a glycosylation site, a proteolytic cleavage site and/or a cysteine residue. Variants also include proteins and peptides with additional amino acid residues before or after the protein or peptide amino acid sequence on linker peptides. The term “variant” also encompasses polypeptides that have the amino acid sequence of the proteins/peptides of the present invention with at

least one and up to 25 (*e.g.*, 5, 10, 15, 20) or more (*e.g.*, 30, 40, 50, 100) additional amino acids flanking either the 3' or 5' end of the amino acid sequence.

The headings provided herein are not limitations of the various aspects or embodiments of the invention, which can be had by reference to the specification as a whole.

- 5 The following examples are illustrative, but not limiting, of the methods and compositions of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered in therapy and that are obvious to those skilled in the art are within the spirit and scope of the embodiments.

EXAMPLES

- 10 The following examples further support, but do not exclusively represent, preferred embodiments of the present invention.

Example 1. IPTG-inducible strain (Zym-IPTG).

- The resulting synthetic oligonucleotide sequences were digested with *EcoRI* and *BamHI* endonucleases and cloned into *EcoRI* and *BamHI* sites of pMMB503EH plasmid (Figure 5). The resulting plasmids containing isoprene synthase genes (Figure 6) were electroporated into *S. degradans* strain 2-40. Selection of the cells containing the plasmid was performed on agar supplemented with 50 µg/mL streptomycin. In the resulting construct the IspS-encoding sequences were under transcriptional regulation of the *tac* promoter. The *S. degradans* strains expressing the isoprene synthases were cultivated in 250 mL shake flasks at 30°C. Expression of the isoprene synthases in the described strains was induced by adding 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the growth medium in the middle of the log-phase. Detection and quantification of the isoprene in the flasks was performed using RAE-106 volatile organic compound detector. Isoprene in the overhead space of the shake flasks also manifested itself in “refinery” smell.
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For more accurate assessment of isoprene production capabilities of the described strains 10L Bioflo fermenters were used. Cultivation of the bacterial cells was performed at 30°C. Composition of the 2x 2-40 growth medium used for that was as follows:

Instant Ocean sea salt — 230 g

Yeast extract — 40 g

Casein peptone — 50 g

Ammonium chloride — 10 g

5 Glucose — 100 g

Water — to 10 L

Concentration of isoprene in the overhead space of the fermenters was constantly monitored using RAE-106 detectors (Figure 7). As an alternative to glucose, 1% (w/v) pretreated corn cob was also used as a major carbon source for isoprene production.

10 **Example 2. The cellulose-inducible strain (Zym-Cel).**

In order to reduce cost of the isoprene production using *S. degradans*-based strain, a novel cellulose-inducible expression system was created. The key element of this system is promoter of *cel9A* gene encoding an endo-1,4- β — glucanase in *S. degradans*. Using qRT-PCR and deep sequencing techniques, *cel9A* gene was identified as the highest

15 transcribed gene among other carbohydrase-encoding genes in *S. degradans*.

Transcription of this gene is highly upregulated in the presence of any cellulose and/or hemicelluloses —containing material in the growth medium. In order to create the cellulose-inducible protein expression system for *S. degradans*, the 500-bp-long nucleotide sequence (Figure 8) upstream from *cel9A* was PCR-amplified. The resulting

20 PCR fragment (Figure 9) contained *cel9A* promoter, operator regions as well as

ribosome-binding site. The PCR primers contained *MluI* and *EcoRI* restriction endonuclease sites. The resulting PCR fragment was digested with *MluI* and *EcoRI* restriction endonucleases and cloned into *MluI* and *EcoRI* sites of pMMB503EH plasmid (Figure 5). Then the isoprene synthase genes were cloned into *EcoRI* and

25 *BamHI* sites of the resulting plasmid (Figure 10).

CLAIMS

1. A saprophytic bacteria comprising an isoprene synthase.
2. The saprophytic bacteria of claim 1, wherein the saprophytic bacteria is *Saccharophagus degradans* 2-40.
3. The saprophytic bacteria of claim 1, wherein the isoprene synthase is selected from the group consisting of quaking aspen isoprene synthase, kudzu isoprene synthase and peanut isoprene synthase.
4. The saprophytic bacteria of claim 1, wherein the saprophytic bacteria comprises a heterologous nucleic acid encoding isoprene synthase.
5. The saprophytic bacteria of claim 4, wherein the heterologous nucleic acid is operably linked to a promoter selected from a *tac* promoter and a *cel9A* promoter.
6. The saprophytic bacteria of claim 4, wherein the heterologous nucleic acid matches codon usage of *Saccharophagus degradans* 2-40.
7. A method of producing isoprene comprising
 - (a) providing a saprophytic bacteria comprising an isoprene synthase; and
 - (b) culturing the saprophytic bacteria in a media comprising a carbon source, thereby producing isoprene.
8. The method of claim 7, wherein the saprophytic bacteria is *Saccharophagus degradans* 2-40.
9. The method of claim 8, wherein the growth media further comprises mineral salts from sea water.
10. The method of claim 7, wherein the growth media further comprises an inorganic nitrogen source.
11. The method of claim 7, wherein the carbon source is selected from the group consisting of glucose, sucrose, lactose, starch, cellulose, hemicellulose and corn cob.

