PRODUCTION AND SEPARATION OF 3-HYDROXYPROPIONIC ACID

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority under 35 U.S.C. §119(e) of U.S. Provisional Application No. 62/577,361, filed on October 26, 2017 and Provisional Application No. 62/594,318, filed on December 4, 2017, the entire contents of each of which are incorporated herein by reference.

TECHNICAL FIELD

This disclosure relates to the production of 3-hydroxypropionic acid, including its production from glycerol by recombinant strains, as well as its removal from an aqueous solution (e.g., aqueous broth), and use thereof for producing various chemicals.

BACKGROUND

3-Hydroxypropionic acid (3-HP) and 3-hydroxypropanoate (a salt of 3-HP) are used in the industrial production of various chemicals, such as acrylic acid, which can be used as a cross-linking agent for polymer coatings, metal lubricants and antistatic agents for textiles.

SUMMARY

The disclosure provides methods and apparatus for producing 3-HP or a salt thereof, for removing 3-HP from aqueous solution (e.g., aqueous broth), and for using it to make various chemicals.

In one aspect of the present disclosure, there is provided an expression system, comprising a first promoter that is either constitutive or inducible by a small molecule, a second promoter that is either constitutive or induced by a small molecule, a first gene encoding a protein involved in the synthesis of 3-HP, a salt of 3-HP, or coenzyme B12, a modified UTR, and a native sequence encoding up to 20 amino acids from the N-terminus of a native Pseudomonas denitrificans protein, wherein the native sequence is operably linked to the 3'-terminus of the modified UTR and operably linked to the 5'-terminus of the first gene, the first and second promoters are operably linked in tandem

upstream of the modified UTR, and the modified UTR comprises a sequence selected from the group consisting of SEQ ID NO: 22-28 and 64 (UTR0-6).

In another aspect of the present disclosure, there is provided a nucleic acid, comprising a first and a second promoter, wherein the first promoter is an inducible promoter and is inducible by a small molecule; the first and second promoters are operably linked to a first gene; and the first gene encodes a protein involved in the synthesis of 3-hydroxypropionic acid (3-HP), a salt of 3-HP, or coenzyme B12 wherein the nucleic acid comprises a sequence selected from the group consisting of SEQ ID NO:22-28 and 64 (UTR 0-6).

In a further aspect of the present disclosure, there is provided a nucleic acid, comprising a first and a second promoter, wherein either the first is an inducible promoter and is inducible by a small molecule; the first and second promoters are operably linked to a first gene; and the first gene encodes a protein involved in the synthesis of 3-hydroxypropionic acid (3-HP), a salt of 3-HP, or coenzyme B12, wherein a second 10 codons at the 5'-terminus of the first gene are optimized for translation in Pseudomonas denitrificans and a codon frequency for each amino acid in the optimized second 10 codons is more than 0.

In another aspect of the present disclosure, there is provided a method, comprising:

evaporating a first solvent;

condensing the evaporated first solvent; and

directing a flow of the first solvent to remove 3-HP from the aqueous solution,

wherein the 3-HP is removed from the aqueous solution without using a counter-current liquid flow.

In some examples, this disclosure provides methods of producing 3-HP or a salt thereoff from glycerol using a genetically modified strain of *Pseudomonas denitrificans.*

In certain examples, this disclosure provides methods to create *Pseudomonas* strains that can produce 3-HP or a salt thereof at a high titer without external supplementation of coenzyme B12.

In some examples, this disclosure provides an expression system (e.g., an expression module or an expression construct) for use in *Pseudomonas* strains. Such an expression system can include, *inter alia,* a tandem promoter system which includes two

or more promoters operably linked to control expression of a downstream gene. Such a promoter can be an inducible promoter or a constitutive promoter. Optionally, an expression system includes at least one inducible promoter. An expression system can be used to produce target proteins/enzymes or to enhance metabolic pathways to produce target products at high titer, such as 3-HP or a salt thereof, by minimizing toxic intermediates, and/or increase vitamin B12 production, a cofactor in the first reaction in the 3-HP synthetic pathway.

In some examples, this disclosure provides nucleic acids that encode these systems, the recombinant bacteria expressing these systems, and/or the methods of culturing the bacteria to produce 3-HP or a salt thereof. These bacteria have been modified to include one or more expression systems that respond to the target product levels, in this case 3-HP or a salt thereof in the media and express the genes used for the production of the target product, e.g., 3-HP or a salt thereof. In some cases, these bacteria have also been modified to include one or more expression systems to increase expression of the genes used for the production of B12. The conditions for culturing said bacterium, to increase 3-HP production (or a salt thereof), are also provided herein.

The disclosure provides for an expression system, comprising, consisting of, or consisting essentially of a first promoter that is inducible by a small molecule, a second promoter, a first gene encoding a protein involved in the synthesis of 3-HP (or a salt thereof) or B12, a modified UTR, and a native sequence encoding up to 20 amino acids from the N-terminus of a native *Pseudomonas denitrificans* protein, wherein the native sequence is operably linked to the 3'-terminus of the modified UTR and operably linked to the 5'-terminus of the first gene, and the first and second promoters are operably linked in tandem upstream of the modified UTR.

In some examples of the present disclosure, the expression system further comprises, consists of, or consists essentially of a third promoter; and a second gene encoding a transcriptional regulator configured to regulate expression of the first gene, wherein the third promoter is operably connected to the second gene. In some instances, the second promoter is inducible by the small molecule. In some cases, the native sequence is operably linked to the 5'-terminus of the first gene to define a fusion gene, the second promoter is a native promoter for the native *Pseudomonas denitrificans* protein, and the

second promoter is operably linked to the 5'-terminus of the fusion gene. In some examples the first promoter is operably linked to the 5'-terminus of the second promoter, the second promoter is operably linked to the 5'-terminus of the modified UTR, and the modified UTR is operably linked to the 5'terminus of the fusion gene.

