

(19) **DANMARK**

(10) **DK/EP 2421973 T3**



(12)

Oversættelse af europæisk patentskrift

Patent- og
Varemærkestyrelsen

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- (51) Int.Cl.: **C 12 N 15/75 (2006.01)** **C 12 N 9/54 (2006.01)**
- (45) Oversættelsen bekendtgjort den: **2018-07-30**
- (80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2018-05-23**
- (86) Europæisk ansøgning nr.: **10714540.1**
- (86) Europæisk indleveringsdag: **2010-04-15**
- (87) Den europæiske ansøgnings publiceringsdag: **2012-02-29**
- (86) International ansøgning nr.: **US2010031269**
- (87) Internationalt publikationsnr.: **WO2010123754**
- (30) Prioritet: **2009-04-24 US 172587 P** **2009-05-13 US 177899 P**
- (84) Designerede stater: **AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO SE SI SK SM TR**
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- (54) Benævnelse: **PROTEASER MED MODIFICEREDE PRO-OMRÅDER**
- (56) Fremdragne publikationer:
WO-A1-2008/141281
WO-A2-2008/112258

DESCRIPTION

FIELD OF THE INVENTION

[0001] The present invention provides methods and uses for the production of mature proteases in bacterial host cells. This involves the use of modified polynucleotides that encode modified proteases, which have at least one mutation in the pro region; the modified serine proteases encoded by the modified polynucleotides; expression cassettes, DNA constructs, and vectors comprising the modified polynucleotides that encode the modified proteases; and the bacterial host cells transformed with the vectors of the invention. The methods include methods for enhancing the production of mature proteases in bacterial host cells *e.g.* *Bacillus sp.* host cells. The produced proteases find use in the industrial production of enzymes, suitable for use in various industries, including but not limited to the cleaning, animal feed and textile processing industry.

BACKGROUND

[0002] Microorganisms, such as the Gram-positive microorganism that are members of the genus *Bacillus*, have been used for large-scale industrial fermentation due, in part, to their ability to secrete their fermentation products into their culture media. Secreted proteins are exported across a cell membrane and a cell wall, and then are subsequently released into the external media.

[0003] Indeed, secretion of heterologous polypeptides is a widely used technique in industry. Typically, cells are transformed with a nucleic acid encoding a heterologous polypeptide of interest to be expressed and secreted to produce large quantities of desired polypeptides. Expression and secretion of desired polypeptides has been controlled through genetic manipulation of the polynucleotides that encode the desired proteins. Despite various advances in protein production methods, there remains a need in the art to provide more efficient methods for extracellular protein secretion with the aim to enhance the production of enzymes such as proteases, which find use in the use in various industries, including but not limited to the cleaning, animal feed and textile processing industry.

[0004] WO 2008/141281 describes a modified secretion system for expressing polypeptides in bacteria. WO 2008/112258 discloses proteases with modified pro regions to improve production.

SUMMARY OF THE INVENTION

[0005] The present invention provides methods and uses for the production of mature proteases in bacterial host cells as set out in the claims. These employ modified polynucleotides that encode modified proteases, which have at least one mutation in the pro region; the modified serine proteases encoded by the modified polynucleotides; expression cassettes, DNA constructs, and vectors comprising the modified polynucleotides that encode the modified proteases; and the bacterial host cells transformed with the vectors of the invention. The methods include methods for enhancing the production of mature proteases in bacterial host cells *e.g.* *Bacillus sp.* host cells. The produced proteases find use in the industrial production of enzymes, suitable for use in various industries, including but not limited to the cleaning, animal feed and textile processing industry.

[0006] In one embodiment, the invention provides the use of a modified polynucleotide encoding a modified full-length protease, said modified polynucleotide comprising:

a first polynucleotide encoding a signal peptide, said first polynucleotide being operably linked to a second polynucleotide encoding the pro region set forth in SEQ ID NO:7, wherein said pro region comprises a combination of substitutions of at least two amino acids at positions chosen from positions 6, 30 and 32 of said pro region, wherein said combination of substitutions is chosen from E6R-A32K, E6N-A32K, E6D-A32K, E6I-A32K, E6K-A32K, E6M-A32K, E6P-A32K, E6T-A32K, E6A-E30G, E6R-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6S-E30G, E6WE30G, E30G-A32R, E30G-A32Q, E30G-A32E, E30G-A32G, E30G-A32H, E30G-A32I, E30G-A32S, E30G-A32T, E30G-A32W, E30G-A32V, E6G-E30G-A32E, E6G-E30G-A32S, E6G-E30G-A32T, E6G-E30G-A32W, E6A-E30G, E6R-E30G, E6N-E30G, E6D-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6M-E30G, E6F-E30G, E6P-E30G, E6S-E30G, E6T-E30G, E6W-E30G, E6V-E30G, E6Y-E30G, E6A-E30S, E6G-E30S, E6L-E30S, E6K-E30S, E6F-E30S, E6P-E30S, E6Y-E30S, E6V-E30S, E30S-A32R, E30S-A32N, E30S-A32D, E30S-A32C, E30S-A32Q, E30S-A32E, E30S-A32G, E30S-A32H, E30S-A32L, E30S-A32M, E30S-A32F, E30S-A32P, E30S-A32S, E30S-A32T, E30S-A32W, E30S-A32Y, and E30S-A32V and wherein the positions are numbered by correspondence with the amino acid sequence of the pro polypeptide of SEQ ID NO:7;

said second polynucleotide being operably linked to a third polynucleotide encoding the mature region of a protease that is at least about 60% identical to the mature protease of SEQ ID NO: 9,

to provide enhanced production of a mature protease that is processed from the modified full-length protease compared to the level of production of the same mature protease when processed from the full-length protease, without the substitutions in the pro region set forth in SEQ ID NO: 7.

[0007] The second polynucleotide may be operably linked to a third polynucleotide that encodes the mature region of a wild-type or variant alkaline serine protease derived from *Bacillus clausii* or *Bacillus lentus* that is at least about 60% identical to the mature protease of SEQ ID NO: 9. Preferably, the substitutions enhance the production of the mature protease by a *Bacillus sp.* host *e.g. Bacillus subtilis*. substitutions of at least two amino acids at positions chosen from positions 6, 30 and 32 of the pro region. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a protease chosen from SEQ ID NOS:9, 11, 13, 15, 17, 19, and 21. Preferably, the substitutions enhance the production of the mature protease by a *Bacillus sp.* host *e.g. Bacillus subtilis*.

