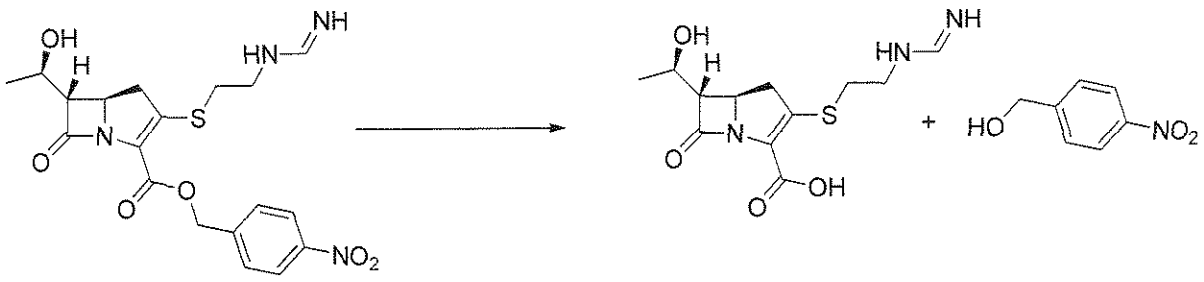
particular, the engineered pNB esterase polypeptides of the present disclosure have been engineered for efficient removal a pNB protecting group from the imipenem precursor compound of compound **(2)** thereby converting it to the product compound **(1)**, imipenem, as shown in Scheme 1.

Scheme 1



(2) **(1)**

[0010] The amino residue differences are located at residue positions affecting various enzyme properties, including among others, activity, stability, product selectivity, and product tolerance. These amino residue differences, although evolved for the removal of pNB protecting groups from the imipenem precursor of compound **(2)**, can also be used to evolve engineered pNB esterase polypeptides having activity useful for removing pNB protecting groups of structurally analogous carbapenem precursor compounds including, but not limited to, pNB-protected precursor compounds of thienamycin meropenem, doripenem, ertapenem, biopenem, and panipenem.

[0011] In one aspect, there is provided an engineered polypeptide having para-nitrobenzyl (pNB) esterase activity, comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 2 and one or more residue differences as compared to SEQ ID NO:2 at residue positions selected from: X108, X115, X116, X130, ~~X193,~~ X214, ~~X219, X273,~~ X276, X321, and X362, further comprising residue differences at positions X193, X219 and X273 selected from X193A/D/E/V, X219L/V and X273A/V. In some embodiments, the residue differences as compared to SEQ ID NO: 2 at the residue positions X108, X115, X116, X130, X193, X214, X219, X273, X276, X321, and X362 are selected from X108L/Y, X115Q/W, X116S, X130T, X164T, X193A/D/E/V, X214G, X219A/D/L/V, X273A/E/T/V, X276A/T/L, X321A, and X362A/D/Q/S/V.

[0012] In some embodiments, the engineered polypeptide having pNB esterase activity comprises an amino acid sequence having at least 80% identity to SEQ ID NO: 2 and one or more residue differences as compared to SEQ ID NO: 2 selected from: X108L/Y, X193A/D/E/V, X219A/D/L/V, X273A/E/T/V, and X362A/D/Q/S/V.

[0013] In some embodiments, the engineered polypeptide having pNB esterase activity comprises an amino acid sequence having at least 80% identity to SEQ ID NO: 2 and a residue difference as compared to SEQ ID NO: 2 at position X193 selected from: X193A/D/E/V. In some embodiments, the amino acid residue difference as compared to SEQ ID NO: 2 at position X193 is X193V.

polypeptides of **Table 2**), but in which the amino acid sequence does not comprise a residue difference as compared to SEQ ID NO: 2 at positions X60, X144, X317, X322, X334, X358, and X370.

[0019] In some embodiments, the engineered polypeptides having pNB esterase activity of the present disclosure having at least 80% identity to SEQ ID NO: 2 and any of the amino acid residue differences disclosed herein, further comprises at least 1.2 fold, 2 fold, 5 fold, 10 fold, 20 fold, 25 fold, 50 fold, 75 fold, 100 fold, or greater increased activity as compared to the polypeptide of SEQ ID NO: 4 in converting compound **(2)** to compound **(1)** under suitable reaction conditions.