12. The method of claim 7, wherein the isoprene synthase is selected from the group consisting of quaking aspen isoprene synthase, kudzu isoprene synthase and peanut isoprene synthase.
13. The method of claim 7, wherein the saprophytic bacteria comprises a heterologous nucleic acid encoding isoprene synthase.
14. The method of claim 13, wherein the heterologous nucleic acid is operably linked to a promoter selected from a *tac* promoter and a *cel9A* promoter.
15. The method of claim 8, wherein the heterologous nucleic acid matches codon usage of *Saccharophagus degradans* 2-40.
16. A method of producing a bacteria that produces isoprene in the presence of a carbon source comprising
 - (a) providing a saprophytic bacteria; and
 - (b) introducing to the saprophytic bacteria a heterologous nucleic acid encoding an isoprene synthase,thereby producing a bacteria that produces isoprene in the presence of a carbon source.
17. The method of claim 16, wherein the saprophytic bacteria is *Saccharophagus degradans* 2-40.
18. The method of claim 16, wherein the carbon source is selected from the group consisting of glucose, sucrose, lactose, starch, cellulose, hemicellulose and corn cob.
19. The method of claim 16, wherein the isoprene synthase is selected from the group consisting of quaking aspen isoprene synthase, kudzu isoprene synthase and peanut isoprene synthase.
20. The method of claim 17, wherein the heterologous nucleic acid is operably linked to a promoter selected from a *tac* promoter and a *cel9A* promoter.
21. The method of claim 17, wherein the heterologous nucleic acid matches codon usage of *Saccharophagus degradans* 2-40.

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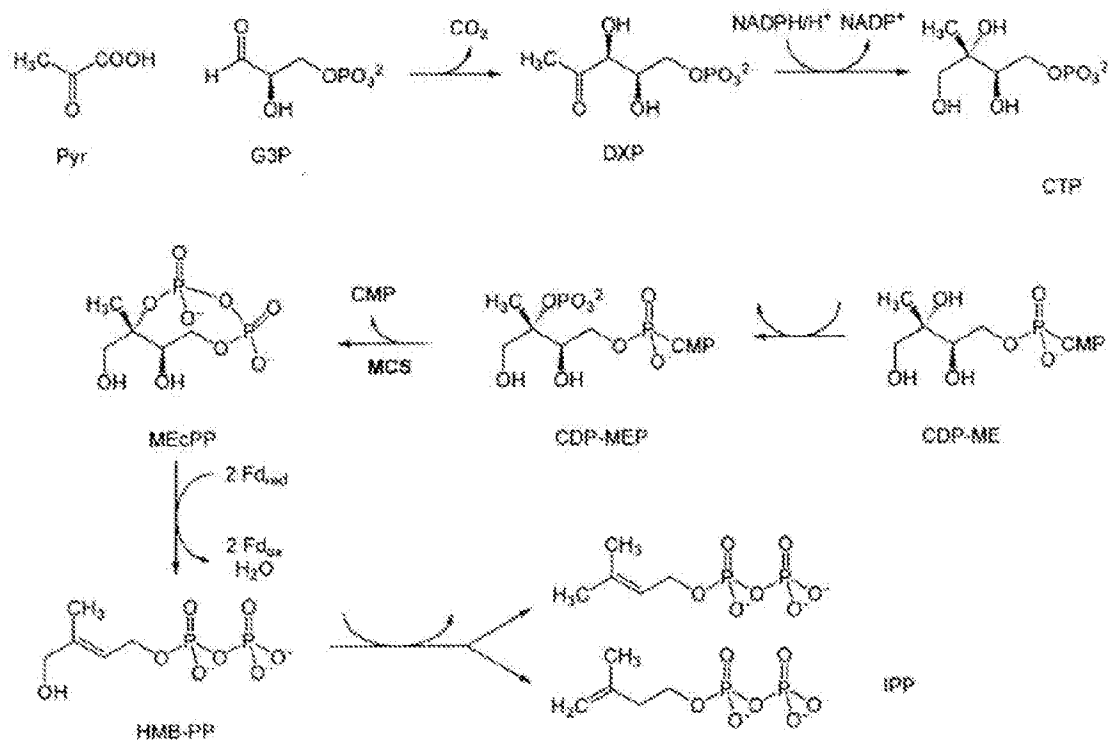