[0008] In another embodiment, the isolated modified polynucleotide comprises a first polynucleotide that encodes a signal peptide, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7, which comprises a combination of substitutions of at least two amino acids chosen from E6X-E30G, E6X-E30S, E6X-A32K, E30X-A32K, E30G-A32X, E30S-A32X and E6G-E30G-A32X. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a protease that is at least about 60% identical to the mature protease of SEQ ID NO: 9. Preferably, the substitutions enhance the production of the mature protease by a *Bacillus sp.* host *e.g. Bacillus subtilis*.

[0009] In another embodiment, the isolated modified polynucleotide comprises a first polynucleotide that encodes a signal peptide, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7, which comprises a combination of substitutions of at least two amino

acids chosen from E6X-E30G, E6X-E30S, E6X-A32K, E30X-A32K, E30G-A32X, E30S-A32X and E6G-E30G-A32X. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a wild-type or variant alkaline serine protease derived from *Bacillus clausii* or *Bacillus lentus* that is at least about 60% identical to the mature protease of SEQ ID NO: 9. Preferably, the substitutions enhance the production of the mature protease by a *Bacillus* sp. host e.g. *Bacillus subtilis*.

[0010] In another embodiment, the isolated modified polynucleotide comprises a first polynucleotide that encodes a signal peptide, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7, which comprises a combination of substitutions of at least two amino acids chosen from E6X-E30G, E6X-E30S, E6X-A32K, E30X-A32K, E30G-A32X, E30S-A32X and E6G-E30G-A32X. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a protease chosen from SEQ ID NOS:9, 11, 13, 15, 17, 19, and 21. Preferably, the substitutions enhance the production of the mature protease by a *Bacillus* sp. host e.g. *Bacillus subtilis*.

[0011] In another embodiment, the isolated modified polynucleotide that encodes a modified protease. The isolated modified polynucleotide comprises a first polynucleotide that encodes a signal peptide chosen from SEQ ID NOS:3 and 5, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7, which comprises a combination of substitutions of at least two amino acids chosen from E6X-E30G, E6X-E30S, E6X-A32K, E30X-A32K, E30G-A32X, E30S-A32X and E6G-E30G-A32X. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a protease that is at least about 60% identical to the mature protease of SEQ ID NO: 9. Preferably, the substitutions enhance the production of the mature protease by a *Bacillus* sp. host e.g. *Bacillus subtilis*.

[0012] In another embodiment, the isolated modified polynucleotide comprises a first polynucleotide that encodes a signal peptide chosen from SEQ ID NOS:3 and 5, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7, which comprises a combination of substitutions of at least two amino acids chosen from E6X-E30G, E6X-E30S, E6X-A32K, E30X-A32K, E30G-A32X, E30S-A32X and E6G-E30G-A32X. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a wild-type or variant alkaline serine protease derived from *Bacillus clausii* or *Bacillus lentus* that is at least about 60% identical to the mature protease of SEQ ID NO: 9. Preferably, the substitutions enhance the production of the mature protease by a *Bacillus* sp. host e.g. *Bacillus subtilis*.

[0013] In another embodiment, the isolated modified polynucleotide comprises a first polynucleotide that encodes a signal peptide chosen from SEQ ID NOS:3 and 5, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7, which comprises a combination of substitutions of at least two amino acids chosen from E6X-E30G, E6X-E30S, E6X-A32K, E30X-A32K, E30G-A32X, E30S-A32X and E6G-E30G-A32X. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a protease chosen from SEQ ID NOS:9, 11, 13, 15, 17, 19, and 21. Preferably, the substitutions enhance the production of the mature protease by a *Bacillus* sp. host e.g. *Bacillus subtilis*.

[0014] As described above, the isolated modified polynucleotide comprises a first polynucleotide that encodes a signal peptide, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7, which comprises a combination of substitutions of at least two amino acids chosen from E6R-A32K, E6N-A32K, E6D-A32K, E6I-A32K, E6K-A32K, E6M-A32K, E6P-A32K, E6T-A32K, E6N-A32K, E6A-E30G, E6R-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G,

E6S-E30G, E6W-E30G, E30G-A32R, E30G-A32Q, E30G-A32E, E30G-A32G, E30G-A32H, E30G-A32I, E30G-A32S, E30G-A32T, E30G-A32W, E30G-A32V, E6G-E30G-A32E, E6G-E30G-A32S, E6G-E30G-A32T, E6G-E30G-A32W, E6A-E30G, E6R-E30G, E6N-E30G, E6D-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6ME30G, E6F-E30G, E6P-E30G, E6S-E30G, E6T-E30G, E6W-E30G, E6V-E30G, E6Y-E30G, E6A-E30S, E6G-E30S, E6L-E30S, E6K-E30S, E6F-E30S, E6P-E30S, E6Y-E30S, E6V-E30S, E30S-A32R, E30S-A32N, E30S-A32D, E30S-A32C, E30S-A32Q, E30S-A32E, E30S-A32G, E30S-A32H, E30S-A32L, E30S-A32M, E30S-A32F, E30S-A32P, E30S-A32S, E30S-A32T, E30S-A32W, E30S-A32Y, and E30S-A32V. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a protease that is at least about 60% identical to the mature protease of SEQ ID NO: 9. Preferably, the substitutions enhance the production of the mature protease by a *Bacillus* sp. host e.g. *Bacillus subtilis*.

[0015] Also described herein is an expression vector comprising an isolated modified polynucleotide, which comprises a first polynucleotide that encodes a signal peptide, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7 that comprises a combination of substitutions of at least two amino acids at positions chosen from positions 6, 30 and 32 of the pro region. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a protease that is at least about 60% identical to the mature protease of SEQ ID NO: 9. Preferably, the mature protease is a wild-type or variant alkaline serine protease derived from *Bacillus clausii* or *Bacillus lentus* e.g. SEQ ID NOS:9, 11, 13, 15, 17, 19, and 21. In some embodiments, the expression of the isolated polynucleotide is driven by the AprE promoter comprised in the expression vector.

[0016] Also described herein is an expression vector that comprises an isolated modified polynucleotide, which comprises a first polynucleotide that encodes a signal peptide chosen from SEQ ID NOS:3 and 5, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7 that comprises a combination of substitutions of at least two amino acids at positions chosen from positions 6, 30 and 32 of the pro region. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a protease that is at least about 60% identical to the mature protease of SEQ ID NO: 9. Preferably, the mature protease is a wild-type or variant alkaline serine protease derived from *Bacillus clausii* or *Bacillus lentus* e.g. SEQ ID NOS:9, 11, 13, 15, 17, 19, and 21. In some embodiments, the expression of the isolated polynucleotide is driven by the AprE promoter comprised in the expression vector.

