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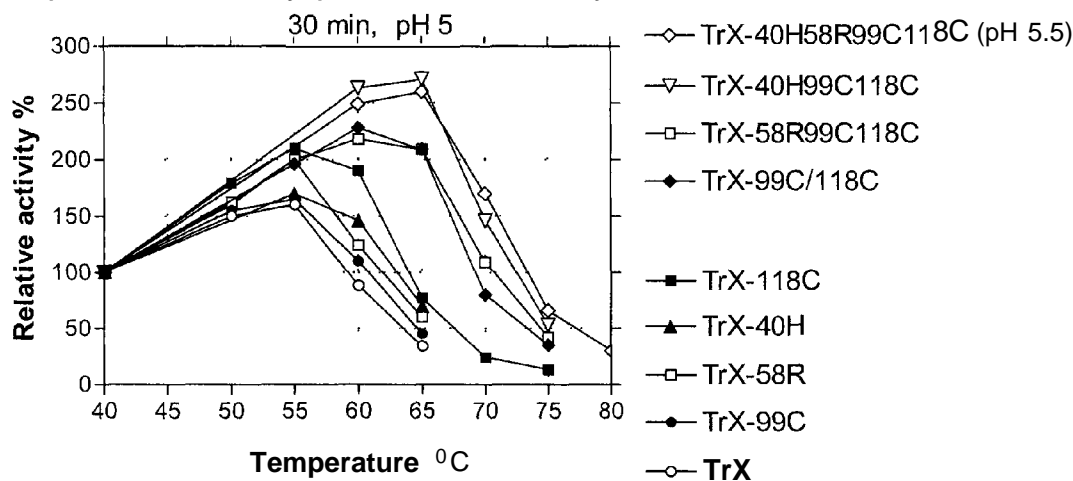
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(54) Title: MODIFICATION OF XYLANASES TO INCREASE THERMOPHILICITY, THERMOSTABILITY AND ALKALOPHILICITY

Temperature/activity profile of mutant xylanases



(57) Abstract: A modified Family 11 xylanase enzyme comprising cysteine residues at positions 99 and 118 to form an intramolecular disulfide bond is provided. The modified xylanase is produced by substitution of an amino acid at position 99, 118 or both positions 99 and 118 with a cysteine to produce the intramolecular disulfide bond. Xylanases of the invention display improved thermophilicity, alkalophilicity or thermostability relative to wild-type xylanases. Such xylanases find use in a variety of applications in industry that require enzyme activities at temperatures and/or pH values above that of the native enzyme.

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AMENDED CLAIMS

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**THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY
OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:**

- 5 1. A modified xylanase comprising cysteine residues at positions 99 and 118 to form an intramolecular disulfide bond, said xylanase produced by substitution of an amino acid at position 99, 118 or both positions 99 and 118 with a cysteine, said positions determined from sequence alignment of said modified xylanase with a *Trichoderma reesei* xylanase II amino acid sequence as defined in SEQ ID NO:16, wherein said modified xylanase is thermophilic,
10 alkalophilic, thermostable, or a combination thereof and wherein said xylanase is a Family 11 xylanase.
2. The modified xylanase of claim 1 further comprising a substituted amino acid at position 40 selected from the group consisting of Arg, Cys, Phe, Tyr and His,
15
3. The modified xylanase of claim 1 further comprising a basic substituted amino acid at position 40.
4. The modified xylanase of claim 3, wherein the basic amino acid at position 40 is a His.
20
5. The modified xylanase of claim 1 further comprising a basic substituted amino acid at position 58.
6. The modified xylanase of claim 5, wherein the basic substituted amino acid at position 58
25 is an Arg.
7. The modified xylanase of claim 1, wherein said Family 11 xylanase is a *Trichoderma reesei* xylanase.

8. The modified xylanase of claim 1, wherein said modified xylanase is not derived from a xylanase with naturally-occurring cysteine residues at positions 99 and 118.

5 9. The modified xylanase of claim 5 further comprising a basic substituted amino acid at position 10, a hydrophobic substituted amino acid at position 27 and a hydrophobic substituted amino acid at position 29.

10 10. The modified xylanase of claim 9, wherein the basic substituted amino acid at position 10 is a His, the hydrophobic substituted amino acid at position 27 is a Met and the hydrophobic substituted amino acid at position 29 is a Leu (HML).

15 11. The modified xylanase of claim 10 further comprising a non-polar substituted amino acid at position 75, a basic substituted amino acid at position 105, a non-polar substituted amino acid at position 125 and an acidic amino acid at position 129.

20 12. The modified xylanase of claim 11, wherein said non-polar substituted amino acid at position 75 is an Ala, said basic substituted amino acid at position 105 is a His, said non-polar substituted amino acid at position 125 is an Ala and said acidic amino acid at position 129 is a Glu.

25 13. The modified xylanase of claim 12 further comprising an acidic amino acid at position 11.

14. The modified xylanase of claim 13, wherein the acidic amino acid at position 11 is an Asp.

15. The modified xylanase of claim 14 further comprising an Asn at position 131.

30 16. The modified xylanase of claim 1 further comprising a basic substituted amino acid at each of positions 40 and 58,

17. The modified xylanase of claim 16, wherein the basic amino acid at position 40 is a His and the basic amino acid at position 58 is an Arg.