The disclosure provides for a nucleic acid, comprising, consisting of, or consisting essentially of a first and a second promoter, wherein the first promoter is an inducible promoter and is inducible by a small molecule; the first and second promoters are operably linked to a first gene; and the first gene encodes a protein involved in the synthesis of 3-hydroxypropionic acid (3-HP)(or a salt thereof) or coenzyme B12.

In some examples of the present disclosure, the first gene encodes a protein involved in the synthesis of 3-hydroxypropionic acid (3-HP) (or a salt thereof) and is selected from the group consisting of a glycerol dehydratase, a glycerol dehydratase reactivase, and an aldehyde dehydrogenase. In some cases, the first gene comprises at least one gene selected from the group consisting of dhaB1, dhaB2, dhaB3, gdrA, gdrB, and kgsA. In some instances, the first gene comprises a dhaB1 gene, a dhaB2 gene, a dhaB3 gene, a gdrA gene, and a gdrB gene.

In some examples, the first gene is dhaB1, and the sequence comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 1. In some cases, the first gene is dhaB2, and the sequence comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 2. In some instances, the first gene is dhaB3, and the sequence comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 3.

In some examples of the present disclosure, the first gene is gdrA, and the sequence comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 4. In some cases, the first gene is grdB and the sequence comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 5.

In some examples of the present disclosure, the first gene comprises, consists of, or consists essentially of an aldehyde dehydrogenase gene. In some instances, the aldehyde dehydrogenase is kgsA. In some cases, kgsA comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 6.

In some examples of the present disclosure, the small molecule is an acid or alcohol. In some cases, the small molecule acid or alcohol is selected from the group consisting of L-lactic acid (LAC), acetic acid (AcOH), propionic acid (PA), 3-hydroxypropionic acid (3-HP), 3-hydroxybutyrate (3-HB), 1,3-propanediol (1,3-PDO), 2,3-butanediol (2,3-BDO), L-valine (L-val), and 3-hydroxyisobutyrate (3-HIB), or salts thereof. In some instances, the small molecule acid or alcohol is selected from the group consisting of 3-hydroxypropionic acid (3-HP), 3-hydroxybutyrate (3-HB), L-valine (L-val), and 3-hydroxyisobutyrate (3-HIB). In some cases, the small molecule is 3-hydroxypropionic acid (3-HP) (or a salt thereof).

In some examples of the present disclosure, the nucleic acid comprises, consists of, or consists essentially of a sequence that is at least 85% identical to SEQ ID NO: 65. In some cases, the nucleic acid comprises, consists of, or consists essentially of SEQ ID NO: 65.

In some examples of the present disclosure, the second promoter is operably linked to the 3'-terminus of the first promoter and the first gene is operably downstream of the second promoter. In some examples of the present disclosure, the first promoter is operably linked to the 3'-terminus of the second promoter and the first gene is operably downstream of the second promoter.

In some examples of the present disclosure, the second promoter is a constitutive promoter. In some instances, the second promoter is a second inducible promoter. In some cases, the intergenic space between the first and the second promoter does not include a terminator sequence. In some instances, first promoter and the second promoter regulate expression of the first gene.

In some examples of the present disclosure, the first promoter is derived from a P_{mmsA} promoter, a P_{hbdH-1} promoter, a P_{hbdH-4} promoter, or a P_{hpdH} promoter. In some instances, the first promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 14 (PmmsA promoter). In some cases, the first promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 13 (PhbdH-1 promoter). In some instances, the first promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 11 (PhbdH-4 promoter). In some cases, the first promoter comprises,

consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 12 (PhpdH promoter). In some instances, the first promoter comprises, consists of, or consists essentially of a sequence that is selected from the group consisting of SEQ ID NO: 11; SEQ ID NO: 12; SEQ ID NO: 13; and SEQ ID NO: 14.

In some examples of the present disclosure, the first promoter comprises, consists of, or consists essentially of a P_{mmsA} promoter and the nucleic acid further comprises an operator site operably linked to the 5'-terminus of the P_{mmsA} promoter. In some cases, the nucleic acid described here in comprises, consists of, or consists essentially of a sequence that is 95% identical to SEQ ID NO: 15. In some instances, the nucleic acid described herein comprises, consists of, or consists essentially of a sequence that is 95% identical to SEQ ID NO: 16 (O1 of MmsA operator) and a sequence that is 95% identical to SEQ ID NO: 17 (O2 of MmsA operator). In some examples of the present disclosure, the nucleic acid described herein comprises, consists of, or consists essentially of a sequence that is 95% identical to SEQ ID NO: 18 (PmmsA and operator Fig. 3). In some cases, the nucleic acid described herein comprises, consists of, or consists essentially of a sequence selected from the group consisting of SEQ ID NO: 15, 18, 19 (PmmsA2a), 20 (PmmsA2b), and 21 (PmmsA2ab).

In some examples of the present disclosure, the second promoter is derived from a P_{mmsA} promoter, a P_{hbdH-1} promoter, a P_{hbdH-4} promoter, a P_{hpdH} promoter, or a P_{zwf} promoter. In some cases, the second promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 11 (PhbdH-4 promoter). In some instances, the second promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 12 (PhpdH promoter). In some examples of the present disclosure, the second promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 14 (PmmsA promoter). In some cases, the second promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 13 (PhbdH-1 promoter). In some examples of the present disclosure, the second promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 7 (Pzwf). In some cases, the second promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 8 (Pzwf-1). In some

instances, the second promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 9 (Pzwf-7). In some examples of the present disclosure, the second promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 62 (Pzwf-7 shorter). In some cases, the second promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 10 (Pzwf-12). In some instances, the second promoter comprises, consists of, or consists essentially of a sequence selected from the group consisting of SEQ ID NO: 7, 8, 9, 10, 11, 12, 13, 14, 52-60, 61, 62, and 63 (e.g., Pzwf promoters).