[0017] Also described herein is an expression vector comprising an isolated modified polynucleotide, which comprises a first polynucleotide that encodes a signal peptide, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7 that comprises a combination of substitutions of at least two amino acids chosen from E6X-E30G, E6X-E30S, E6X-A32K, E30X-A32K, E30G-A32X, E30S-A32X and E6G-E30G-A32X. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a protease that is at least about 60% identical to the mature protease of SEQ ID NO: 9. Preferably, the mature protease is a wild-type or variant alkaline serine protease derived from *Bacillus clausii* or *Bacillus lentus* e.g. SEQ ID NOS:9, 11, 13, 15, 17, 19, and 21. In some embodiments, the expression of the isolated polynucleotide is driven by the AprE promoter comprised in the expression vector.

[0018] Also described herein is an expression vector that comprises an isolated modified polynucleotide, which comprises a first polynucleotide that encodes a signal peptide chosen from SEQ ID NOS:3 and 5, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7 that comprises a combination of substitutions of at least two amino acids chosen from E6X-E30G, E6X-

E30S, E6X-A32K, E30X-A32K, E30G-A32X, E30S-A32X and E6G-E30G-A32X. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a protease that is at least about 60% identical to the mature protease of SEQ ID NO: 9. Preferably, the mature protease is a wild-type or variant alkaline serine protease derived from *Bacillus clausii* or *Bacillus lentus* e.g. SEQ ID NOS:9, 11, 13, 15, 17, 19, and 21. In some embodiments, the expression of the isolated polynucleotide is driven by the AprE promoter comprised in the expression vector.

[0019] Also described herein is an expression vector comprising an isolated modified polynucleotide, which comprises a first polynucleotide that encodes a signal peptide, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7 that comprises a combination of substitutions of at least two amino acids chosen from E6R-A32K, E6N-A32K, E6D-A32K, E6I-A32K, E6K-A32K, E6M-A32K, E6P-A32K, E6S-A32K, E6T-A32K, E6N-A32K, E30W-A32K, E30V-A32K, E6A-E30G, E6R-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6S-E30G, E6W-E30G, E30G-A32R, E30G-A32Q, E30G-A32E, E30G-A32G, E30G-A32H, E30G-A32I, E30G-A32K, E30G-A32S, E30G-A32T, E30G-A32W, E30G-A32V, E6G-E30G-A32E, E6G-E30G-A32S, E6G-E30G-A32T, E6G-E30G-A32W, E6A-E30G, E6R-E30G, E6N-E30G, E6D-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6M-E30G, E6F-E30G, E6P-E30G, E6S-E30G, E6T-E30G, E6W-E30G, E6V-E30G, E6Y-E30G, E6A-E30S, E6G-E30S, E6L-E30S, E6K-E30S, E6F-E30S, E6P-E30S, E6Y-E30S, E6V-E30S, E30S-A32R, E30S-A32N, E30S-A32D, E30S-A32C, E30S-A32Q, E30S-A32E, E30S-A32G, E30S-A32H, E30S-A32L, E30S-A32K, E30S-A32M, E30S-A32F, E30S-A32P, E30S-A32S, E30S-A32T, E30S-A32W, E30S-A32Y, and E30S-A32V. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a protease that is at least about 60% identical to the mature protease of SEQ ID NO: 9. Preferably, the mature protease is a wild-type or variant alkaline serine protease derived from *Bacillus clausii* or *Bacillus lentus* e.g. SEQ ID NOS:9, 11, 13, 15, 17, 19, and 21. In some embodiments, the expression of the isolated polynucleotide is driven by the AprE promoter comprised in the expression vector.

[0020] Also described herein is an expression vector that comprises an isolated modified polynucleotide, which comprises a first polynucleotide that encodes a signal peptide chosen from SEQ ID NOS:3 and 5, which is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7 that comprises a combination of substitutions of at least two amino acids chosen from E6R-A32K, E6N-A32K, E6D-A32K, E6I-A32K, E6K-A32K, E6M-A32K, E6P-A32K, E6S-A32K, E6T-A32K, E6N-A32K, E30W-A32K, E30V-A32K, E6A-E30G, E6R-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6S-E30G, E6W-E30G, E30G-A32R, E30G-A32Q, E30G-A32E, E30G-A32G, E30G-A32H, E30G-A32I, E30G-A32K, E30G-A32S, E30G-A32T, E30G-A32W, E30G-A32V, E6G-E30G-A32E, E6G-E30G-A32S, E6G-E30G-A32T, E6G-E30G-A32W, E6A-E30G, E6R-E30G, E6N-E30G, E6D-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6M-E30G, E6F-E30G, E6P-E30G, E6S-E30G, E6T-E30G, E6W-E30G, E6V-E30G, E6Y-E30G, E6A-E30S, E6G-E30S, E6L-E30S, E6K-E30S, E6F-E30S, E6P-E30S, E6Y-E30S, E6V-E30S, E30S-A32R, E30S-A32N, E30S-A32D, E30S-A32C, E30S-A32Q, E30S-A32E, E30S-A32G, E30S-A32H, E30S-A32L, E30S-A32K, E30S-A32M, E30S-A32F, E30S-A32P, E30S-A32S, E30S-A32T, E30S-A32W, E30S-A32Y, and E30S-A32V. In turn, the second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of a protease that is at least about 60% identical to the mature protease of SEQ ID NO: 9. Preferably, the mature protease is a wild-type or variant alkaline serine protease derived from *Bacillus clausii* or *Bacillus lentus* e.g. SEQ ID NOS:9, 11, 13, 15, 17, 19, and 21. In some embodiments, the expression of the isolated polynucleotide is driven by the AprE promoter comprised in the expression vector.

[0021] Also described herein is a *Bacillus* sp. host cell e.g. *Bacillus subtilis*, which comprises any one of the expression vectors described above. Preferably, the substitutions comprised in the pro region of the

modified polynucleotide enhance the production of the mature protease from the *Bacillus* host cell. In addition to *Bacillus subtilis*, other host cells that can be used to express the modified polynucleotides from the expression vectors include *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus clausii*, *Bacillus halodurans*, *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, and *Bacillus thuringiensis*.

[0022] In another embodiment, the invention provides a method for producing a mature protease in a *Bacillus sp.* host cell. The method includes providing any one of the expression vectors described above, transforming the expression vector into a *Bacillus sp.* host cell, and culturing the transformed host cell under suitable conditions to produce the protease. Preferably, the host cell is a *Bacillus subtilis* host cell. However, in addition to *Bacillus subtilis*, other host cells that can be used to produce the mature proteases from the expression vector include *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus clausii*, *Bacillus halodurans*, *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, and *Bacillus thuringiensis*.