Figure 1

ATAGCgaattcAGAAGGAGATATACC

ATG GCG ACC GAA TTA TTA TGC TTA CAC CGC CCA ATT AGC TTA ACC CAC AAA TTA TTT
 CGC AAT CCA TTA CCA AAA GTA ATT CAA GCG ACC CCA TTA ACC TTA AAA TTA CGC TGC
 AGC GTA AGC ACC GAA AAT GTA AGC TTT AGC GAA ACC GAA ACC GAA ACC CGC CGC AGC
 GCG AAT TAC GAA CCA AAT AGC TGG GAT TAC GAT TAC TTA TTA AGC AGC GAT ACC GAT
 GAA AGC ATT GAA GTA CAC AAA GAT AAA GCG AAA AAA TTA GAA GCG GAA GTA CGC CGC
 GAA ATT AAT AAT GAA AAA GCG GAA TTT TTA ACC TTA TTA GAA TTA ATT GAT AAT GTA
 CAA CGC TTA GGC TTA GGC TAC CGC TTT GAA AGC GAT ATT CGC CGC GCG TTA GAT CGC
 TTT GTA AGC AGC GGC GGC TTT GAT GGC GTA ACC AAA ACC AGC TTA CAC GGC ACC GCG
 TTA AGC TTT CGC TTA TTA CGC CAA CAC GGC TTT GAA GTA AGC CAA GAA GCG TTT AGC
 GGC TTT AAA GAT CAA AAT GGC AAT TTT TTA GAA AAT TTA AAA GAA GAT ATT AAA GCG
 ATT TTA AGC TTA TAC GAA GCG AGC TTT TTA GCG TTA GAA GGC GAA AAT ATT TTA GAT
 GAA GCG AAA GTA TTT GCG ATT AGC CAC TTA AAA GAA TTA AGC GAA GAA AAA ATT GGC
 AAA GAA TTA GCG GAA CAA GTA AGC CAC GCG TTA GAA TTA CCA TTA CAC CGC CGC ACC
 CAA CGC TTA GAA GCG GTA TGG AGC ATT GAA GCG TAC CGC AAA AAA GAA GAT GCG AAT
 CAA GTA TTA TTA GAA TTA GCG ATT TTA GAT TAC AAT ATG ATT CAA AGC GTA TAC CAA
 CGC GAT TTA CGC GAA ACC AGC CGC TGG TGG CGC CGC GTA GGC TTA GCG ACC AAA TTA
 CAC TTT GCG CGC GAT CGC TTA ATT GAA AGC TTT TAC TGG GCG GTA GGC GTA GCG TTT
 GAA CCA CAA TAC AGC GAT TGC CGC AAT AGC GTA GCG AAA ATG TTT AGC TTT GTA ACC
 ATT ATT GAT GAT ATT TAC GAT GTA TAC GGC ACC TTA GAT GAA TTA GAA TTA TTT ACC
 GAT GCG GTA GAA CGC TGG GAT GTA AAT GCG ATT AAT GAT TTA CCA GAT TAC ATG AAA
 TTA TGC TTT TTA GCG TTA TAC AAT ACC ATT AAT GAA ATT GCG TAC GAT AAT TTA AAA
 GAT AAA GGC GAA AAT ATT TTA CCA TAC TTA ACC AAA GCG TGG GCG GAT TTA TGC AAT
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 TTT GCG GTA GTA CAA AAT ATT AAA AAA GAA GAA ATT GAA AAT TTA CAA AAA TAC CAC
 GAT ATT ATT AGC CGC CCA AGC CAC ATT TTT CGC TTA TGC AAT GAT TTA GCG AGC GCG
 AGC GCG GAA ATT GCG CGC GGC GAA ACC GCG AAT AGC GTA AGC TGC TAC ATG CGC ACC
 AAA GGC ATT AGC GAA GAA TTA GCG ACC GAA AGC GTA ATG AAT TTA ATT GAT GAA ACC
 TGG AAA AAA ATG AAT AAA GAA AAA TTA GGC GGC AGC TTA TTT GCG AAA CCA TTT GTA
 GAA ACC GCG ATT AAT TTA GCG CGC CAA AGC CAC TGC ACC TAC CAC AAT GGC GAT GCG
 CAC ACC AGC CCA GAT GAA TTA ACC CGC AAA CGC GTA TTA AGC GTA ATT ACC GAA CCA
 ATT TTA CCA TTT GAA CGC TAA
 ggatccATAT

