93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more identical to SEQ ID NO:2, 4, 6, 8, 10, and/or 12. In some embodiments of the methods, the penicillin G acylase is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more identical to SEQ ID NO:2, 4, 6, 8, 10, and/or 12. In some further embodiments of the methods, , the penicillin G acylase comprises SEQ ID NO:2, 4, 6, 8, 10, and/or 12. In some embodiments, the engineered penicillin G acylase produces more than 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more free insulin. The present invention also provides compositions comprising free insulin produced according to the method(s) provided herein.

[0009A] The invention also provides an engineered variant penicillin G acylase capable of removing the A1/B1/B29 tri-phenyl acetate protecting groups from insulin to produce free insulin, wherein said engineered variant penicillin G acylase lacks the 54 amino acid spacer region of the wild-type penicillin G acylase of SEQ ID NO:1; wherein said engineered variant is at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to SEQ ID NO:2, 4, 6, 8, 10, and/or 12; and wherein said engineered variant has a Fold Improvement Over Positive Control (FIOPC) of at least 0.7.

[0009B] The invention also provides a vector comprising the polynucleotide sequence of the invention.

[0009C] The invention also provides a host cell comprising the vector of the invention.

[0009D] The invention also provides a method for producing free insulin, comprising: i) providing the engineered variant of the invention, and insulin comprising A1/B1/B29 tri-phenyl acetate protecting groups; and ii) exposing said engineered variant to said insulin comprising A1/B1/B29 tri-phenyl acetate protecting groups, under conditions such that said engineered variant removes the A1/B1/B29 tri-phenyl acetate protecting groups and free insulin is produced.

[0009D] The invention also provides a composition comprising free insulin produced according to the method of any one of the invention.

BRIEF DESCRIPTION OF THE FIGURES

[0010] Figure 1 provides a graph showing the substrate inhibition activity observed with Variant 1.

[0011] Figure 2 provides a graph showing the amount of free insulin produced using seven variant PGAs.

[0012] Figure 3 provides a graph showing % yield of free insulin produced using three variant PGAs.

[0013] Figure 4 provides a graph showing % yield of free insulin produced using three variant PGAs in the presence of DMSO in the reactions.

DESCRIPTION OF THE INVENTION

[0014] The present invention provides engineered penicillin G acylases (PGA) that are capable of cleaving penicillin to phenylacetic acid and 6-aminopenicillanic acid (6-APA), which is a key intermediate in the synthesis of a large variety of β-lactam antibiotics. In particular, the present invention provides engineered PGAs that are capable of removing the A1/B1/B29 tri-phenyl acetate protecting groups to release free insulin.

[0015] Generally, naturally occurring PGAs are heterodimeric enzymes composed of an alpha subunit and a beta-subunit. Wild-type PGA is naturally synthesized as a pre-pro-PGA polypeptide, containing an N-terminal signal peptide that mediates translocation to the periplasm and a linker region connecting the C-terminus of the alpha subunit to the N-terminus of the beta subunit. Proteolytic processing leads to the mature heterodimeric enzyme. The intermolecular linker region can also function in promoting proper folding of the enzyme. The PGAs described herein are based on the PGA from *Kluyvera citrophila* in which various modifications have been introduced to generate improved enzymatic properties as described in detail below.

[0016] For the descriptions provided herein, the use of the singular includes the plural (and vice versa) unless specifically stated otherwise. For instance, the singular forms “a”, “an” and “the” include plural referents unless the context clearly indicates otherwise. Similarly, “comprise,” “comprises,” “comprising” “include,” “includes,” and “including” are interchangeable and not intended to be limiting. It is to be further understood that where descriptions of various embodiments

The Claims defining the Invention are as follows:

1. An engineered variant penicillin G acylase capable of removing the A1/B1/B29 tri-phenyl acetate protecting groups from insulin to produce free insulin, wherein said engineered variant penicillin G acylase lacks the 54 amino acid spacer region of the wild-type penicillin G acylase of SEQ ID NO:1; wherein said engineered variant is at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to SEQ ID NO:2, 4, 6, 8, 10, and/or 12; and wherein said engineered variant has a Fold Improvement Over Positive Control (FIOPC) of at least 0.7.

2. The engineered variant of Claim 1, wherein said engineered variant comprises at least one mutation as provided in Table 5.1, Table 6.2, and/or Table 6.3.

3. The engineered variant of Claim 1, wherein engineered variant comprises SEQ ID NO:4, 6, 8, 10, or 12.

4. The engineered variant of Claim 1, wherein said engineered variant is encoded by a polynucleotide sequence selected from SEQ ID NOS:3, 5, 7, 9, and 11.

5. A vector comprising the polynucleotide sequence of Claim 4.

6. A host cell comprising the vector of Claim 5.

7. A method for producing free insulin, comprising: i) providing the engineered variant of Claim 1, and insulin comprising A1/B1/B29 tri-phenyl acetate protecting groups; and ii) exposing said engineered variant to said insulin comprising A1/B1/B29 tri-phenyl acetate protecting groups, under conditions such that said engineered variant removes the A1/B1/B29 tri-phenyl acetate protecting groups and free insulin is produced.

8. The method of Claim 7, wherein said engineered variant produces more than 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more free insulin.

9. The method of Claim 7 or Claim 8, wherein said engineered variant comprises SEQ ID NO:4, 6, 8, 10, or 12.

10. A composition comprising free insulin produced according to the method of any one of Claims 7 to 9.