[0023] In another embodiment, the method produces the mature protease of SEQ ID NO:9 by providing an expression vector that expresses an isolated modified polynucleotide comprising a first polynucleotide encoding the signal peptide of SEQ ID NO:3, a second polynucleotide encoding the pro region of SEQ ID NO:7 that includes a combination of substitutions chosen from E6R-A32K, E6N-A32K, E6D-A32K, E6I-A32K, E6K-A32K, E6M-A32K, E6P-A32K, E6S-A32K, E6T-A32K, E6N-A32K, E30W-A32K, E30V-A32K, and the third polynucleotide, which encodes the mature protease of SEQ ID NO:9. The expression vector is transformed into a *Bacillus sp.* host cell e.g. *Bacillus subtilis*, which is grown under suitable conditions to produce the mature protease.

[0024] In another embodiment, the method produces the mature protease of SEQ ID NO:17 by providing an expression vector that expresses an isolated modified polynucleotide comprising a first polynucleotide encoding the signal peptide of SEQ ID NO:3, a second polynucleotide encoding the pro region of SEQ ID NO:7 that includes a combination of substitutions chosen from E6A-E30G, E6R-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6S-E30G, E6W-E30G, E30G-A32R, E30G-A32Q, E30G-A32E, E30G-A32G, E30G-A32H, E30G-A32I, E30G-A32K, E30G-A32S, E30G-A32T, E30G-A32W, E30G-A32V, E6G-E30G-A32E, E6G-E30G-A32S, E6G-E30G-A32T, and E6G-E30G-A32W, and the third polynucleotide, which encodes the mature protease of SEQ ID NO:17. The expression vector is transformed into a *Bacillus sp.* host cell e.g. *Bacillus subtilis*, which is grown under suitable conditions to produce the mature protease.

[0025] In another embodiment, the method produces the mature protease of SEQ ID NO:19 by providing an expression vector that expresses an isolated modified polynucleotide comprising a first polynucleotide encoding the signal peptide of SEQ ID NO:3, a second polynucleotide encoding the pro region of SEQ ID NO:7 that includes a combination of substitutions chosen from E6A-E30G, E6R-E30G, E6N-E30G, E6D-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6M-E30G, E6F-E30G, E6P-E30G, E6S-E30G, E6T-E30G, E6W-E30G, E6V-E30G, and E6Y-E30G, and the third polynucleotide, which encodes the mature protease of SEQ ID NO:19. The expression vector is transformed into a *Bacillus sp.* host cell e.g. *Bacillus subtilis*, which is grown under suitable conditions to produce the mature protease.

[0026] In another embodiment, the method produces the mature protease of SEQ ID NO:21 by providing an expression vector that expresses an isolated modified polynucleotide comprising a first polynucleotide encoding the signal peptide of SEQ ID NO:3, a second polynucleotide encoding the pro

region of SEQ ID NO:7 that includes a combination of substitutions chosen from E6A-E30S, E6G-E30S, E6L-E30S, E6K-E30S, E6F-E30S, E6P-E30S, E6Y-E30S, E6V-E30S, E30S-A32R, E30S-A32N, E30S-A32D, E30S-A32C, E30S-A32Q, E30S-A32E, E30S-A32G, E30S-A32H, E30S-A32L, E30S-A32K, E30S-A32M, E30S-A32F, E30S-A32P, E30S-A32S, E30S-A32T, E30S-A32W, E30S-A32Y, and E30S-A32V, and the third polynucleotide, which encodes the mature protease of SEQ ID NO:21. The expression vector is transformed into a *Bacillus sp.* host cell e.g. *Bacillus subtilis*, which is grown under suitable conditions to produce the mature protease.

[0027] In other embodiments, the isolated modified polynucleotides comprise one amino acid substitution, which preferably enhances the production of a mature protease from a *Bacillus sp.* host cell. In one embodiment, the isolated modified polynucleotide comprises a first polynucleotide encoding the signal peptide of SEQ ID NO:3, and that is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7, which comprises the substitution of an amino acid chosen from E6A, E6R, E6C, E6Q, E6H, E6I, E6K, E6M, E6S, E6Y, E30A, E30R, E30N, E30D, E30Q, E30G, E30L, E30M, E30P, E30S, E30T, E30W, E30Y, E30V, A32, A32R, A32C, A32E, A32G, A32L, A32K, A32F, A32T, A32Y, and A32V. The second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of the protease of SEQ ID NO:17.

[0028] In another embodiment, the isolated modified polynucleotide comprises a first polynucleotide encoding the signal peptide of SEQ ID NO:3, and that is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7, which comprises the substitution of an amino acid chosen from E6A, E6R, E6N, E6C, E6Q, E6G, E6H, E6M, E6F, E6P, E6S, E6T, E6W, E6V, A32K, A32T, and A32V. The second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of the protease of SEQ ID NO:9.

[0029] In another embodiment, the isolated modified polynucleotide comprises a first polynucleotide encoding the signal peptide of SEQ ID NO:3, and that is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7, which comprises the substitution of an amino acid chosen from E6A, E6H, E6K, and E6R, E30A, E30R, E30N, E30D, E30G, E30H, E30L, E30K, E30F, E30S, E30T, and E30V. The second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of the protease of SEQ ID NO:19.

[0030] In another embodiment, the isolated modified polynucleotide comprises a first polynucleotide encoding the signal peptide of SEQ ID NO:3, and that is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7, which comprises the substitution of an amino acid chosen from E6A, E6R, E6Q, E6G, E6L, E6K, E6M, E6F, E6T, E6V, E30R, E30Q, E30G, E30I, E30L, E30M, E30F, E30P, E30T, E30W, E30Y, E30V, A32Q, A32S, A32T, and A32V. The second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of the protease of SEQ ID NO:11.

[0031] In another embodiment, the isolated modified polynucleotide comprises a first polynucleotide encoding the signal peptide of SEQ ID NO:3, and that is operably linked to a second polynucleotide that encodes the pro region set forth in SEQ ID NO:7, which comprises the substitution of an amino acid chosen from E30A, E30R, E30N, E30D, E30C, E30G, E30H, E30M, E30F, E30S, E30W, A32L, A32F, and A32V. The second polynucleotide is operably linked to a third polynucleotide that encodes the mature region of the protease of SEQ ID NO:21.

[0032] The method for producing the mature proteases expressed from modified polynucleotides comprising two or three substitutions in the pro region is also used for producing mature proteases

expressed from modified polynucleotides comprising single amino acid substitutions.