Figure 2

ATAGCgaattcAGAAGGAGATATACC

ATG GCG ACC AAT TTA TTA TGC TTA AGC AAT AAA TTA AGC AGC CCA ACC CCA ACC CCA
 AGC ACC CGC TTT CCA CAA AGC AAA AAT TTT ATT ACC CAA AAA ACC AGC TTA GCG AAT
 CCA AAA CCA TGG CGC GTA ATT TGC GCG ACC AGC AGC CAA TTT ACC CAA ATT ACC GAA
 CAC AAT AGC CGC CGC AGC GCG AAT TAC CAA CCA AAT TTA TGG AAT TTT GAA TTT TTA
 CAA AGC TTA GAA AAT GAT TTA AAA GTA GAA AAA TTA GAA GAA AAA GCG ACC AAA TTA
 GAA GAA GAA GTA CGC TGC ATG ATT AAT CGC GTA GAT ACC CAA CCA TTA AGC TTA TTA
 GAA TTA ATT GAT GAT GTA CAA CGC TTA GGC TTA ACC TAC AAA TTT GAA AAA GAT ATT
 ATT AAA GCG TTA GAA AAT ATT GTA TTA TTA GAT GAA AAT AAA AAA AAT AAA AGC GAT
 TTA CAC GCG ACC GCG TTA AGC TTT CGC TTA TTA CGC CAA CAC GGC TTT GAA GTA AGC
 CAA GAT GTA TTT GAA CGC TTT AAA GAT AAA GAA GGC GGC TTT AGC GGC GAA TTA AAA
 GGC GAT GTA CAA GGC TTA TTA AGC TTA TAC GAA GCG AGC TAC TTA GGC TTT GAA GGC
 GAA AAT TTA TTA GAA GAA GCG CGC ACC TTT AGC ATT ACC CAC TTA AAA AAT AAT TTA
 AAA GAA GGC ATT AAT ACC AAA GTA GCG GAA CAA GTA AGC CAC GCG TTA GAA TTA CCA
 TAC CAC CAA CGC TTA CAC CGC TTA GAA GCG CGC TGG TTT TTA GAT AAA TAC GAA CCA
 AAA GAA CCA CAC CAC CAA TTA TTA TTA GAA TTA GCG AAA TTA GAT TTT AAT ATG GTA
 CAA ACC TTA CAC CAA AAA GAA TTA CAA GAT TTA AGC CGC TGG TGG ACC GAA ATG GGC
 TTA GCG AGC AAA TTA GAT TTT GTA CGC GAT CGC TTA ATG GAA GTA TAC TTT TGG GCG
 TTA GGC ATG GCG CCA GAT CCA CAA TTT GGC GAA TGC CGC AAA GCG GTA ACC AAA ATG
 TTT GGC TTA GTA ACC ATT ATT GAT GAT GTA TAC GAT GTA TAC GGC ACC TTA GAT GAA
 TTA CAA TTA TTT ACC GAT GCG GTA GAA CGC TGG GAT GTA AAT GCG ATT AAT ACC TTA
 CCA GAT TAC ATG AAA TTA TGC TTT TTA GCG TTA TAC AAT ACC GTA AAT GAT ACC AGC
 TAC AGC ATT TTA AAA GAA AAA GGC CAC AAT AAT TTA AGC TAC TTA ACC AAA AGC TGG
 CGC GAA TTA TGC AAA GCG TTT TTA CAA GAA GCG AAA TGG AGC AAT AAT AAA ATT ATT
 CCA GCG TTT AGC AAA TAC TTA GAA AAT GCG AGC GTA AGC AGC AGC GGC GTA GCG TTA
 TTA GCG CCA AGC TAC TTT AGC GTA TGC CAA CAA CAA GAA GAT ATT AGC GAT CAC GCG
 TTA CGC AGC TTA ACC GAT TTT CAC GGC TTA GTA CGC AGC AGC TGC GTA ATT TTT CGC
 TTA TGC AAT GAT TTA GCG ACC AGC GCG GCG GAA TTA GAA CGC GGC GAA ACC ACC AAT
 AGC ATT ATT AGC TAC ATG CAC GAA AAT GAT GGC ACC AGC GAA GAA CAA GCG CGC GAA
 GAA TTA CGC AAA TTA ATT GAT GCG GAA TGG AAA AAA ATG AAT CGC GAA CGC GTA AGC
 GAT AGC ACC TTA TTA CCA AAA GCG TTT ATG GAA ATT GCG GTA AAT ATG GCG CGC GTA
 AGC CAC TGC ACC TAC CAA TAC GGC GAT GGC TTA GGC CGC CCA GAT TAC GCG ACC GAA
 AAT CGC ATT AAA TTA TTA TTA ATT GAT CCA TTT CCA ATT AAT CAA TTA ATG TAC GTA
 TAA