[0020] In some embodiments, the engineered polypeptides having pNB esterase activity comprises an amino acid sequence having at least 80%, 85%, 87%, 88%, 89%, 90% 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to a sequence selected from SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38,40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120.

[0021] In some embodiments, that disclosure further provides any of the engineered polypeptides having pNB esterase activity as disclosed herein, wherein the polypeptide is immobilized on a solid support. In some embodiments, the solid support is a bead or resin comprising polymethacrylate with epoxide functional groups, polymethacrylate with amino epoxide functional groups, styrene/DVB copolymer or polymethacrylate with octadecyl functional groups

[0022] *Exemplary engineered polypeptides having pNB esterase activity and amino acid sequences* incorporating the residue differences disclosed herein, including various combinations thereof, and having improved properties (*e.g.*, capable of converting compound **(2)** to compound **(1)** under suitable reaction conditions) are disclosed in **Table 2**, and the Examples. The amino acid sequences are provided in the Sequence Listing and include SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38,40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120.

[0023] The present disclosure also provides polynucleotides encoding the engineered polypeptides having pNB esterase activity, as well as expression vectors comprising the polynucleotides, and host cells capable of expressing the polynucleotides encoding the engineered polypeptides.

In one aspect, there is provided a polynucleotide encoding the engineered pNB esterase polypeptide as defined herein.

In one aspect, there is provided a polynucleotide encoding the engineered pNB esterase polypeptide as defined herein comprising a nucleotide sequence selected from SEQ ID NO: ~~3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41,~~ 43, 45, ~~47, 49, 51.~~ 53, ~~55, 57, 59, 61, 63,~~ 65, 67, ~~69, 71,~~ 73, 75, ~~77,~~ 79, 81, ~~83, 85,~~ 87, 89, 91, 93, 95, 97, 99, 101, ~~103,~~ 105, 107, 109, 111, 113, 115, 117, and 119. Exemplary polynucleotide sequences are provided in the Sequence Listing incorporated

What is claimed is:

1. An engineered polypeptide having para-nitrobenzyl (pNB) esterase activity, comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 2 and one or more residue differences as compared to SEQ ID NO:2 at residue positions selected from: X108, X115, X116, X130, X214, X276, X321, and X362, further comprising residue differences at positions X193, X219 and X273 selected from X193A/D/E/V, X219L/V and X273A/V.

2. The engineered polypeptide of **claim** 1 in which the residue differences as compared to SEQ ID NO: 2 at the residue positions X108, X115, X116, X130, X214, X276, X321, and X362 are selected from X108L/Y, X115Q/W, X116S, X130T, X214G, X276A/T/L, X321A, and X362A/D/Q/S/V.

3. The engineered polypeptide of **claim** 1 or 2 in which the amino acid sequence comprises one or more residue differences as compared to SEQ ID NO: 2 selected from: X108L/Y, and X362A/D/Q/S/V.

4. The engineered polypeptide of **claims 1 or 2** in which the amino acid sequence further comprises residue differences as compared to SEQ ID NO: 2 at positions X108 and X362 selected from X108L/Y and X362A/D/Q/S/V.

5. The engineered polypeptide of **claim 4** in which the amino acid sequence further comprises a residue difference as compared to SEQ ID NO: 2 at position X115 selected from X115Q/W.

6. The engineered polypeptide of **claim 2** in which the amino acid sequence comprises a combination of residue differences as compared to SEQ ID NO: 2 selected from:
   X193V, X219V, and X273A;
   X108Y, X193D, X219V, X273A, and X362S;
   X108Y, X193V, X219V, X273A, and X362Q;
   X108Y, X115Q, X193V, X219L, X273A, and X362Q; and
   X108Y, X115Q, X193V, X219V, X273A, and X362Q.

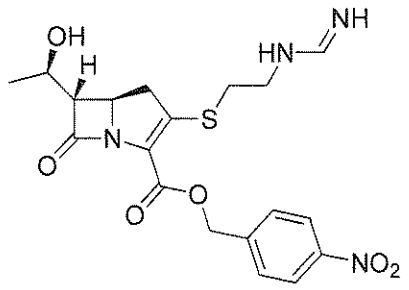
7. The engineered polypeptide of any one of **claims** 1 to **6** in which the amino acid sequence further comprises one or more residue differences as compared to SEQ ID NO: 2 selected from: X116S, X130T, X164T, X214G, X276A/T/L, and X321A.