5 18. The modified xylanase of claim 16 further comprising a basic substituted amino acid at position 10, a hydrophobic substituted amino acid at position 27 and a hydrophobic substituted amino acid at position 29.

10 19. The modified xylanase of claim 18, wherein the basic substituted amino acid at position 10 is a His, the hydrophobic substituted amino acid at position 27 is a Met and the hydrophobic substituted amino acid at position 29 is a Leu (HML).

15 20. The modified xylanase of claim 19, further comprising a non-polar substituted amino acid at position 75, a basic substituted amino acid at position 105, a non-polar substituted amino acid at position 125 and an acidic amino acid at position 129.

20 21. The modified xylanase of claim 20, wherein said non-polar substituted amino acid at position 75 is Ala, said basic substituted amino acid at position 105 is a His, said non-polar substituted amino acid at position 125 is an Ala and said acidic amino acid at position 129 is GIu.

22. The modified xylanase of claim 21, further comprising an acidic amino acid at position 11.

25 23. The modified xylanase of claim 22, wherein the acidic amino acid at position 11 is an Asp.

24. The modified xylanase of claim 23 further comprising an Asn at position 131,

30 25. The modified xylanase of claim 23, further comprising a Cys at position 52.

26. The modified xylanase of claim 25, further comprising a basic substituted amino acid at each of positions 144 and 161.

27. The modified xylanase of claim 26, wherein the basic substituted amino acid at each of
5 positions 144 and 161 is an Arg.

28. A modified xylanase according to claim 1, wherein said modified xylanase has a maximum effective temperature (MET) between about 65°C and about 85°C.

10 29. A modified xylanase according to claim 1, wherein said modified xylanase has a maximum effective pH (MEP) between about pH 6.5 and about pH 8.0.

30. The modified xylanase according to claim 1, wherein said modified xylanase is derived from *Aspergillus*, *Bacillus*, *Cellulomonas*, *Chainia*, *Clostridium*, *Fibrobacter*, *Neocallimastix*,
15 *Ruminococcus*, *Schizophyllum*, *Streptomyces*, *Thermobifida*, *Thermomyces*, *Piromyces*, *Talaromyces*, *Orpinomyces*, *Phanerochaete*, *Gibberella*, *Cochliobolus*, *Aureobasidium*, *Chaetomium*, *Diclyoglossum*, *Magnaporthe*, *Penicillium*, *Fusarium*, *Humicola*, *Trichoderma* or *Hypocrea*,

31. A modified Family 11 xylanase selected from the group consisting of:
20

TrX-99C-1 18C (SEQ K) NO:66);

TrX-40H-9->C-1 18C (SEQ ID NO:67);

TrX-5SR-99C-1 18C (SEQ ID NO:68);

TrX-40H-58R-99C-1 18C (SEQ ID NO:69);

25 TrX-10H-27M-29L-40R-58R-99C-1 18C (SEQ ID NO:70);

TrX-10H-27M-29L-40R-58R-75A-99C-1 18C (SEQ ID NO:71);

TTX-10H-27M-29L-75A-99C-105H-1 18C-125A-129E (SEQ ID NO:72);

TrX-10H-27M-29L-5SR-75A-99C-105H-1 18C-125A-129E (SEQ ID NO:73);

TrX-10H-1 1D-27M-29L-58R-75A-99C-105H-1 18C-125A-129E (SEQ ID NO:74);

$Y_7X-10H-n D-21M-29L-40X-58R-75A-99C-105E-118C-n 5A-i 29E$, wherein X is C, F, H, Y or R (SEQ ID NOs:75, 76, 77, 78 and 79, respectively);

TrX-10H-11D-27M-29L-40H-52C-58R-75A-99C-105H-118C-125A-129E (SEQ ID NO:80);

5 TrX-10H-11D-~~27M-29L-40R-58R-75A-99C-105H-118C-125A-129E-144R161R~~ (SEQ ID NO:81); and

T₁X-10H-11D-27M-29L-58R-75A-99C-105H-118C-125A-129E-131N (SEQ ID NO:82).

10 32. A modified Family 11 xylanase comprising cysteine residues at positions 99 and 118 to form an intramolecular disulfide bond, said xylanase produced by substitution of an amino acid at position 99, 118 or both positions 99 and 118 with a cysteine, said positions determined from sequence alignment of said modified xylanase with a *Trichoderma reesei* xylanase II amino acid sequence as defined in SEQ ID NO: 16.

15 33. A modified Family 11 xylanase comprising a substituted basic amino acid at position 40, said position determined from sequence alignment of said modified xylanase with a *Trichoderma reesei* xylanase II amino acid sequence as defined in SEQ ID NO: 16.

20 34. A modified Family 11 xylanase comprising a basic substituted amino acid at position 40, said position determined from sequence alignment of said modified xylanase with a *Trichoderma reesei* xylanase II amino acid sequence as defined in SEQ ID NO: 16 and an intramolecular disulfide bond enclosing a loop having between 10 and 24 amino acids.

25 35. The modified Family 11 xylanase of claim 34, wherein the intramolecular disulfide bond is produced by substitution of an amino acid at position 99, 118 or both positions 99 and 118 with a cysteine.

30 36. The modified Family 11 xylanase of claim 34, wherein the basic amino acid at position 40 is a His.