ggatccATAT

Figure 3

ATAGCgaattcAGAAGGAGATATACC

AAT AAA AAT CGC ATT ATG GGC GGC ACC CAA CAA TTT AGC AGC GCG ACC TTA ATT GCG
 AGC GCG AAT GCG AGC TAC CGC TTA CCA TTA ATT CGC AAT TTT CCA CAA CGC AGC ACC
 TTT TTA GTA AAT CCA AAT AAT AAA GCG TTA AGC TTA ACC ACC ACC TGC GCG GTA AGC
 ACC ACC CAA GAT GCG CAA TTT ACC GAA AAT AAT ACC CGC CGC AGC GCG AAT TAC CAA
 CCA AAT TTA TGG GAT TTT GAA TTT TTA CAA AGC GTA GAA AAT GAT TTA CAA GTA GAA
 CGC TTA GAA GAA CGC GCG CGC AAA TTA GAA GAA GAA GTA CGC GGC TTA ATG AAA AAA
 GTA GAA ATT GAA CCA TTA AGC TTA TTA GAA TTA ATG GAT AAT GTA GAA CGC TTA GGC
 TTA ACC TAC AAA TTT GAA GAA GAT ATT AAA AGC GCG TTA AAT AAT CGC ATT GTA CCA
 TTA TTA CAC CAC CAC ACC ATT AAT AAA TAC GGC TTA CAC GCG ACC GCG TTA AGC TTT
 CGC TTT TTA CGC CAA CAC GCG TTT CAC GTA AGC CCA GAT GTA TTT GAA AGC TTT AAA
 GAA GAA GGC AAA TTT AAA AAA GAA ATT AGC GGC GAT GTA TTA GGC TTA TTA AAT TTA
 TAC GAA ACC AGC TAC TTA GGC TTT GAA GGC GAA ACC ATT TTA GAT GAA GCG CGC GCG
 TTT AGC GCG ACC CAC TTA AAA AAT TTA TTA CAA ACC AAT CAA GTA CAA AAT AAA GTA
 ATT GCG GAA AAA GTA CGC CAC GCG TTA GAA TTA CCA TAC CAC CGC CGC GTA CAC CGC
 TTA GAA GCG CGC TGG TTT ATT GAA CGC TAC GAA CAA AAA GAA GCG CAC GAT GGC GCG
 TTA TTA GAA TTA GCG AAA TTA GAT TTT AAT ATG GTA CAA AGC GTA ATG AAA AAA GAA
 TTA CAA GAA TTA AGC CGC TGG TGG CGC GAA ATT GGC TTA ACC AGC AAA TTA GAT TTT
 GTA CGC GAT CGC TTA ATG GAA GTA TAC TTT TGG GCG TTA GGC ATG GCG CCA CAC CCA
 CAA TTA ACC GAA TGC CGC AAA GCG GTA ACC AAA ATG TTT GGC TTA GTA ACC ATT ATT
 GAT GAT GTA TAC GAT GTA TAC GGC ACC TTA GAT GAA TTA CAA TTA TTT ACC GAT GCG
 GTA GAT CGC TGG GAT GTA AAT GCG GTA GAA ACC TTA CCA GAT TAC ATG AAA TTA TGC
 TAC TTA GCG TTA TAC AAT AGC GTA AAT GAT ACC GCG TAC AGC ACC TTA CGC GAA AAA
 GGC GAT AAT AGC TTA CCA CAC TTA GCG AAA AGC TGG CGC GAT TTA TGC AAA GCG TTT
 TTA CAA GAA GCG AAA TGG AGC AAT AAT AAA ATT ATT CCA CCA TTT GAT GCG TAC ATT
 CGC AAT GCG AGC GTA AGC AGC AGC GGC GGC GCG TTA TTA GCG CCA TGC TAC TTT AGC
 GTA ACC CAA GAT AGC ACC AGC CAA GCG ATT GAT AGC ATT ACC AAT TAC CAC GGC ATT
 GTA CGC AGC AGC TGC GCG ATT TTT CGC TTA TGC AAT GAT TTA GCG ACC AGC GCG GCG
 GAA TTA GAA CGC GGC GAA ACC ACC AAT AGC ATT ACC AGC TAC ATG ACC GAA AAT GGC
 ACC ACC GAA GAA GAA GCG CGC GAA AGC TTA GGC AAA TTA ATT GAT CAA GAA TGG AAA
 AAA ATG AAT CGC GAT GTA GTA TTA GAA AGC GCG TAC CCA AAT GTA TTT AAA GAA ATT
 GCG ATT AAT ATG GCG CGC GTA AGC CAC TGC ACC TAC CAA TAC GGC GAT GGC TTA GGC
 CGC CCA GAT GAT ACC GCG GAA AAT CGC ATT AAA TTA AGC TTA ATT GAA CCA ATT CCA
 ATT AAT TAA
 ggatccATAT