8. The engineered polypeptide of any one of **claims** 1 to 7in which the amino acid sequence further

comprises a residue difference as compared to SEQ ID NO: 2 selected from: X49G, X94G, X96S, X227T, X251V, X267R, X271L, X274L, X313F, X322C/Y, X343V, X356R, X359A, X398L, X412E, X437T, X464A, and X481R.

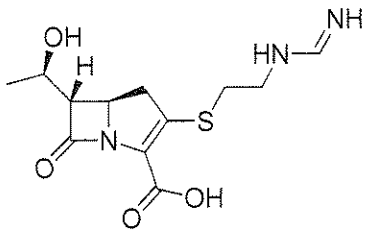
9. The engineered polypeptide of any one of **claims** 1 to **8** in which the amino acid sequence does not comprise a residue difference as compared to SEQ ID NO: **2** at positions X60, X144, X317, X322, X334, X358, and X370.

10. The engineered polypeptide of **claim 2** in which the pNB esterase has at least 1.2 fold, 2 fold, 5 fold, 10 fold, 20 fold, 25 fold, 50 fold, 75 fold, 100 fold, or greater increased activity as compared to the polypeptide of SEQ ID NO: 4 in converting compound **(2);**



(2)

to compound **(1);**



(1)

under suitable reaction conditions.

11. The engineered polypeptide of **claim 1** in which the amino acid sequence comprises a sequence selected from SEQ ID NO: 44, 46, 54, 66, 68, 74, 76, 80, 82, 88, 90, 92, 94, 96, 98, 100, 102, 106, 108, 110, 112, 114, 116, 118, and 120.

12. The engineered polypeptide of any one of **claims** 1 to **11** in which the polypeptide is immobilized on a solid support.

13. The engineered polypeptide of **claim 12** in which the solid support is a bead or resin comprising

polymethacrylate with epoxide functional groups, polymethacrylate with amino epoxide functional groups, styrene/DVB copolymer or polymethacrylate with octadecyl functional groups.

14. A polynucleotide encoding the engineered pNB esterase polypeptide of any one of **claims** 1 to **11**.

15. A polynucleotide encoding the engineered pNB esterase polypeptide of **claim** 1, comprising a nucleotide sequence selected from SEQ ID NO: 43, 45, 53, 65, 67, 73, 75, 79, 81, 87, 89, 91, 93, 95, 97, 99, 101, 105, 107, 109, 111, 113, 115, 117, and 119.

16. An expression vector comprising the polynucleotide of **claim 14** or **15**.

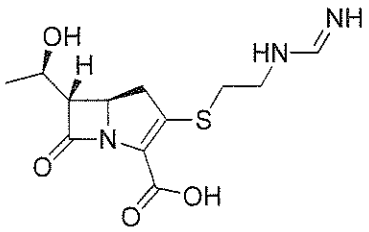
17. The expression vector of **claim 16** comprising a control sequence.

18. A host cell comprising the polynucleotide of **claim 20** or **21** or the expression vector of **claim 16** or **17**.

19. A method of preparing the engineered polypeptide of any one of **claims** 1 to **11**, comprising culturing the host cell of **claim 18** under conditions suitable for expression of the polypeptide.

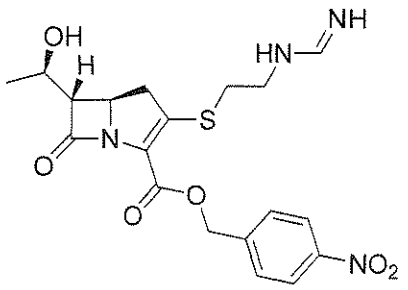
20. The method of **claim 19**, further comprising isolating the engineered polypeptide.

21. A process for preparing compound **(1)**, or a salt or hydrate of compound **(1)**



(1)

wherein the method comprises contacting a substrate compound **(2)**, or a salt or hydrate of compound **(2)**



(2)

with an engineered pNB esterase polypeptide of any one of **claims** 1 to **13** under suitable reaction conditions.

22. The process of **claim 21** in which the substrate of compound **(2)** is at a loading of from 1 to 200 g/L, from 2 to 100 g/L, or 5 to 50 g/L.

23. The process of **claim 21** in which the substrate of compound **(2)** is at a loading of at least 0.5 g/L, 1 g/L, 5 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L, 100 g/L, 150 g/L, or 200 g/L.

