AOX1 promoters described herein can be used for efficient expression of operably linked coding sequences in *Pichia*, for example.

In one example, provided herein is a nucleic acid construct comprising a first alcohol oxidase promoter element, wherein the first alcohol oxidase promoter element includes a mutation at one or more nucleotide positions corresponding to any of nucleotide positions 668-734 relative to SEQ ID NO: 28.

Implementations can have one or more of the following features. The first alcohol oxidase promoter element can include a mutation at one or more nucleotide positions corresponding to any of nucleotide positions 673-729 relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include a mutation at one or more nucleotide positions corresponding to any of nucleotide positions 678-724 relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include a mutation at one or more nucleotide positions corresponding to any of nucleotide positions 683-719 relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include a mutation at one or more nucleotide positions corresponding to any of nucleotide positions 688-714 relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include two or more mutations at nucleotide positions corresponding to any of nucleotide positions 668-734 relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include three or more mutations at nucleotide positions corresponding to any of nucleotide positions 668-734 relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include four or more mutations at nucleotide positions corresponding to any of nucleotide positions 668-734 relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include five or more mutations at nucleotide positions corresponding to any of nucleotide positions 668-734 relative to SEQ ID NO: 28.

In another example, provided herein is a nucleic acid construct comprising a first alcohol oxidase promoter element, wherein the first alcohol oxidase promoter element can include one or more mutations at a nucleotide position selected from the group consisting of nucleotide positions corresponding to T146, C154, T303, T426, A433, A435, T530, C572, T596, T617, T688, A696, T702, A709, A712, T714, A790, A841, and T862 relative to SEQ ID NO: 28. In another aspect, the present disclosure provides a method of producing a protein, the method comprising culturing a cell, wherein the cell comprises (i) a nucleic acid construct encoding the protein, wherein the nucleic acid construct comprises a first alcohol oxidase promoter element, wherein the first alcohol oxidase promoter element comprises a first nucleotide sequence comprising a mutation at one or more nucleotide positions selected from the group consisting of T146, C154, T303, T426, A433, A435, T530, C572, T596, T688, A696, T702, A709, A712, T714, A790, A841, and T862 relative to SEQ ID NO: 28, or (ii) an expression vector comprising the nucleic acid construct; wherein the method is carried out in the absence of added methanol.

Implementations can include one or more of the following features. The first alcohol oxidase promoter element can include two or more mutations at nucleotide positions selected

C154T, T303C, T426A, A433T, A435G, T530A, C572T, T596C, T617C, T688C, A696T, T702C, A709G, A712G, T714G, A790G, A841T, and T862A relative to SEQ ID NO: 28. In one aspect, the present disclosure provides a method of producing a protein, the method comprising culturing a cell, wherein the cell comprises (i) a nucleic acid construct encoding the protein, wherein the nucleic acid construct comprises a first alcohol oxidase promoter element, wherein the first alcohol oxidase promoter element comprises a first nucleotide sequence comprising one or more mutations selected from the group consisting of T146C, C154T, T303C, T426A, A433T, A435G, T530A, C572T, T596C, T617C, T688C, A696T, T702C, A709G, A712G, T714G, A790G, A841T, and T862A relative to SEQ ID NO: 28, or (ii) an expression vector comprising the nucleic acid construct; wherein the method is carried out in the absence of added methanol. In one aspect, the present disclosure provides a nucleic acid construct comprising a first alcohol oxidase promoter element, wherein the first alcohol oxidase promoter element comprises a first nucleotide sequence comprising the mutations T688C, A696T, T702C, A712G, and T714G relative to SEQ ID NO: 28.

Implementations can include one or more of the following features. The first alcohol oxidase promoter element can include two or more mutations selected from the group consisting of mutations corresponding to T146C, C154T, T303C, T426A, A433T, A435G, T530A, C572T, T596C, T617C, T688C, A696T, T702C, A709G, A712G, T714G, A790G, A841T, and T862A relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include three or more mutations selected from the group consisting of mutations corresponding to T146C, C154T, T303C, T426A, A433T, A435G, T530A, C572T, T596C, T617C, T688C, A696T, T702C, A709G, A712G, T714G, A790G, A841T, and T862A relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include four or more mutations selected from the group consisting of mutations corresponding to T146C, C154T, T303C, T426A, A433T, A435G, T530A, C572T, T596C, T617C, T688C, A696T, T702C, A709G, A712G, T714G, A790G, A841T, and T862A relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include five or more mutations selected from the group consisting of mutations corresponding to T146C, C154T, T303C, T426A, A433T, A435G, T530A, C572T, T596C, T617C, T688C, A696T, T702C, A709G, A712G, T714G, A790G, A841T, and T862A relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include one or more mutations selected from the group consisting of T688C, A696T, T702C, A712G, and T714G relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include two or more mutations selected from the group consisting of T688C, A696T, T702C, A712G, and T714G relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include three or more mutations selected from the group consisting of T688C, A696T, T702C, A712G, and T714G relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include four or more mutations selected from the group consisting of T688C, A696T, T702C, A712G, and T714G relative to SEQ ID NO: 28. The first alcohol oxidase promoter element can include the mutations T688C, A696T, T702C, A712G, and T714G relative to SEQ ID NO: 28.

Implementations of any of the nucleic acid constructs described herein can have one or more of the following features. The first alcohol oxidase promoter element can be an alcohol oxidase 1 promoter element. The first alcohol oxidase promoter element can have at least 90%

CLAIMS

1. A method of producing a protein, the method comprising culturing a cell, wherein the cell comprises (i) a nucleic acid construct encoding the protein, wherein the nucleic acid construct comprises a first alcohol oxidase promoter element, wherein the first alcohol oxidase promoter element comprises a first nucleotide sequence comprising a mutation at one or more nucleotide positions selected from the group consisting of T146, C154, T303, T426, A433, A435, T530, C572, T596, T688, A696, T702, A709, A712, T714, A790, A841, and T862 relative to SEQ ID NO: 28, or (ii) an expression vector comprising the nucleic acid construct; wherein the method is carried out in the absence of added methanol.