In some examples of the present disclosure, the first promoter comprises, consists of, or consists essentially of the P_{mmsA} promoter and the second promoter comprises, consists of, or consists essentially of the P_{hbdH-4} promoter. In some cases, the first promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 14 (PmmsA promoter sequence), and the second promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 11 (PhbdH-4 promoter). In some instances, the first promoter comprises, consists of, or consists essentially of the PhbdH-1 promoter and the second promoter comprises, consists of, or consists essentially of the PhpdH promoter. In some cases, the first promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 13 (PhbdH-1 promoter sequence), and the second promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 12 (PhpdH promoter).

In some examples of the present disclosure, the second promoter comprises, consists of, or consists essentially of a PhbdH promoter and the nucleic acid further comprises, consists of, or consists essentially of an operator site operably linked to the N-terminus of the PhbdH promoter. In some cases, the operator site comprises, consists of, or consists essentially of one or more sequence that is at least 95% identical to SEQ ID NO: RBS-1, RBS-2, ABS-1, ABS-2, and ABS-3. In some instances, the operator site comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NOs: RBS-1 site and ABS-2. In some cases, the operator site comprises, consists of, or consists essentially of SEQ ID NOs: RBS-1 site and ABS-2.

In some examples of the present disclosure, the nucleic acid further comprises, consists of, or consists essentially of a gene encoding a transcriptional regulator that regulates expression of the first gene. In some instances, the transcriptional regulator binds to the first or second promoter. In some cases, the transcriptional regulator is a LysR-type transcriptional regulator (LTTR). In some examples, the transcriptional regulator is MmsR or HpdR. In some cases, the transcriptional regulator is MmsR.

In some examples of the present disclosure, the transcriptional regulator binds to the first promoter. In some cases, the transcriptional regulator is derived from a mmsR protein and the first promoter is derived from a PmmsA promoter. In some cases, the transcriptional regulator comprises, consists of, or consists essentially of a mmsR protein and the first promoter comprises, consists of, or consists essentially of a PmmsA promoter.

In some examples of the present disclosure, the nucleic acid described herein further comprises, consists of, or consists essentially of an operator site, wherein the MmsR protein binds to the operator site. In some cases, the nucleic acid described herein comprises, consists of, or consists essentially of a sequence that is at least 95% identical to SEQ ID NO: 19 (PmmsA2a). In some instances, the nucleic acid described herein comprises, consists of, or consists essentially of a sequence that is selected from the group consisting of SEQ ID NO: 19-21 (PmmsA2a, A2b, A2ab). In some cases, the transcriptional regulator binds to the second promoter. In some examples of the present disclosure, the second promoter is a constitutive promoter. In some cases, the transcriptional regulator is a HpdR protein and the second promoter is derived from a hpdH promoter. In some instances, the second promoter comprises, consists of, or consists essentially of a sequence that is at least 95% identical to the sequence of P_{hpdH} SEQ ID NO: 12.

In some examples of the present disclosure, the transcriptional regulator binds to the first promoter and has enhanced binding to the first promoter in the presence of the small molecule. In some cases, the transcriptional regulator binds to the second promoter and has enhanced binding to the second promoter in the presence of the small molecule. In some instances, the transcriptional regulator is self-regulating. In some cases, the nucleic

acid described herein further comprises, consists of, or consists essentially of a third promoter that is operably linked to the gene encoding the first transcriptional regulator.

In some examples of the present disclosure, the third promoter comprises, consists of, or consists essentially of a sequence that is 95% identical to the sequence selected from the group consisting of SEQ ID NO: 7-10 and 52-63. In some cases, the second constitutive promoter is selected from the group consisting of SEQ ID NOs: 9, 10, 57-60, 62, and 63; the transcriptional regulator comprises, consists of, or consists essentially of a MmsR protein; and the first promoter comprises, consists of, or consists essentially of P_{mmsA}. In some instances, the second constitutive promoter is selected from the group consisting of SEQ ID NOs: 9, 10, 57-60, 62, and 63; the transcriptional regulator comprises, consists of, or consists essentially of a HpdR protein; and the first promoter comprises, consists of, or consists essentially of P_{hbdH}. In some cases, the third promoter comprises, consists of, or consists essentially of SEQ ID NO: 10. In some cases, the third promoter comprises, consists of, or consists essentially of SEQ ID NO: 62.

In some examples of the present disclosure, the first gene comprises, consists of, or consists essentially of a modified 5' UTR of the gene. In some instances, the first gene comprises, consists of, or consists essentially of a sequence selected from the group consisting of SEQ ID NO: 22-28 and 64. (UTR 0-6). In some cases, the 5' UTR comprises, consists of, or consists essentially of SEQ ID NO: 28 (UTR-6). In some instances, the first gene comprises, consists of, or consists essentially of a gene encoding kgsA and the 5'UTR comprises, consists of, or consists essentially of SEQ ID NO: 28 (UTR-6).

In some examples of the present disclosure, at least the 10 codons at the 5'terminus of the first gene are optimized for translation in *Pseudomonas denitrificans*. In some cases, up to 10 codons at the 5'terminus of the first gene are optimized for translation in *Pseudomonas denitrificans*. In some instances, codon optimization comprises, consists of, or consists essentially of: measuring a codon frequency of each amino acid in a gene encoding a native *Pseudomonas denitrificans* protein; using the codon frequency of the native protein to replace the 10 codons at the 5'-terminus of the first gene with an optimized 10 codons, wherein the codon for each amino acid of the optimized 10 codons is present at the same frequency as the codon frequency of the native protein. In some

cases, the codon frequency for each amino acid in the optimized second 10 codons is more than 0. In some instances, the codon frequency is measured of each amino acid in the 10 codons at the 5' terminus of the gene encoding the native *Pseudomonas denitrificans* protein.