24. The process of **claim 21** in which the suitable reaction conditions comprise a buffer selected from borate, phosphate, carbonate, triethanolamine (TEA), Tris, and 2-(N-morpholino)ethanesulfonic acid (MES).

25. The process of **claim 24** in which the buffer comprises MES, wherein the MES concentration is 0.01 to 0.4 M, 0.05 to 0.4 M, 0.1 to 0.3 M, or 0.1 to 0.2 M.

26. The process of **claim 25** in which the MES concentration is about 0.01 M, 0.02 M, 0.03 M, 0.04 M, 0.05 M, 0.07 M, 0.1 M, 0.12 M, 0.14 M, 0.16 M, 0.18 M, 0.2 M, 0.3 M, or 0.4 M.

27. The process of **claim 21** in which the suitable reaction conditions comprise a pH from 5 to 10, pH from 6 to 9, or pH from 7 to 8.

28. The process of **claim 27** in which the suitable reaction conditions comprise a pH of about 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10.

29. The process of **claim 27** in which the suitable reaction conditions comprise a temperature of 5°C to 65°C, 10°C to 60°C, 15°C to 55°C, 15°C to 45°C, 15°C to 35°C, 20°C to 55°C, or 30°C to 60°C.

30. The process of **claim 29** in which the suitable reaction conditions comprise a temperature of about 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, or 70°C.

31. The process of **claim 21** in which the suitable reaction conditions comprise dimethylformamide (DMF) as a co-solvent present at a concentration of from 2% to 30% v/v; or from 5% to 20% v/v.

32. The process of **claim 31** in which the DMF is present at a concentration of about 2% v/v, 5% v/v, 10% v/v, 15% v/v, 20% v/v, 25% v/v, or 30% v/v.

33. The process of **claim 21** in which the pNB esterase polypeptide is at a concentration of 0.01 to 50 g/L, 0.05 to 50 g/L, 0.1 to 40 g/L, 1 to 40 g/L, 2 to 40 g/L, 5 to 40 g/L, 5 to 30 g/L, 0.1 to 10 g/L, 0.5 to 10 g/L, 1 to 10 g/L, 0.1 to 5 g/L, 0.5 to 5 g/L, or 0.1 to 2 g/L.

34. The process of **claim 33** in which the pNB esterase polypeptide is at a concentration of about 0.01 g/L, 0.05 g/L, 0.1 g/L, 0.2 g/L, 0.5 g/L, 1 g/L, 2 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, or 50 g/L.

35. The process of **claim 21** in which the suitable reaction conditions comprise:

(a) substrate loading at 2 g/L to 200 g/L;

(b) 0.1 to 10 g/L of engineered pNB esterase polypeptide;

(c) 0.05 to 0.5 M MES buffer;

(d) 5% to 20% (v/v) DMF co-solvent;

(e) pH of 6 to 8; and

(f) temperature of 10 to 35°C.

36. The process of **claim 21** in which the suitable reaction conditions comprise:

(a) substrate loading at 5 g/L to 100 g/L;

(b) 2 to 5 g/L of engineered pNB esterase polypeptide;

(c) about 0.1 M MES buffer;

(d) about 15% (v/v) DMF co-solvent;

(e) pH of about 7; and

(f) temperature of about 15°C.

What is claimed is:

1. An engineered polypeptide having pNB esterase activity, comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 2 and one or more residue differences as compared to SEQ ID NO:2 at residue positions selected from: X108, X115, X116, X130, X193, X214, X219, X273, X276, X321, and X362.

2. The engineered polypeptide of **claim** 1 in which the residue differences as compared to SEQ ID NO: 2 at the residue positions X108, X115, X116, X130, X193, X214, X219, X273, X276, X321, and X362 are selected from X108L/Y, X115Q/W, X116S, X130T, X164T, X193A/D/E/V, X214G, X219A/D/L/V, X273A/E/T/V, X276A/T/L, X321A, and X362A/D/Q/S/V.

3. The engineered polypeptide of **claim** 1 or 2 in which the amino acid sequence comprises one or more residue differences as compared to SEQ ID NO: 2 selected from: X108L/Y, X193A/D/E/V, X219A/D/L/V, X273A/E/T/V, and X362A/D/Q/S/V.