[0033] In one embodiment, the method includes providing any one of the expression vectors described above and containing a modified polynucleotide comprising a single amino acid substitution in the pro region, transforming the expression vector into a *Bacillus sp.* host cell, and culturing the transformed host cell under suitable conditions to produce the protease. Preferably, the host cell is a *Bacillus subtilis* host cell. However, in addition to *Bacillus subtilis*, other host cells that can be used to produce the mature proteases from the expression vector include *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus clausii*, *Bacillus halodurans*, *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, and *Bacillus thuringiensis*.

[0034] In one embodiment, the method produces the mature protease of SEQ ID NO:17 by providing an expression vector that expresses an isolated modified polynucleotide comprising a first polynucleotide encoding the signal peptide of SEQ ID NO:3, a second polynucleotide encoding the pro region of SEQ ID NO:7 that includes a single amino acid substitution chosen from E6A, E6R, E6C, E6Q, E6H, E6I, E6K, E6M, E6S, E6Y, E30A, E30R, E30N, E30D, E30Q, E30G, E30L, E30M, E30P, E30S, E30T, E30W, E30Y, E30V, A32, A32R, A32C, A32E, A32G, A32L, A32K, A32F, A32T, A32Y, and A32V, and the third polynucleotide, which encodes the mature protease of SEQ ID NO:17. The expression vector is transformed into a *Bacillus sp.* host cell *e.g.* *Bacillus subtilis*, which is grown under suitable conditions to produce the mature protease.

[0035] In another embodiment, the method produces the mature protease of SEQ ID NO:9 by providing an expression vector that expresses an isolated modified polynucleotide comprising a first polynucleotide encoding the signal peptide of SEQ ID NO:3, a second polynucleotide encoding the pro region of SEQ ID NO:7 that includes a single amino acid substitution chosen from E6A, E6R, E6N, E6C, E6Q, E6G, E6H, E6M, E6F, E6P, E6S, E6T, E6W, E6V, A32K, A32T, and A32V, and the third polynucleotide, which encodes the mature protease of SEQ ID NO:9. The expression vector is transformed into a *Bacillus sp.* host cell *e.g.* *Bacillus subtilis*, which is grown under suitable conditions to produce the mature protease.

[0036] In another embodiment, the method produces the mature protease of SEQ ID NO:19 by providing an expression vector that expresses an isolated modified polynucleotide comprising a first polynucleotide encoding the signal peptide of SEQ ID NO:3, a second polynucleotide encoding the pro region of SEQ ID NO:7 that includes a single amino acid substitution chosen from E6A, E6H, E6K, and E6R, E30A, E30R, E30N, E30D, E30G, E30H, E30L, E30K, E30F, E30S, E30T, and E30V, and the third polynucleotide, which encodes the mature protease of SEQ ID NO:19. The expression vector is transformed into a *Bacillus sp.* host cell *e.g.* *Bacillus subtilis*, which is grown under suitable conditions to produce the mature protease.

[0037] In another embodiment, the method produces the mature protease of SEQ ID NO:11 by providing an expression vector that expresses an isolated modified polynucleotide comprising a first polynucleotide encoding the signal peptide of SEQ ID NO:3, a second polynucleotide encoding the pro region of SEQ ID NO:7 that includes a single amino acid substitution chosen from E6A, E6R, E6Q, E6G, E6L, E6K, E6M, E6F, E6T, E6V, E30R, E30Q, E30G, E30I, E30L, E30M, E30F, E30P, E30T, E30W, E30Y, E30V, A32Q, A32S, A32T, and A32V, and the third polynucleotide, which encodes the mature protease of SEQ ID NO:11. The expression vector is transformed into a *Bacillus sp.* host cell *e.g.* *Bacillus subtilis*, which is grown under suitable conditions to produce the mature protease.

[0038] In another embodiment, the method produces the mature protease of SEQ ID NO:21 by

providing an expression vector that expresses an isolated modified polynucleotide comprising a first polynucleotide encoding the signal peptide of SEQ ID NO:3, a second polynucleotide encoding the pro region of SEQ ID NO:7 that includes a single amino acid substitution chosen from E30A, E30R, E30N, E30D, E30C, E30G, E30H, E30M, E30F, E30S, E30W, A32 L, A32F, and A32V, and the third polynucleotide, which encodes the mature protease of SEQ ID NO:21. The expression vector is transformed into a *Bacillus* sp. host cell e.g. *Bacillus subtilis*, which is grown under suitable conditions to produce the mature protease.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039]

Figure 1 shows an alignment of the amino acid sequences of the mature region of *B. lentus* wild-type serine protease of SEQ ID NO:9 (GG36), the *B. lentus* variant serine protease of SEQ ID NO:11, the *B. clausii* serine protease of SEQ ID NO:13, and the *B. clausii* variant serine proteases of SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23 and SEQ ID NO:25.

Figure 2 shows an alignment of the amino acid sequence of the unmodified pro region of SEQ ID NO:7 with that of unmodified pro regions of proteases from various *Bacillus* sp. resulting from a Blast search.

Figure 3 shows an alignment of the amino acid sequence of the mature protease (SEQ ID NO:9) with that of mature regions of proteases from various *Bacillus* sp. resulting from a Blast search.

Figure 4 provides the map of the pJH-Pn plasmid (A) and the map of the pBN3-Pn (B) vector comprising the aprE signal sequence (SEQ ID NO:3), the pro sequence of SEQ ID NO:7 and the polynucleotide encoding the mature serine protease Pn.

Figure 5 provides an alignment of exemplary polynucleotides (SEQ ID NOS:8, 10, 12, 14, 16, 18, 20, 22, and 24) that encode the mature proteases of SEQ ID NOS:9, 11, 13, 15, 17, 19, 21, 23, and 25, respectively. It is understood that a polypeptide may be coded for by more than one nucleotide sequence due to the degeneracy of the genetic code.

DESCRIPTION OF THE INVENTION

[0040] This invention provides modified polynucleotides encoding modified proteases, and methods for enhancing the production of proteases in microorganisms. In particular, the modified polynucleotides comprise one or more mutations that encode proteases having modifications e.g. amino acid substitutions, of the pro region to enhance the production of the active enzyme. The present invention further relates to methods for altering the expression of proteases in microorganisms, such as *Bacillus* species.

[0041] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains (e.g. Singleton and Sainsbury, Dictionary of Microbiology and Molecular Biology, 2d Ed., John Wiley and Sons, NY [1994]; and Hale and Markham, The Harper Collins Dictionary of Biology, Harper Perennial, NY [1991]). Although any methods and materials similar or equivalent to those described herein find use in

the practice of the present invention, the preferred methods and materials are described herein. Accordingly, the terms defined immediately below are more fully described by reference to the Specification as a whole. Also, as used herein, the singular "a", "an" and "the" includes the plural reference unless the context clearly indicates otherwise. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary, depending upon the context they are used by those of skill in the art.

