CLAIMS

We claim:

1. An engineered penicillin G acylase variant having 95% or more sequence identity to SEQ ID NO:6, and a substitution at two ore more positions to include position 254 and positin 255 and at least one additional substitution selected from the group consisting of 54C, 62G, 115A/P, 125L/T, 127S/V, 185V, 253K/V, 256Q, 257I, 257V, 260A/P, 268S/V, 322P, 325G, 348C, 348Q, 369L, 369P, 369V, 369W, 370F/G/S, 372A/H/L, 373F/M, 377P, 378H, 384A, 384F/513Q/536M, 384G/L, 388T, 389L, 391P/S, 435R, 461A, 517L/P, 530C/Y, 554A/E/P/V, 556G, 557G/S, 559P/S, 560I, 600T/623V, 623A/G/R/W, 624A, 626G, 627G/H, 705G/P, 706G, 707S, 723A/G, 740L, 748G, and 752E, and wherein said engineered penicillin G acylase variant is capable of deacylating a di or tetra-protected insulin dimer, wherein said positions are numbered with reference to SEQ ID NO:6.

2. The engineered penicillin G acylase variant of Claim 1, wherein said engineered penicillin G acylase variant comprises a histidine tag.

3. The engineered penicillin G acylase variant of Claim 2, wherein said histidine tag is present at the C-terminus of said engineered penicillin G acylase variant.

4. A method for producing free insulin, comprising i) providing at least one engineered penicillin G acylase variant of Claim 1, and a composition comprising insulin-dimer comprising A1/B1/ A1'/B1'-tetra-phenyl acetate protecting groups; and ii) exposing the engineered penicillin G acylase to the insulin-dimer comprising A1/B1/A1'/B1'- tetra-phenyl acetate protecting groups, under conditions such that the engineered penicillin G acylase variant removes the A1/B1/ A1'/B1'-tetra-phenyl acetate protecting groups and free insulin is produced.

5. A method for producing free insulin, comprising i) providing at least one engineered penicillin G acylase variant of Claim 1, and a composition comprising insulin-dimer comprising A1/A1'-di-phenyl acetate protecting groups; and ii) exposing the engineered penicillin G acylase variant to the insulin-dimer comprising A1/A1'- di-phenyl acetate protecting groups, under conditions such that the engineered penicillin G acylase variant removes the A1/A1'-di-phenyl acetate protecting groups and free insulin is produced.

6. A method for producing free insulin, comprising i) providing at least one engineered penicillin G acylase variant of Claim 1, and a composition comprising insulin comprising a A1/A1'-di-phenyl acetate protecting groups; and ii) exposing the engineered penicillin G acylase variant to the insulin comprising A1/A1'- di-phenyl acetate protecting groups, under conditions such that the engineered penicillin G acylase variant removes the A1/A1'-di-phenyl acetate protecting groups and free insulin is produced.