2. The method of claim 1, wherein the first nucleotide sequence comprises a mutation at one or more nucleotide positions selected from the group consisting of T688, A696, T702, A712, and T714 relative to SEQ ID NO: 28.

3. The method of claim 1, wherein the first nucleotide sequence comprises a mutation at each of nucleotide positions T688, A696, T702, A712, and T714 relative to SEQ ID NO: 28.

4. A method of producing a protein, the method comprising culturing a cell, wherein the cell comprises (i) a nucleic acid construct encoding the protein, wherein the nucleic acid construct comprises a first alcohol oxidase promoter element, wherein the first alcohol oxidase promoter element comprises a first nucleotide sequence comprising one or more mutations selected from the group consisting of T146C, C154T, T303C, T426A, A433T, A435G, T530A, C572T, T596C, T617C, T688C, A696T, T702C, A709G, A712G, T714G, A790G, A841T, and T862A relative to SEQ ID NO: 28, or (ii) an expression vector comprising the nucleic acid construct; wherein the method is carried out in the absence of added methanol.

5. The method of claim 4, wherein the first nucleotide sequence comprises one or more mutations selected from the group consisting of T688C, A696T, T702C, A712G, and T714G relative to SEQ ID NO: 28.

6. A nucleic acid construct comprising a first alcohol oxidase promoter element, wherein the first alcohol oxidase promoter element comprises a first nucleotide sequence comprising the mutations T688C, A696T, T702C, A712G, and T714G relative to SEQ ID NO: 28.

7. The nucleic acid construct of claim 6, wherein the first alcohol oxidase promoter element is an alcohol oxidase 1 (AOX1) promoter element.

8. The nucleic acid construct of claim 6 or 7, wherein the first nucleotide sequence has at least 90% sequence identity to SEQ ID NO: 28.

9. The nucleic acid construct of claim 6 or 7, wherein the first nucleotide sequence has at least 95% sequence identity to SEQ ID NO: 28.

10. The nucleic acid construct of claim 6 or 7, wherein the first nucleotide sequence has at least 97% sequence identity to SEQ ID NO: 28.

11. The nucleic acid construct of any one of claims 6-10, wherein the first alcohol oxidase promoter element comprises a recognition sequence for a transcription factor.

12. The nucleic acid construct of any one of claims 6-11, further comprising a second nucleotide sequence, wherein the second nucleotide sequence is operably linked to the first alcohol oxidase promoter element.

13. The nucleic acid construct of claim 12, wherein the second nucleotide sequence encodes a first protein.

14. The nucleic acid construct of claim 13, wherein the first protein is selected from the group consisting of an antibody or fragment thereof, an enzyme, a regulatory protein, a peptide hormone, a blood clotting protein, a cytokine, a cytokine inhibitor, and a heme-binding protein.

15. The nucleic acid construct of claim 13, wherein the first protein is a heme-binding protein.

16. The nucleic acid construct of claim 15, wherein the heme-binding protein comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of any one of SEQ ID NOs: 1-27.

17. An expression vector comprising the nucleic acid construct of any one of claims 6 to 16.

18. A cell comprising the nucleic acid construct of any one of claims 6-16 or the expression vector of claim 17.

19. The cell of claim 18, further comprising (a) a second nucleic acid construct comprising a third nucleotide sequence, wherein the third nucleotide sequence is operably linked to the first alcohol oxidase promoter element or to a second promoter element, or (b) a second expression vector comprising the second nucleic acid construct.

20. The cell of claim 18 or 19, which is a yeast cell.

21. The cell of claim 18 or 19, which is a methylotrophic yeast cell.

22. The cell of claim 18 or 19, which is a *Pichia pastoris* cell.

23. A method of producing a protein, the method comprising culturing the cell of any one of claims 18-22, wherein the nucleic acid construct encodes the protein.

24. The method of claim 23, wherein the method further comprises purifying the protein.

25. The method of claim 23 or 24, wherein the method is carried out in the absence of added methanol.

26. The method of claim 1, wherein the first alcohol oxidase promoter element comprises four or more mutations selected from the group consisting of T146, C154, T303, T426, A433, A435, T530, C572, T596, T688, A696, T702, A709, A712, T714, A790, A841, and T862 relative to SEQ ID NO: 28.

27. The method of claim 4, wherein the first alcohol oxidase promoter element comprises two or more mutations selected from the group consisting of mutations corresponding to T146C, C154T, T303C, T426A, A433T, A435G, T530A, C572T, T596C, T617C, T688C, A696T, T702C, A709G, A712G, T714G, A790G, A841T, and T862A relative to SEQ ID NO: 28.

28. The method of any one of claims 1-5, 26, and 27, wherein the method further comprises purifying the protein.

29. The method of any one of claims 1-5 and 26-28, wherein the cell further comprises (a) a second nucleic acid construct comprising a third nucleotide sequence, wherein the third nucleotide sequence is operably linked to the first alcohol oxidase promoter element or to a second promoter element, or (b) a second expression vector comprising the second nucleic acid construct.

30. The method of any one of claims 1-5 and 26-29, wherein the cell is a yeast cell.

31. The method of any one of claims 1-5 and 26-29, wherein the cell is a methylotrophic yeast cell.

32. The method of any one of claims 1-5 and 26-29, wherein the cell is a *Pichia pastoris* cell.