In some examples of the present disclosure, the nucleic acid comprises, consists of, or consists essentially of a sequence that is 95% identical to a sequence selected from the group consisting of SEQ ID NOs: 29, 30, and 31. (Opt1-3). In some cases, the first gene comprises, consists of, or consists essentially of a gene encoding kgsA and comprises, consists of, or consists essentially of a sequence selected from the group consisting of SEQ ID NO: 29-31. In some instances, the first gene is fused at its 5'-terminus to a sequence encoding up to 20 amino acids that are derived from the 5'terminus of a second gene encoding a native *Pseudomonas denitrificans* protein, thereby creating a fusion gene. In some cases, the mRNA of the fusion gene has a higher stability than the mRNA of the first gene alone. In some instances, the fusion gene increases gene translation when compared to the first gene alone. In some examples of the present disclosure, the mRNA of the fusion gene is more resistant to ribonuclease degradation than the mRNA of the first gene alone. In some cases, the fusion gene increases gene translation in comparison to the first gene alone.

In some examples of the present disclosure, the first gene is fused at its 5'-terminus to a sequence encoding up to 20 (5, 10, 15, or 20) amino acids that are derived from the 5'terminus of a second gene encoding a native *Pseudomonas denitrificans* protein, thereby creating a fusion gene, and the second promoter is derived from the native promoter for the second gene. In some cases, the second promoter is derived from the P_{mmsA} promoter and the fusion gene comprises, consists of, or consists essentially of a sequence encoding at least 5, 10, 15, or 20 amino acids in the N-terminus of a native MmsA protein. In some instances, the second promoter comprises, consists of, or consists essentially of the P_{mmsA} promoter and the fusion gene comprises, consists of, or consists essentially of a sequence encoding five or more amino acids from the N-terminus of a native MmsA gene, and the fusion gene is operably linked to the 3' end of the P_{mmsA} promoter. In some cases, the nucleic acid comprises, consists of, or consists essentially of a sequence selected from the group consisting of SEQ ID NOs: 32-35.

In some examples of the present disclosure, the first gene is fused at its 5'-terminus to a sequence encoding up to 20 (5, 10, 15, or 20) amino acids that are derived from a sequence encoding the N-terminus of a native *Pseudomonas denitrificans* MmsA protein. In some cases, the nucleic acid described herein comprises, consists of, or consists essentially of a sequence selected from the group consisting of SEQ ID NOs: 32-35 (Pcm-mmsA(5)(10)(15)(20)).

In some examples of the present disclosure, the first gene encodes a protein involved in the synthesis of vitamin B12. In some cases, the first gene comprises, consists of, or consists essentially of a gene selected from the group consisting of cobJ, cobI, cobH, cobG, cobL, cobF, cobK, gst, xre,chlD, chlI, dahp, cobN, cobW, cbtBA, cobE, cobM, btuB, cobO, cob, cobR, cobD, cobC, cobQ, cobU, cobP, bgpM, and cobV. In some instances, the first gene comprises, consists of, or consists essentially of bgpM. In some cases, the first gene is derived from a *Pseudomonas* species selected from the group consisting of *P. denitrifcans; P. aeruginosa; P. entomophila; P. putida; P. syringae; P. fluorescens; P mendocina;* and *P. stutzeri.*

The disclosure provides for a nucleic acid comprising, consisting of, or consisting essentially of a first promoter operably linked to a first gene that encodes a protein involved in the synthesis of coenzyme B12. In some cases, the first promoter is a constitutive promoter. In some instances, the nucleic acid comprises, consists of, or consists essentially of a sequence selected from SEQ ID NOs: 7-10 and 52-63. In some examples of the present disclosure, the first gene comprises, consists of, or consists essentially of one or more genes selected from the group consisting of cobJ, cobI, cobH, cobG, cobL, cobF, cobK, gst, xre,chlD, chlI, dahp, cobN, cobW, cbtBA, cobE, cobM, btuB, cobO, cob, cobR, cobD, cobC, cobQ, cobU, cobP, bgpM, and cobV. In some cases, the first gene is from a *Pseudomonas* species selected from the group consisting of *P. denitrifcans; P. aeruginosa; P. entomophila; P. putida; P. syringae; P. fluorescens; P mendocina;* and *P. stutzeri.*

In some examples of the present disclosure, the nucleic acid described herein further comprises, consists of, or consists essentially of a second promoter operably linked to the first promoter. In some cases, the first and the second promoter regulate expression of

the first gene. In some instances, the first promoter is not a native promoter for the first gene.

The disclosure provides for a nucleic acid comprising a cobG gene, a P_{edd} promoter, a P_{sucA} promoter, and a cobL gene; wherein the P_{edd} promoter is operably linked to the cobG gene and the P_{sucA} promoter, and the P_{sucA} promoter is operably linked to the cobL gene. In some cases, the P_{edd} promoter comprises, consists of, or consists essentially of the sequence SEQ ID NO: 36; and the P_{sucA} promoter comprises, consists of, or consists essentially of the sequence SEQ ID NO: 37. In some examples, the nucleic acid described herein comprises, consists of, or consists essentially of the sequence SEQ ID NO: 38.

The disclosure provides for a nucleic acid comprising, consisting of, or consisting essentially of a cobG gene, a P_{sp9} promoter, a P_{zwf} promoter, and a cobL gene; wherein the P_{sp9} promoter is operably linked to the cobG gene and the P_{zwf} promoter, and the P_{zwf} promoter is operably linked to the cobL gene. In some cases, the P_{sp9} promoter comprises, consists of, or consists essentially of SEQ ID NO: 39; and the P_{zwf} promoter comprises SEQ ID NO: 40. In some instances, the nucleic acid described herein comprises, consists of, or consists essentially of SEQ ID NO: 41.