Figure 4

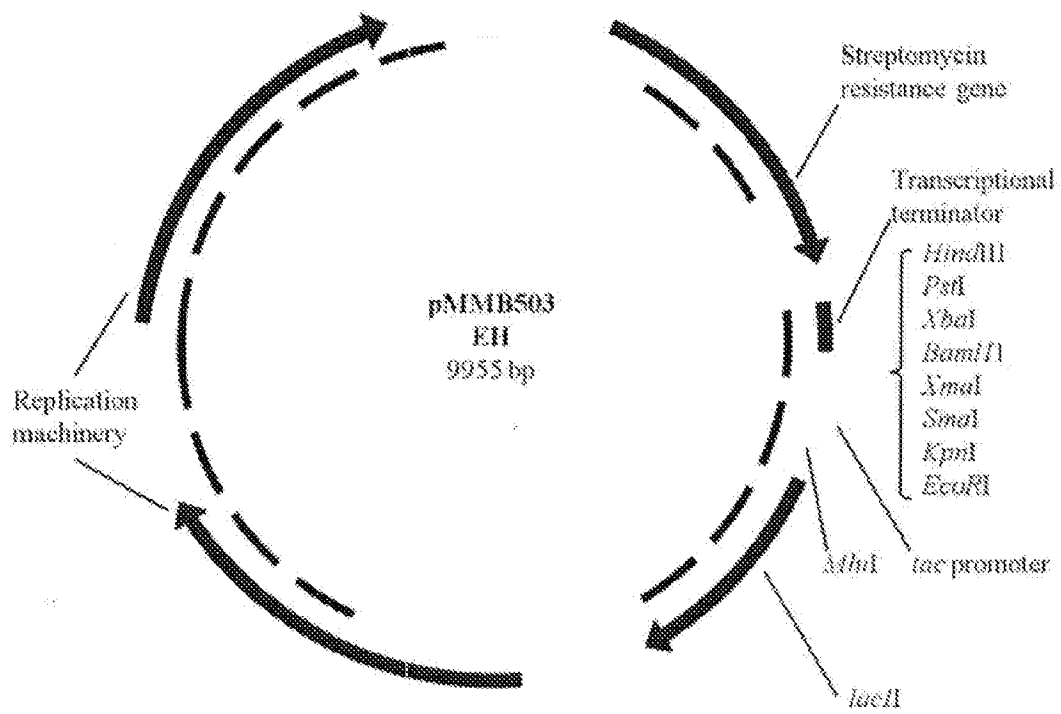


Figure 5