[0042] It is intended that every maximum numerical limitation given throughout this specification include every lower numerical limitation, as if such lower numerical limitations were expressly written herein. Every minimum numerical limitation given throughout this specification will include every higher numerical limitation, as if such higher numerical limitations were expressly written herein. Every numerical range given throughout this specification will include every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

[0043] All patents, patent applications, articles and publications mentioned herein, both supra and infra, are hereby expressly incorporated herein by reference.

[0044] Furthermore, the headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole. Nonetheless, in order to facilitate understanding of the invention, a number of terms are defined below.

Definitions

[0045] As used herein, the terms "isolated" and "purified" refer to a nucleic acid or amino acid (or other component) that is removed from at least one component with which it is naturally associated.

[0046] The term "modified polynucleotide" herein refers to a polynucleotide sequence that has been altered to contain at least one mutation to encode a "modified" protein.

[0047] As used herein, the terms "protease" and "proteolytic activity" refer to a protein or peptide exhibiting the ability to hydrolyze peptides or substrates having peptide linkages. Many well known procedures exist for measuring proteolytic activity (Kalisz, "Microbial Proteinases," In: Fiechter (ed.), *Advances in Biochemical Engineering/Biotechnology*, [1988]). For example, proteolytic activity may be ascertained by comparative assays which analyze the produced protease's ability to hydrolyze a commercial substrate. Exemplary substrates useful in such analysis of protease or proteolytic activity, include, but are not limited to di-methyl casein (Sigma C-9801), bovine collagen (Sigma C-9879), bovine elastin (Sigma E-1625), and bovine keratin (ICN Biomedical 902111). Colorimetric assays utilizing these substrates are well known in the art (See e.g., WO 99/34011; and U.S. Pat. No. 6,376,450, both of which are incorporated herein by reference. The AAPF assay (See e.g., Del Mar et al., *Anal. Biochem.*, 99:316-320 [1979]) also finds use in determining the production of mature protease. This assay measures the rate at which p-nitroaniline is released as the enzyme hydrolyzes the soluble synthetic substrate, succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (sAAPF-pNA). The rate of production of yellow color from the hydrolysis reaction is measured at 410 nm on a spectrophotometer and is proportional to the active enzyme concentration. In particular, the term "protease" herein refers to a

"serine protease".

[0048] As used herein, the terms "subtilisin" and "serine protease" refer any member of the S8 serine protease family as described in MEROPS - The Peptidase Data base (Rawlings et al., MEROPS: the peptidase database, Nucleic Acids Res, 34 Database issue, D270-272, 2006, at the website merops.sanger.ac.uk/cgi-bin/merops.cgi?id=s08;action=.). The following information was derived from MEROPS - The Peptidase Data base as of November 6, 2008 "Peptidase family S8 contains the serine endopeptidase serine protease and its homologues (Biochem J, 290:205-218, 1993). Family S8, also known as the subtilase family, is the second largest family of serine peptidases, and can be divided into two subfamilies, with subtilisin (S08.001) the type-example for subfamily S8A and kexin (S08.070) the type-example for subfamily S8B. Tripeptidyl-peptidase II (TPP-II; S08.090) was formerly considered to be the type-example of a third subfamily, but has since been determined to be misclassified.

[0049] The term "parent protease" herein refers to a full-length protease comprising pre, pro and mature regions that are naturally expressed in combination. In some embodiments, the pre and/or pro and/or mature regions of a parent protease serve to originate the pre and/or pro and/or mature regions of a precursor protease.

[0050] The term "precursor protease" herein refers to an unmodified full-length protease comprising a signal peptide, a pro region and a mature region. The precursor protease can be derived from naturally-occurring *i.e.* wild-type proteases, or from variant proteases. It is the pro region of a precursor protease that is modified to generate a modified protease. In some embodiments, the precursor protease comprises a pro region and a mature region that are derived from one parent protease. In other embodiments, the precursor protease is a chimeric protein that comprises a pro region that is derived from one parent protease and a mature region that is derived from a different parent protease.

[0051] The term "chimeric" or "fusion" when used in reference to a protein, herein refer to a protein created through the joining of two or more polynucleotides which originally coded for separate proteins. Translation of this fusion polynucleotide results in a single chimeric polynucleotide with functional properties derived from each of the original proteins. Recombinant fusion proteins are created artificially by recombinant DNA technology. A "chimeric polypeptide," or "chimera" means a protein containing sequences from more than one polypeptide. A modified protease can be chimeric in the sense that it contains a portion, region, or domain from one protease fused to one or more portions, regions, or domains from one or more other protease. By way of example, a chimeric protease might comprise the mature region of one protease linked to the pro peptide of another protease. The skilled artisan will appreciate that chimeric polypeptides and proteases need not consist of actual fusions of the protein sequences, but rather, polynucleotides with the corresponding encoding sequences can also be used to express chimeric polypeptides or proteases.

[0052] "Naturally-occurring" or "wild-type" herein refer to a protease, or a polynucleotide encoding a protease having the unmodified amino acid sequence identical to that found in nature. Naturally occurring enzymes include native enzymes, those enzymes naturally expressed or found in the particular microorganism. A sequence that is wild-type or naturally-occurring refers to a sequence from which a variant is derived. The wild-type sequence may encode either a homologous or heterologous protein.

[0053] As used herein, "variant" refers to a mature protein which differs from its corresponding wild-type mature protein by the addition of one or more amino acids to either or both the C- and N-terminal end, substitution of one or more amino acids at one or a number of different sites in the amino acid sequence, deletion of one or more amino acids at either or both ends of the protein or at one or more sites in the

amino acid sequence, and/or insertion of one or more amino acids at one or more sites in the amino acid sequence of the mature protein. Variant proteins encompass naturally-occurring variants and genetically engineered variant proteins. A variant protein in the context of the present invention is exemplified by the *B. lentus* protease of SEQ ID NO:11, which is a variant of the naturally-occurring protein *B. lentus* protease GG36 (SEQ ID NO:9), from which it differs by three amino acid substitutions at positions 74, 101 and 102 of the mature region. Another example of a variant protease is the *B. clausii* protease SEQ ID NO:19, which is a variant of the naturally-occurring protein *B. clausii* protease Maxacal (SEQ ID NO:13), from which it differs by two amino acid substitutions at positions 99 and 102 of the mature region (Figure 1).

[0054] As used herein, "homolog" and "homologous protein" refers to a protein (e.g., protease) that has similar action and/or structure, as a protein of interest (e.g., a protease from another source). It is not intended that homologs be necessarily related evolutionarily. Thus, it is intended that the term encompass the same or similar enzyme(s) (i.e., in terms of structure and function) obtained from different species.