The disclosure provides for a nucleic acid comprising, consisting of, or consisting essentially of a cobW gene, a P_{zwf} promoter, a P_{sp9} promoter, and a cbtB gene, wherein the P_{zwf} promoter is operably linked to the cobW gene and the P_{sp9} promoter, and the P_{sp9} promoter is operably linked to the cbtB gene. In some cases, the Pzwf promoter comprises SEQ ID NO: 42; and the Psp9 promoter comprises SEQ ID NO: 43. In some instance, the nucleic acid described herein comprises, consists of, or consists essentially of SEQ ID NO: 44.

The disclosure provides for a nucleic acid comprising, consisting of, or consisting essentially of a cobW gene, a P_{tkt} promoter, a P_{sp2} promoter, and a cbtB gene, wherein the P_{tkt} promoter is operably linked to the cobW gene and the P_{sp2} promoter, and the P_{sp2} promoter is operably linked to the cbtB gene. In some instances, the P_{tkt} promoter comprises, consists of, or consists essentially of SEQ ID NO: 45; and the Psp2 promoter comprises, consists of, or consists essentially of SEQ ID NO: 46. In

some cases, the nucleic acid described herein comprises, consists of, or consists essentially of SEQ ID NO: 47.

The disclosure provides for a nucleic acid comprising, consisting of, or consisting essentially of a P_{zwf} promoter operably linked to a tonB gene (e.g., a butB gene). In some cases, the Pzwf promoter comprises, consists of, or consists essentially of SEQ ID NO: 48. In some instances, the nucleic acid described herein comprises, consists of, or consists essentially of SEQ ID NO: 49.

The disclosure provides for a nucleic acid comprising, consisting of, or consisting essentially of a P_{sp9} promoter operably linked to a tonB gene (e.g., a butB gene). In some cases, the Psp9 promoter comprises, consists of, or consists essentially of SEQ ID NO: 50. In some instances, the nucleic acid described herein comprises, consists of, or consists essentially of SEQ ID NO: 51.

In some examples of the present disclosure, the nucleic acid comprises, consists of, or consists essentially of one or more sequences derived from *Pseudomonas denitrificans*. In some cases, the first or the second promoters are derived from *Pseudomonas denitrificans*. In some instances, the nucleic acid comprises, consists of, or consists essentially of one or more sequences derived from an Enterobacter, Lactobacillus, Pseudomonas, or Azospirillum bacterium. In some cases, the first gene is derived from an Enterobacter, Lactobacillus, Pseudomonas, or Azospirillum bacterium.

The disclosure provides for a nucleic acid comprising a DhaB expression module that comprises, consists of, or consists essentially of a sequence that is at least 85% identical to SEQ ID NO: 66 (DhaB expression module). In some cases, the sequence is at least 90% identical to SEQ ID NO: 66. In some instances, the nucleic acid described herein comprises, consists of, or consists essentially of SEQ ID NO: 66.

The disclosure provides for a nucleic acid comprising a DhaB expression module that comprises, consists of, or consists essentially of a sequence that is at least 85% identical to SEQ ID NO: 67 (kgsA expression module). In some cases, the sequence is at least 90% identical to SEQ ID NO: 67. In some instances, the sequence comprises, consists of, or consists essentially of SEQ ID NO: 67.

The present disclosure provides for a nucleic acid comprising a DhaB expression module comprising, consisting of, or consisting essentially of a UTR of the

gdrA gene comprising, consisting of, or consisting essentially of SEQ ID NO: 68 and a UTR of the *gdrB* gene comprising, consisting of, or consisting essentially of SEQ ID NO: 69. In some cases, the nucleic acid comprises, consists of, or consists essentially of a sequence that is at least 85% identical to a sequence selected from the group consisting of SEQ ID NO: 70-72. In some instances, the nucleic acid comprises, consists of, or consists essentially of a sequence selected from the group consisting of SEQ ID NO: 70-72.

The present disclosure provides for a nucleic acid comprising a coenzyme B12 sensor comprising, consisting of, or consisting essentially of a sequence that is at least 85% identical to a sequence selected from the group consisting of SEQ ID NO: 73 and 74. In some cases, the nucleic acid described herein comprises, consists of, or consists essentially of a sequence selected from the group consisting of SEQ ID NO: 73 and 74.

The present disclosure provides for a nucleic acid comprising, consisting of, or consisting essentially of a coenzyme B12 expression module that comprises, consists of, or consists essentially of a first gene involved in the synthesis of coenzyme B12, a first promoter that is induced by a small molecule, and a second promoter, wherein the first and second promoters are operably linked in tandem upstream of the first gene such that they regulate expression of the first gene. In some cases, the nucleic acid further comprises, consists of, or consists essentially of a second gene involved in the synthesis of coenzyme B12, a third promoter that is induced by a small molecule, and a fourth promoter, wherein the third and fourth promoters are operably linked in tandem upstream of the second gene such that they regulate expression of the second gene. In some examples of the present disclosure, the nucleic acid further comprises, consists of, or consists essentially of a third gene involved in the synthesis of coenzyme B12, a fifth promoter that is induced by a small molecule, and a sixth promoter, wherein the fifth and sixth promoters are operably linked in tandem upstream of the third gene such that they regulate expression of the third gene. In some cases, the nucleic acid further comprises, consists of, or consists essentially of a fourth gene involved in the synthesis of coenzyme B12, a seventh promoter that is induced by a small molecule, and a eighth promoter, wherein the seventh and eighth promoters are operably linked in tandem upstream of the

fourth gene such that they regulate expression of the fourth gene. In some instances, the first, second, third, and fourth genes each comprise, consist of, or consist essentially of one or more genes selected from the group consisting of cobJ, cobI, cobH, cobG, cobL, cobF, cobK, gst, xre,chlD, chlI, dahp, cobN, cobW, cbtBA, cobE, cobM, btuB, cobO, cob, cobR, cobD, cobC, cobQ, cobU, cobP, bgpM, and cobV. In some cases, the first gene comprises, consists of, or consists essentially of bgpM.