4. The engineered polypeptide of **claim** 1 or **2** in which the amino acid sequence comprises a residue difference as compared to SEQ ID NO: 2 at position X193 selected from: X193A/D/E/V.

5. The engineered polypeptide of **claim 4** in which the amino acid residue difference as compared to SEQ ID NO: 2 at position X193 is X193V.

6. The engineered polypeptide of **claim 5** in which the amino acid sequence further comprises residue differences as compared to SEQ ID NO: 2 at positions X219 and X273 selected from X219L/V and X273A/V.

7. The engineered polypeptide of **claim 6** in which the amino acid sequence further comprises residue differences as compared to SEQ ID NO: 2 at positions X108 and X362 selected from X108L/Y and X362A/D/Q/S/V.

8. The engineered polypeptide of **claim 7** in which the amino acid sequence further comprises a residue difference as compared to SEQ ID NO: 2 at position X115 selected from X115Q/W.

9. The engineered polypeptide of **claim 2** in which the amino acid sequence comprises a combination of residue differences as compared to SEQ ID NO: 2 selected from:

   X193V, X219V, and X273A;

   X108Y, X193D, X219V, X273A, and X362S;

   X108Y, X193V, X219V, X273A, and X362Q;

X108Y, X115Q, X193V, X219L, X273A, and X362Q; and
X108Y, X115Q, X193V, X219V, X273A, and X362Q.

10. The engineered polypeptide of any one of **claims** 1 to **9** in which the amino acid sequence further comprises one or more residue differences as compared to SEQ ID NO: 2 selected from: X116S, X130T, X164T, X214G, X276A/T/L, and X321A.

11. The engineered polypeptide of any one of **claims** 1 to **10** in which the amino acid sequence further comprises a residue difference as compared to SEQ ID NO: 2 selected from: X49G, X94G, X96S, X227T, X251V, X267R, X271L, X274L, X313F, X322C/Y, X343V, X356R, X359A, X398L, X412E, X437T, X464A, and X481R.

12. The engineered polypeptide of any one of **claims** 1 to **11** in which the amino acid sequence does not comprise a residue difference as compared to SEQ ID NO: **2** at positions X60, X144, X317, X322, X334, X358, and X370.

13. The engineered polypeptide of **claim 2** in which the pNB esterase has at least 1.2 fold, 2 fold, 5 fold, 10 fold, 20 fold, 25 fold, 50 fold, 75 fold, 100 fold, or greater increased activity as compared to the polypeptide of SEQ ID NO: 4 in converting compound **(2)** to compound **(1)** under suitable reaction conditions.

14. The engineered polypeptide of **claim 1** in which the amino acid sequence comprises a sequence selected from SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38,40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120.

15. The engineered polypeptide of any one of **claims** 1 to **14** in which the polypeptide is immobilized on a solid support.

16. The engineered polypeptide of **claim 15** in which the solid support is a bead or resin comprising polymethacrylate with epoxide functional groups, polymethacrylate with amino epoxide functional groups, styrene/DVB copolymer or polymethacrylate with octadecyl functional groups.

17. A polynucleotide encoding the engineered pNB esterase polypeptide of any one of **claims** 1 to **14**.

18. A polynucleotide encoding the engineered pNB esterase polypeptide of **claim** 1, comprising a nucleotide sequence selected from SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29,

31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, and 119.

19. An expression vector comprising the polynucleotide of **claim 17** or **18**.

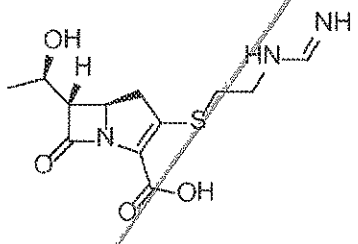
20. The expression vector of **claim 19** comprising a control sequence

21. A host cell comprising the polynucleotide of **claim 17** or **18** or the expression vector of **claim 19** or **20**.

22. A method of preparing the engineered polypeptide of any one of **claims 1** to **14**, comprising culturing the host cell of **claim 21** under conditions suitable for expression of the polypeptide.

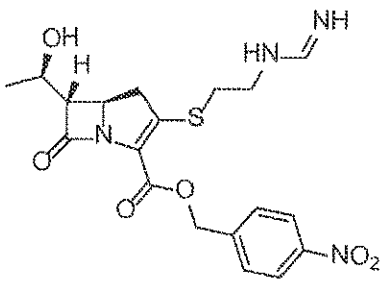
23. The method of **claim 22**, further comprising isolating the engineered polypeptide.