[0055] The terms "derived from" and "obtained from" refer to not only a protease produced or producible by a strain of the organism in question, but also a protease encoded by a DNA sequence isolated from such strain and produced in a host organism containing such DNA sequence. Additionally, the term refers to a protease which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the protease in question. To exemplify, "proteases derived from *Bacillus*" refers to those enzymes having proteolytic activity which are naturally-produced by *Bacillus*, as well as to serine proteases like those produced by *Bacillus* sources but which through the use of genetic engineering techniques are produced by non-*Bacillus* organisms transformed with a nucleic acid encoding said serine proteases.

[0056] A "modified full-length protease", a "modified precursor protease" or a "modified protease" are interchangeably used to refer to a full-length protease that comprises a signal peptide, a mature region and a pro region that are derived from a parent or precursor protease, wherein the pro region is modified to contain at least one mutation. In some embodiments, the pro region and the mature region are derived from the same parent protease. In other embodiments, the pro region and the mature region are derived from different parent proteases. The modified protease comprises a pro region that is modified to contain at least one mutation, and it is encoded by a modified polynucleotide. The amino acid sequence of the modified protease is said to be "generated" from the parent protease amino acid sequence by introducing into the pro region of the parent amino acid sequence at least one mutation e.g. a substitution, deletion or insertion of one or more amino acids. In some embodiments, one or more amino acids of the pro region of the precursor protease are substituted to generate the modified full-length protease. Such modification is of the "precursor" DNA sequence which encodes the amino acid sequence of the "precursor" protease rather than manipulation of the precursor protease per se.

[0057] The term "unmodified" when used in reference to a protease polypeptide or polynucleotide, herein refers to a protease comprising a pro region that has not been modified to comprise at least one mutation e.g. a substitution.

[0058] The terms "full-length protein" and "pre-pro-protein" herein refer to a gene product comprising a signal peptide, a pro sequence and a mature sequence. For example, the full-length protease of SEQ ID NO:59 comprises the signal peptide (pre region) (SEQ ID NO:3, encoded for example by the pre polynucleotide of SEQ ID NO:2), the pro region (SEQ ID NO:7, encoded for example by the pre polynucleotide of SEQ ID NO:6), and the mature region (SEQ ID NO:9 encoded by the polynucleotide of

SEQ ID NO:8).

[0059] The term "signal sequence", "signal peptide" or "pre region" refers to any sequence of nucleotides and/or amino acids which may participate in the secretion of the mature or precursor forms of the protein. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by the N-terminal portion of the protein gene, which participate in the effectuation of the secretion of protein. To exemplify, a pre peptide of a protease of the present invention at least includes the amino acid sequence identical to residues 1-29 of SEQ ID NO:3.

[0060] The term "pro sequence" or "pro region" is an amino acid sequence between the signal sequence and mature protease that is necessary for the secretion/production of the protease. Cleavage of the pro sequence will result in a mature active protease. To exemplify, a pro region of a protease of the present invention at least includes the amino acid sequence identical to residues 1-84 of the pro region of SEQ ID NO:7, which correspond to amino acids 30-113 of the full-length protease of SEQ ID NO:59.

[0061] The terms "mature form" or "mature region" refer to the final functional portion of the protein. To exemplify, a mature form of the protease of the present invention includes the amino acid sequence identical to residues 1-269 of SEQ ID NO:9. In this context, the "mature form" is "processed from" a full-length protease, wherein the processing of the full-length protease encompasses the removal of the signal peptide and the removal of the pro region.

[0062] The terms "pro-protein", "pro-polypeptide" and "pro-protease", herein refer to a protein comprising the mature form operably linked to a pro-polypeptide. A "pro-polypeptide" is encoded by a "pro-polynucleotide".

[0063] As used herein, the term "heterologous protein" refers to a protein or polypeptide that does not naturally occur in the host cell. Similarly, a "heterologous polynucleotide" refers to a polynucleotide that does not naturally occur in the host cell. Heterologous polypeptides and/or heterologous polynucleotides include chimeric polypeptides and/or polynucleotides.

[0064] As used herein, "substituted" and "substitutions" refer to replacement(s) of an amino acid residue or nucleic acid base in a parent sequence. In some embodiments, the substitution involves the replacement of a naturally occurring residue or base. The modified proteases herein encompass the substitution of any one of the 84 amino acids of the pro region of the precursor protease by any one of the remaining nineteen amino acids. For example, the substitution at position 6 (E6) is a replacement of a glutamic acid (E) with one of the group consisting of alanine (A), cysteine (C), aspartic acid (D), glycine (G), phenylalanine (F), histidine (H), isoleucine (I), lysine (K), leucine (L), methionine (M), asparagine (N), proline (P), glutamine (Q), arginine (R), serine (S), threonine (T), valine (V), thryptophan (W), and tyrosine (Y). A substitution of an amino acid e.g. E6, for any other amino acid at the same position is denoted by E6X, wherein X is one of the remaining 19 amino acids that substitutes E at position 6. In some embodiments, two or more amino acids are substituted to generate a modified protease that comprises a combination of amino acid substitutions. For example, a combination of a substitution of amino acid E at position 6 for amino acid A in combination with the substitution of amino acid E at position 30 for amino acid T is denoted as E6A-E30T. Amino acid positions for the substitutions in the pro region are numbered corresponding to the numbered position in the pro region of SEQ ID NO:7.

[0065] As used herein, "by correspondence to", "corresponding to," or "equivalent to" refers to a residue at the enumerated position in a protein or peptide, or a residue that is analogous, homologous, or

equivalent to an enumerated residue in a protein or peptide. As used herein, "corresponding region," generally refers to an analogous position along related proteins or a reference protein.

[0066] The terms "pre polynucleotide", "pro nucleotide" and "mature polynucleotide" herein refer to the polynucleotide sequences that respectively encode for the pre, pro and mature regions of a protein *e.g.* a protease.

[0067] The term "production" with reference to a protease, encompasses the two processing steps of a full-length protease including: 1. the removal of the signal peptide, which is known to occur during protein secretion; and 2. the removal of the pro region, which creates the active mature form of the enzyme and which is known to occur during the maturation process (Wang et al., *Biochemistry* 37:3165-3171 (1998); Power et al., *Proc Natl Acad Sci USA* 83:3096-3100 [1986]). The term "enhanced production" herein refers to the production of a mature protease that is processed from a modified full-length protease, and which occurs at a level that is greater than the level of production of the same mature protease when processed from an unmodified full-length protease.