In some examples of the present disclosure the nucleic acid does not include any native riboswitches that regulate genes involved in the production of coenzyme B12. In some cases, the nucleic acid does not include SEQ ID NOs: 75 or 76.

The disclosure provides for a recombinant bacterium comprising, consisting of, or consisting essentially of the nucleic acid described herein. In some cases, the bacterium is a *Pseudomonas* species. In some instances, the bacterium is *Pseudomonas denitrificans*.

In some examples of the present disclosure, the nucleic acid is on an expression plasmid. In some cases, the nucleic acid is integrated into the bacterium's chromosome. In some instances, the nucleic acid is on an episome.

The disclosure provides for a recombinant bacterium comprising, consisting of, or consisting essentially of a first nucleic acid comprising, consisting of, or consisting essentially of a DhaB expression module and a second nucleic acid comprising, consisting of, or consisting essentially of a ALDH expression module. In some cases, the DhaB expression module comprises, consists of, or consists essentially of any one of the nucleic acids described herein. In some instances, the ALDH expression module comprises, consists of, or consists essentially of any one of the nucleic acids described herein.

In some examples of the present disclosure, the first and the second nucleic acids are on expression plasmids. In some cases, the first and the second nucleic acids are on episomes. In some instances, the first and the second nucleic acids are integrated into the bacterium's chromosome. In some cases, the first nucleic acid is integrated into the bacterium's chromosome and the second nucleic acid is on an expression plasmid. In some instances, the first nucleic acid is on an expression plasmid and the second nucleic acid is integrated into the bacterium's chromosome. In some examples of the present disclosure,

the first nucleic acid is integrated into the bacterium's chromosome and the second nucleic acid is on an episome. In some instances, the first nucleic acid is on an episome and the second nucleic acid is integrated into the bacterium's chromosome.

In some examples of the present disclosure, the first nucleic acid is integrated into the bacterium's chromosome at a first position and the second nucleic acid is integrated into the bacterium's chromosome at a second position; and the first and second positions are within 2500 kilo base pairs of each other. In some cases, the first and second positions are within 100 kilo base pairs of each other. In some instances, the first and second positions are within 50 kilo base pairs of each other. In some cases, the first and second positions are within 1000 base pairs of each other.

In some examples of the present disclosure, the first nucleic acid is integrated into the bacterium's chromosome at a first position that is within 4000 base pairs of the origin of replication. In some cases, the first position is within 1000 base pairs of the origin of replication.

The disclosure provides for a recombinant bacterium comprising, consisting of, or consisting essentially of a coenzyme B12 expression module that comprises, consists of, or consists essentially of a first gene involved in the synthesis of coenzyme B12, a first promoter that is induced by a small molecule, and a second promoter, wherein the first and second promoters are operably linked in tandem upstream of the first gene.

In some examples of the present disclosure, the bacterium is a *Pseudomonas denitrificans* bacterium. In some cases, the bacterium comprises, consists of, or consists essentially of a deletion at a first position of a first riboswitch which regulates expression of the first gene, and the first and second promoters are integrated into the bacterium's chromosome at the first position such that they regulate expression of the first gene. In some instances, the bacterium further comprises, consists of, or consists essentially of a second deletion at a second position of a second riboswitch which regulates expression of a second gene involved in the synthesis of coenzyme B12, a third deletion at a third position of a third riboswitch which regulates expression of a third gene involved in the synthesis of coenzyme B12, and a fourth deletion at a fourth position of a fourth riboswitch which regulates expression of a fourth gene involved in the synthesis of coenzyme B12. In

some cases, the recombinant bacterium further comprises, consists of, or consists essentially of a third promoter and a fourth promoter which are operably linked in tandem and integrated into the chromosome at the second position such that they regulate expression of the second gene; a fifth promoter and a sixth promoter which are operably linked in tandem and integrated into the chromosome at the third position such that they regulate expression of the third gene; and a seventh and eighth promoter which are operably linked in tandem and integrated into the chromosome at the fourth position such that they regulate expression of the fourth gene. In some examples of the present disclosure, the bacterium produces more coenzyme B12 than native *Pseudomonas denitrificans*. In some examples of the present disclosure, the recombinant bacterium described herein further comprises, consists of, or consists essentially of a coenzyme B12 expression module.

The disclosure provides for a method of producing 3-HP or a salt thereof, comprising, consisting of, or consisting essentially of culturing a recombinant bacterium described herein under conditions sufficient to produce 3-HP or a salt thereof. In some cases, the conditions are sufficient to produce at least 85 g/L of 3-HP or a salt thereof. In some instances, the conditions are sufficient to produce at least 90 g/L of 3-HP or a salt thereof. In some examples of the present disclosure, the conditions are sufficient to produce at least 95 g/L of 3-HP or a salt thereof. In some cases, the conditions are sufficient to produce at least 100 g/L of 3-HP or a salt thereof.

In some examples of the present disclosure, culturing the bacterium does not include the addition of external coenzyme B12. In some cases, culturing the bacterium comprises addition external coenzyme B12 to the culture.

In some cases, culturing comprises, consists of, or consists essentially of growing the bacterium in a media comprising a carbon source selected from the group consisting of glycerol, glucose, gluconate, glutamate, and citric acid. In some instances culturing comprising, consisting of, or consisting essentially of inducing the bacterium to produce 3-HP or a salt thereof by adding glycerol. In some cases, the glyercol is add mid-log phase.

In some examples of the present disclosure, culturing comprises, consists of, or consists essentially of growing the bacterium in a media comprising a nitrogen source selected

from the group consisting of yeast extract, corn steep liquor powder, and corn steep liquor paste.