24. A process for preparing compound **(1)**, or a salt or hydrate of compound **(1)**



(1)

wherein the method comprises contacting a substrate compound **(2)**, or a salt or hydrate of compound **(2)**



(2)

with an engineered pNB esterase polypeptide of any one of **claims 1** to **16** under suitable reaction conditions.

25. The process of **claim 24** in which the substrate of compound **(2)** is at a loading of from about 1 to

about 200 g/L, from about 2 to about 100 g/L, or about 5 to about 50 g/L.

26. The process of **claim 25** in which the substrate of compound **(2)** is at a loading of at least about 0.5 g/L, 1 g/L, 5 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L, 100 g/L, 150 g/L, or 200 g/L.

27. The process of **claim 24** in which the suitable reaction conditions comprise a buffer selected from borate, phosphate, carbonate, triethanolamine (TEA), Tris, and 2-(N-morpholino)ethanesulfonic acid (MES).

28. The process of **claim 27** in which the buffer comprises MES, wherein the MES concentration is about 0.01 to about 0.4 M, about 0.05 to about 0.4 M, about 0.1 to about 0.3 M, or about 0.1 to about 0.2 M.

29. The process of **claim 28** in which the MES concentration is about 0.01 M, 0.02 M, 0.03 M, 0.04 M, 0.05 M, 0.07 M, 0.1 M, 0.12 M, 0.14 M, 0.16 M, 0.18 M, 0.2 M, 0.3 M, or 0.4 M.

30. The process of **claim 24** in which the suitable reaction conditions comprise a pH from about 5 to about 10, pH from about 6 to about 9, or pH from about 7 to about 8.

31. The process of **claim 30** in which the suitable reaction conditions comprise a pH of about 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10.

32. The process of **claim 24** in which the suitable reaction conditions comprise a temperature of about 5°C to about 65°C, about 10°C to about 60°C, about 15°C to about 55°C, about 15°C to about 45°C, about 15°C to about 35°C, about 20°C to about 55°C, or about 30°C to about 60°C.

33. The process of **claim 32** in which the suitable reaction conditions comprise a temperature of about 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, or 70°C.

34. The process of **claim 24** in which the suitable reaction conditions comprise dimethylformamide (DMF) as a co-solvent present at a concentration of from about 2% to about 30% v/v; or from about 5% to about 20% v/v.

35. The process of **claim 34** in which the DMF is present at a concentration of about 2% v/v, 5% v/v, 10% v/v, 15% v/v, 20% v/v, 25% v/v, or 30% v/v.

36. The process of **claim 24** in which the pNB esterase polypeptide is at a concentration of about 0.01

to about 50 g/L, about 0.05 to about 50 g/L, about 0.1 to about 40 g/L, about 1 to about 40 g/L, about 2 to about 40 g/L, about 5 to about 40 g/L, about 5 to about 30 g/L, about 0.1 to about 10 g/L, about 0.5 to about 10 g/L, about 1 to about 10 g/L, about 0.1 to about 5 g/L, about 0.5 to about 5 g/L, or about 0.1 to about 2 g/L.

37. The process of **claim 36** in which the pNB esterase polypeptide is at a concentration of about 0.01 g/L, 0.05 g/L, 0.1 g/L, 0.2 g/L, 0.5 g/L, 1 g/L, 2 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, or 50 g/L.

38. The process of **claim 24** in which the suitable reaction conditions comprise:

(a) substrate loading at about 2 g/L to 200 g/L;

(b) about 0.1 to 10 g/L of engineered pNB esterase polypeptide;

(c) about 0.05 to 0.5 M MES buffer;

(d) about 5% to about 20% (v/v) DMF co-solvent;

(e) pH of about 6 to 8; and

(f) temperature of about 10 to 35°C.

39. The process of **claim 24** in which the suitable reaction conditions comprise:

(a) substrate loading at about 5 g/L to 100 g/L;

(b) about 2 to 5 g/L of engineered pNB esterase polypeptide;

(c) about 0.1 M MES buffer;

(d) about 15% (v/v) DMF co-solvent;

(e) pH of about 7; and

(f) temperature of about 15°C.