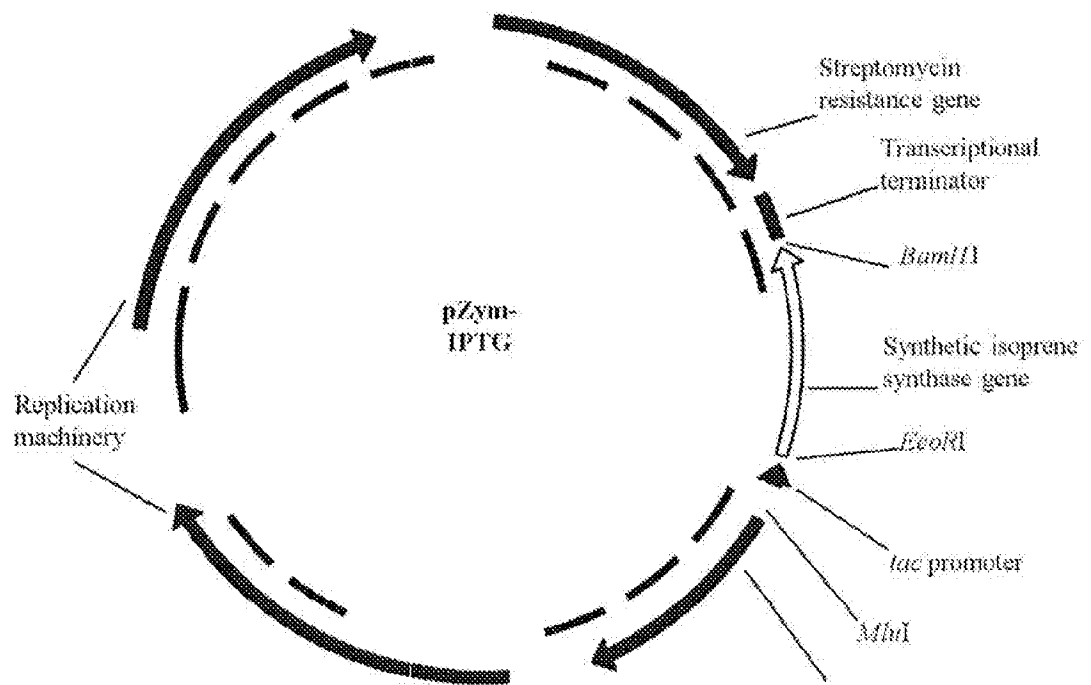
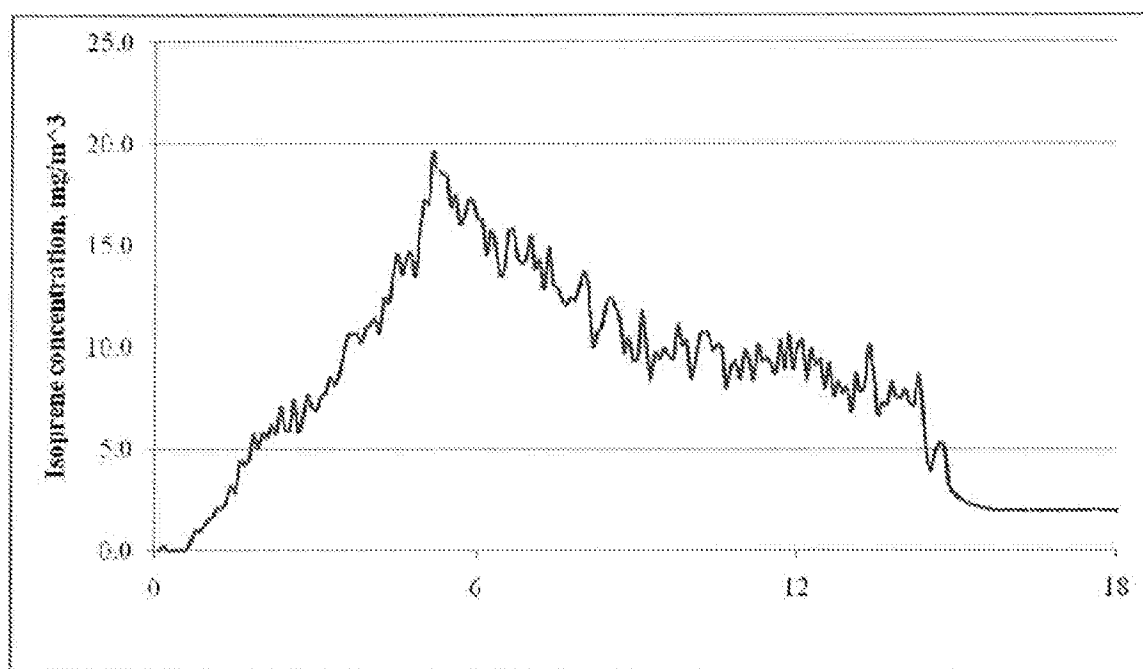