In some examples of the present disclosure, culturing comprises, consists of, or consists essentially of growing the bacterium at a pH between 6.8 and 7.8. In some cases, culturing comprises, consists of, or consists essentially of adding one or more bases selected from the group consisting of NaOH, KOH, $NaHCO_3$, NH_4HCO_3, NH_4OH, $(NH4)_2CO_3$, and Na_2CO_3. In some instances, culturing comprises, consists of, or consists essentially of adding at least one base selected from the group consisting of NaOH, KOH, $NaHCO_3$, NH_4HCO_3, NH_4OH, $(NH_4)_2CO_3$, and Na_2CO_3. In some cases, culturing comprises, consists of, or consists essentially of diluting the base concentration and increasing the acid concentration (see FIG. 46 and FIG. 49).

In some examples of the present disclosure, culturing comprises, consists of, or consists essentially of growing the bacterium at a temperature between 28 and 40 degrees Celsius. In some cases, culturing comprises, consists of, or consists essentially of growing the bacterium at 33 degrees Celsius.

The disclosure provides for a method of producing 3-HP or a salt thereof, the method comprising, consisting of, or consisting essentially of: providing the recombinant bacterium described herein in a culture media suitable for growth of the bacterium and production of 3-HP or a salt thereof. In some cases the method described herein comprises, consists of, or consists essentially of separating 3-HP or a salt thereof from the bacterium or culture media.

In some instances, the culture media comprises, consists of, or consists essentially of a dissolved oxygen concentration that is approximately 2-20% dissolved oxygen. In some examples of the present disclosure the dissolved oxygen concentration is no more than 20%. In some cases, the dissolved oxygen concentration is at least 2%.

Further, this disclosure relates to methods of providing and reacting 3-hydroxypropionic acid.

The disclosure provides methods for removing 3-hydroxypropionic acid (3-HP) from an aqueous solution. The methods can be relatively simple and inexpensive to perform. The methods can provide relatively high yields of 3-HP.

The disclosure also provides methods for making acrylic acid. The methods can be relatively simple and inexpensive to perform. The methods can be performed with little, or no, acrylic acid polymerization taking place. The methods can provide relatively high yields of acrylic acid.

The present disclosure provides a method removing 3-HP from an aqueous solution without using a counter-current liquid flow.

Thepresent disclosure provides a method that includes evaporating a first solvent, condensing the evaporated first solvent, and directing a flow of the first solvent to remove 3-HP from the aqueous solution.

The present disclosure provides a method including using a water-immiscible solvent to remove 3-HP from an aqueous solution with a yield of at least 50%.

The present disclosure provides a method including using an organic liquid containing at least two solvents to remove 3-hydroxypropionic acid (3-HP) from an aqueous solution.

The present disclosure provides a method including using a liquid to remove 3-HP from an aqueous solution, wherein the liquid includes a two different solvents (e.g., a water-miscible solvent and a water-immiscible solvent).

The present disclosure provides a method including removing 3-HP from an aqueous solution, wherein pH of the aqueous solution is at least about 3.

The present disclosure provides a method that includes reacting 3-HP to form acrylic acid at a pressure of less than about one atmosphere.

The present disclosure provides a method that includes reacting 3-HP to form acrylic acid in a reaction mixture, and removing gaseous acrylic acid from the reaction mixture.

The present disclosure provides a method that includes reacting a liquid containing 3-HP to form acrylic acid.

The present disclosure provides a method that includes reacting 3-HP to form acrylic acid with a yield of at least about 70%.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this

CLAIMS

1. An expression system, comprising a first promoter that is either constitutive or inducible by a small molecule, a second promoter that is either constitutive or induced by a small molecule, a first gene encoding a protein involved in the synthesis of 3-HP, a salt of 3-HP, or coenzyme B12, a modified UTR, and a native sequence encoding up to 20 amino acids from the N-terminus of a native Pseudomonas denitrificans protein, wherein the native sequence is operably linked to the 3'-terminus of the modified UTR and operably linked to the 5'-terminus of the first gene, the first and second promoters are operably linked in tandem upstream of the modified UTR, and the modified UTR comprises a sequence selected from the group consisting of SEQ ID NO: 22-28 and 64 (UTR0-6).

2. The expression system of claim 1, further comprising a third promoter; and a second gene encoding a transcriptional regulator configured to regulate expression of the first gene, wherein the third promoter is operably connected to the second gene, and the third promoter comprises a sequence that is 95% identical to the sequence selected from the group consisting of SEQ ID NO: 7-10 and 52-63.

3. The expression system of claim 1, wherein the first promoter is operably linked to the 5'-terminus of the second promoter, the second promoter is operably linked to the 5'-terminus of the modified UTR, and the modified UTR is operably linked to the 5'terminus of the fusion gene.

4. A nucleic acid, comprising a first and a second promoter, wherein the first promoter is an inducible promoter and is inducible by a small molecule; the first and second promoters are operably linked to a first gene; and the first gene encodes a protein involved in the synthesis of 3-hydroxypropionic acid (3-HP), a salt of 3-HP, or coenzyme B12 wherein the nucleic acid comprises a sequence selected from the group consisting of SEQ ID NO:22-28 and 64 (UTR 0-6).

5. The nucleic acid of claim 4, wherein the first gene encodes a protein involved in the synthesis of 3-hydroxypropionic acid (3-HP) and is selected from the group consisting of a glycerol dehydratase, a glycerol dehydratase reactivase, and an aldehyde dehydrogenase,

wherein:

the glycerol dehydratase is selected from the group consisting of dhaB1, dhaB2, dhaB3;

the glycerol dehydratase reactivase is selected from the group consisting of gdrA and gdrB; and

the aldehyde dehydrogenase is selected from the group consisting of kgsA.