Figure 6

**Figure 7**

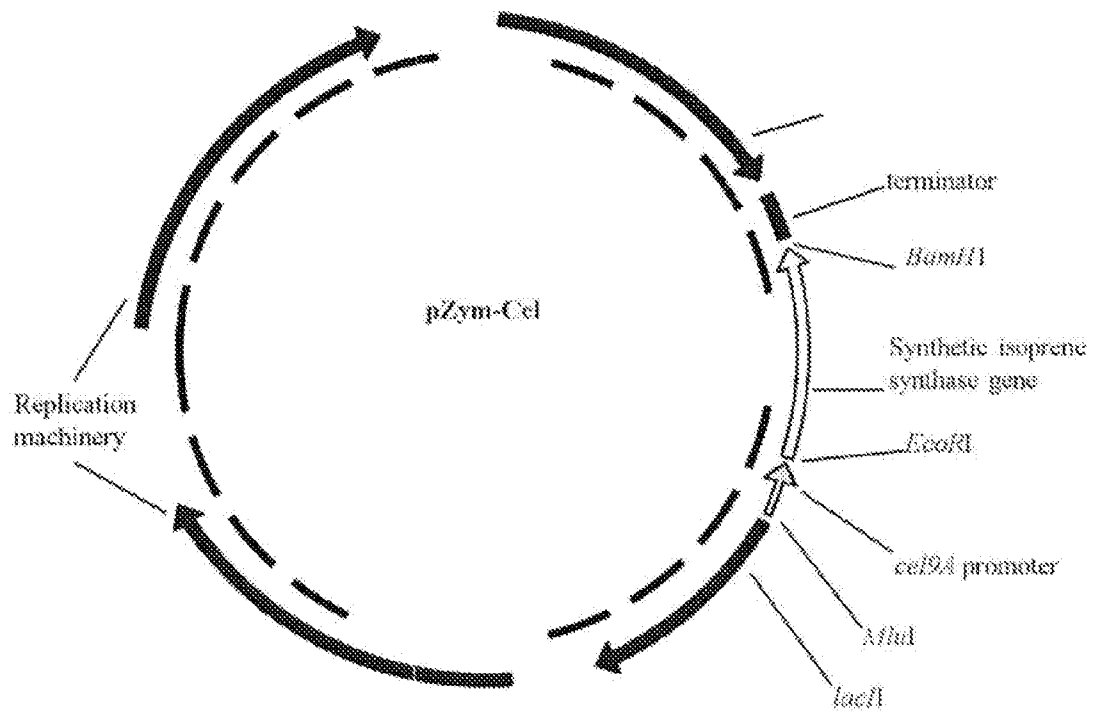
Agtgtttttattcagagccgacccaaatatgtccttgtaaattccatgtatagctacttggttgcaa
gttgcttagcgaacgcagtgcgtaacaagcaagggcgccattgttgtagttgggtggtcaag
cccagcttatggctcttatctgtaatttagttataagtgataagacacatattgggcgatata
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gatgtgtggcgaatttggctaatttatgtgtgttttatcgccgatcgccgcgataaggcgat
tcgctgctaggctaattcttgaagaggggcacaacctgtaggcggtgttatagggttaagcgc
tttaaacaatgtgaaagcagggtaaaaaagcccggaattgtgccgtgtatagtccaccgctagtt
gccatttgtatggcaattcttaagcggttttagcctgttgtgtgggaaaaata

Figure 8

PromD: ATAacgcgtAGIGTTTTATTTCAGAGCCGACCAAATAT

PromR: ATAgattcTATTTTCCACACAACAGGCTAAAACC

Figure 9

**Figure 10**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2012/046610

A. CLASSIFICATION OF SUBJECT MATTER		<i>C12N 1/21 (2010.01)</i> <i>C12N 15/52 (2010.01)</i> <i>C12P 5/02 (2010.01)</i> <i>C07C 11/18 (2010.01)</i> <i>C12R 1/01 (2010.01)</i>																				
According to International Patent Classification (IPC) or to both national classification and IPC																						
B. FIELDS SEARCHED																						
Minimum documentation searched (classification system followed by classification symbols)																						
C12N 1/21, 15/52, C12P 5/02, C07C 11/18, C12R 1/01																						
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched																						
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)																						
PAJ, Esp@cenet, PCT Online, USPTO DB, CIPO (Canada PO), SIPO DB, PatSearch																						
C. DOCUMENTS CONSIDERED TO BE RELEVANT																						
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																				
X	WO 2010/031068 A1 (DANISCO US INC. et al.) 18.03.2010, claims, paragraphs [0208], [0231], [0246]-[0248], [0263], [0273], [0275], [0281], [0358]-[0360]	1, 3-5, 7, 9-14, 16, 18-20																				
Y		2, 5, 6, 8, 9, 14, 15, 17, 20, 21																				
Y	EA 200900096 A1 (DCM AIPI ASSETS B.V.) 30.06.2009. claims, p. 64	2, 6, 8, 15, 17, 21																				
Y	BRIAN J. WATSON et al., Processive Endoglucanases Mediate Degradation of Cellulose by <i>Saccharophagus degradans</i> , J. Bacteriol, 2009,191(18):5697-5705	5, 14, 20																				
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.																						
* Special categories of cited documents: <table border="0"> <tr> <td>"A"</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E"</td> <td>earlier document but published on or after the international filing date</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>"&"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family	"P"	document published prior to the international filing date but later than the priority date claimed		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																			
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																			
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																			
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family																			
"P"	document published prior to the international filing date but later than the priority date claimed																					
Date of the actual completion of the international search		Date of mailing of the international search report																				
11 September 2012 (11.09.2012)		01 November 2012 (01.11.2012)																				
Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1		Authorized officer S. Ilchenko																				
Facsimile No. +7 (499) 243-33-37		Telephone No. (495)531-65-15																				

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2012/046610

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LA ENSOR et al., Expression of multiple complex polysaccharide-degrading enzyme systems by marine bacterium strain 2-40, Journal of Industrial Microbiology & Biotechnology, 1999, 23:123-126	9
A	V. V. LISAK, Microbiologiya, Minsk, BGU, 2007, p. 363, lines 3-6	1-21
A	WO 2011/044279 A2 (BIO ARCHITECTURE LAB, INC) 14.04.2011, claims 124, 162, p. 6, last paragraph- p. 7, paragraph 1	1-21