6. The nucleic acid of claim 4, wherein the first gene comprises a dhaB1 gene, a dhaB2 gene, a dhaB3 gene, a gdrA gene, and a gdrB gene, and the sequence comprises a sequence that is at least 95% identical to a SEQ ID selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 5 and SEQ ID NO: 6, and/or

wherein the small molecule acid or alcohol is selected from the group consisting of L-lactic acid (LAC), acetic acid (AcOH), propionic acid (PA), 3-hydroxypropionic acid (3-HP), 3-hydroxybutyrate (3-HB), 1,3-propanediol (1,3-PDO), 2,3-butanediol (2,3-BDO), L-valine (L-val), and 3-hydroxyisobutyrate (3-HIB), and/or

wherein the second promoter is derived from a PmmsA promoter, a PhbdH-1 promoter, a PhbdH-4 promoter, a PhpdH promoter, or a Pzwf promoter, and the promoter comprises a sequence that it at least 95% identical to a SEQ ID selected from the group consisting of: SEQ ID NO: 7; SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 10; SEQ ID NO: 11; SEQ ID NO: 12; SEQ ID NO: 13; SEQ ID NO: 14; SEQ ID NO: 15; SEQ ID NO: 16; SEQ ID NO: 17; SEQ ID NO: 18; SEQ ID NO: 19; SEQ ID NO: 52-63; and SEQ ID NO: 65-72.

7. The nucleic acid of claim 4, wherein the nucleic acid further comprises a gene encoding a transcriptional regulator that regulates expression of the first gene, wherein the transcriptional regulator binds to the first or second promoter,

optionally wherein the transcriptional regulator is a LysR-type transcriptional regulator (LTTR), a MmsR regulator or a HpdR regulator

8. The nucleic acid of claim 4, wherein the first gene is fused at its 5'-terminus to a sequence encoding up to 20 (5, 10, 15, or 20) amino acids that are derived from the 5'terminus of a second gene encoding a native Pseudomonas denitrificans protein, thereby creating a fusion gene, and the second promoter is derived from the native promoter for the second gene, and the fusion gene comprises a sequence encoding at least 5, 10, 15, or 20 amino acids in the N-terminus of a native protein, and/or

wherein the second promoter comprises the PmmsA promoter and the fusion gene comprises a sequence encoding five or more amino acids from the N-terminus of a native MmsA gene, and the fusion gene is operably linked to the 3' end of the PmmsA promoter, and wherein the nucleic acid comprises a sequence selected from the group consisting of SEQ ID NOs: 32-35,

optionally wherein the mRNA of the fusion gene has a higher stability and increases the gene translation when compared to the first gene alone.

9. A nucleic acid, comprising a first and a second promoter, wherein either the first is an inducible promoter and is inducible by a small molecule; the first and second promoters are operably linked to a first gene; and the first gene encodes a protein involved in the synthesis of 3-hydroxypropionic acid (3-HP), a salt of 3-HP, or coenzyme B12, wherein a second 10 codons at the 5'-terminus of the first gene are optimized for translation in Pseudomonas denitrificans and a codon frequency for each amino acid in the optimized second 10 codons is more than 0.

10. The nucleic acid of claims 9, wherein codon optimization comprises:

measuring a codon frequency of each amino acid in a gene encoding a native Pseudomonas denitrificans protein; and

using the codon frequency of the native proteins to replace the second 10 codons at the 5'-terminus of the first gene with an optimized 10 codons, wherein the codon for each amino acid of the optimized 10 codons is present at the same frequency as the codon frequency of the native proteins,

optionally wherein the nucleic acid comprises a sequence that is 95% identical to a sequence selected from the group consisting of SEQ ID Nos: 29, 30, and 31 (Opt1-3).

11. A method, comprising:

evaporating a first solvent;

condensing the evaporated first solvent; and

directing a flow of the first solvent to remove 3-HP from the aqueous solution,

wherein the 3-HP is removed from the aqueous solution without using a counter-current liquid flow.

12. The method of claim 11, wherein the first solvent has density of less than about 1 g/mL, or wherein the first solvent has a density of greater than about 1 g/mL.

13. The method of claim 11, wherein the first solvent comprises a water-immiscible solvent.

14. The method of claim 11, wherein the pH of the aqueous solution is from about 3 to about 7.

15. The method of claim 11, wherein the first solvent comprises at least one solvent selected from the group consisting of a C1-6 alkyl acetate, a C4-6 alcohol, and a C1-6 alkyl ether, and wherein the first solvent comprises at least one solvent selected from the group consisting of methyl acetate, ethyl acetate, n-propyl acetate, isopropyl acetate, n-butyl acetate, isobutyl acetate, sec-butyl acetate, tert-butyl acetate, n-pentyl acetate, and n-hexyl acetate, or

wherein the first solvent comprises at least one solvent selected from the group consisting of n-butyl alcohol, isobutyl alcohol, tert-butyl alcohol, n-pentyl alcohol, n-hexyl alcohol, diethyl ether, dipropyl ether, ethyl propyl ether, methyl tert-butyl ether, methyl hexyl ether, chloroform, methylene chloride and carbon tetrachloride

16. The method of claim 1, comprising using a second solvent to remove 3-HP from the aqueous solution,

optionally wherein the second solvent comprises a water-miscible solvent.

17. The method of claim 16, wherein the first solvent comprises a water-immiscible solvent, and the second solvent comprises a water-miscible solvent.

18. The method of claim 16, wherein the second solvent comprises a C1-3 alcohol,

optionally, wherein the second solvent comprises at least one solvent selected from the group consisting of methanol, ethanol, n-propanol, and isopropanol.

19. The method of claim 16, wherein an amount of the second solvent is from about 5 v/v% to about 50 v/v% of an amount of the aqueous solution,

optionally wherein an amount of the second solvent is about 20 v/v% of an amount of the aqueous solution.

20. The method of claim 16, wherein a ratio of 1) a combined volume of the first and second solvents to 2) a volume of the aqueous solution is in from 1:1 to about 2:1.