[0068] The term "processed" with reference to a mature protease refers to the maturation process that a full-length protein *e.g.* a full-length protease, undergoes to become an active mature enzyme.

[0069] "Activity" with respect to enzymes means "catalytic activity" and encompasses any acceptable measure of enzyme activity, such as the rate of activity, the amount of activity, or the specific activity. Catalytic activity refers to the ability to catalyze a specific chemical reaction, such as the hydrolysis of a specific chemical bond. As the skilled artisan will appreciate, the catalytic activity of an enzyme only accelerates the rate of an otherwise slow chemical reaction. Because the enzyme only acts as a catalyst, it is neither produced nor consumed by the reaction itself. The skilled artisan will also appreciate that not all polypeptides have a catalytic activity. "Specific activity" is a measure of activity of an enzyme per unit of total protein or enzyme. Thus, specific activity may be expressed by unit weight (*e.g.* per gram, or per milligram) or unit volume (*e.g.* per ml) of enzyme. Further, specific activity may include a measure of purity of the enzyme, or can provide an indication of purity, for example, where a standard of activity is known, or available for comparison. The amount of activity reflects to the amount of enzyme that is produced by the host cell that expresses the enzyme being measured.

[0070] The term "relative activity" or "ratio of production" are used herein interchangeably to refer to the ratio of the enzymatic activity of a mature protease that was processed from a modified protease to the enzymatic activity of a mature protease that was processed from an unmodified protease. The ratio of production is determined by dividing the value of the activity of the protease processed from a modified precursor by the value of the activity of the same protease when processed from an unmodified precursor. The relative activity is the ratio of production expressed as a percentage.

[0071] As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

[0072] The term "percent (%) identity" is defined as the percentage of amino acid /nucleotide residues in a candidate sequence that are identical with the amino acid residues/ nucleotide residues of the precursor sequence (*i.e.*, the parent sequence). A % amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. Amino acid sequences may be similar, but are not "identical" where an amino acid is substituted, deleted, or inserted in the subject sequence relative to the reference sequence. For proteins, the percent sequence identity is preferably measured between sequences that

are in a similar state with respect to posttranslational modification. Typically, the "mature sequence" of the subject protein, i.e., that sequence which remains after processing to remove a signal sequence, is compared to a mature sequence of the reference protein. In other instances, a precursor sequence of a subject polypeptide sequence may be compared to the precursor of the reference sequence.

[0073] As used herein, the term "promoter" refers to a nucleic acid sequence that functions to direct transcription of a downstream gene. In some embodiments, the promoter is appropriate to the host cell in which the target gene is being expressed. The promoter, together with other transcriptional and translational regulatory nucleic acid sequences (also termed "control sequences") is necessary to express a given gene. In general, the transcriptional and translational regulatory sequences include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences.

[0074] A nucleic acid or a polypeptide is "operably linked" when it is placed into a functional relationship with another nucleic acid or polypeptide sequence, respectively. For example, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation; or a modified pro region is operably linked to a mature region of a protease if it enables the processing of the full-length protease to produce the mature active form of the enzyme. Generally, "operably linked" means that the DNA or polypeptide sequences being linked are contiguous.

[0075] A "host cell" refers to a suitable cell that serves as a host for an expression vector comprising DNA according to the present invention. A suitable host cell may be a naturally occurring or wild-type host cell, or it may be an altered host cell. In one embodiment, the host cell is a Gram positive microorganism. In some embodiments, the term refers to cells in the genus *Bacillus*.

[0076] As used herein, "*Bacillus* sp." includes all species within the genus "*Bacillus*," as known to those of skill in the art, including but not limited to *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. pumilis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. clausii*, *B. halodurans*, *B. megaterium*, *B. coagulans*, *B. circulans*, *B. lautus*, and *B. thuringiensis*. It is recognized that the genus *Bacillus* continues to undergo taxonomical reorganization. Thus, it is intended that the genus include species that have been reclassified, including but not limited to such organisms as *B. stearothermophilus*, which is now named "*Geobacillus stearothermophilus*." The production of resistant endospores in the presence of oxygen is considered the defining feature of the genus *Bacillus*, although this characteristic also applies to the recently named *Alicyclobacillus*, *Amphibacillus*, *Aneurinibacillus*, *Anoxybacillus*, *Brevibacillus*, *Filobacillus*, *Gracilibacillus*, *Halobacillus*, *Paenibacillus*, *Salibacillus*, *Thermobacillus*, *Ureibacillus*, and *Virgibacillus*.

[0077] The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length. These terms include, but are not limited to, a single-, double-stranded DNA, genomic DNA, cDNA, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. Non-limiting examples of polynucleotides include genes, gene fragments, chromosomal fragments, ESTs, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. It will be understood that, as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding a given protein may be produced.

[0078] As used herein, the terms "DNA construct" and "transforming DNA" are used interchangeably to

refer to DNA used to introduce sequences into a host cell or organism. The DNA construct may be generated in vitro by PCR or any other suitable technique(s) known to those in the art. In some embodiments, the DNA construct comprises a sequence of interest (e.g., a sequence encoding a modified protease). In some embodiments, the sequence is operably linked to additional elements such as control elements (e.g., promoters, etc.). The DNA construct may further comprise a selectable marker. In some embodiments, the DNA construct comprises sequences homologous to the host cell chromosome. In other embodiments, the DNA construct comprises non-homologous sequences. Once the DNA construct is assembled in vitro it may be used to mutagenize a region of the host cell chromosome (i.e., replace an endogenous sequence with a heterologous sequence).

[0079] As used herein, the term "expression cassette" refers to a nucleic acid construct generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a vector such as a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid sequence to be transcribed and a promoter. In some embodiments, expression vectors have the ability to incorporate and express heterologous DNA fragments in a host cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those of skill in the art. The term "expression cassette" is used interchangeably herein with "DNA construct," and their grammatical equivalents. Selection of appropriate expression vectors is within the knowledge of those of skill in the art.

[0080] As used herein, the term "heterologous DNA sequence" refers to a DNA sequence that does not naturally occur in a host cell. In some embodiments, a heterologous DNA sequence is a chimeric DNA sequence that is comprised of parts of different genes, including regulatory elements.

[0081] As used herein, the term "vector" refers to a polynucleotide construct designed to introduce nucleic acids into one or more cell types. Vectors include cloning vectors, expression vectors, shuttle vectors, and plasmids. In some embodiments, the polynucleotide construct comprises a DNA sequence encoding the full-length protease (e.g., modified protease or unmodified precursor protease). As used herein, the term "plasmid" refers to a circular double-stranded (ds) DNA construct used as a cloning vector, and which forms an extrachromosomal self-replicating genetic element in some eukaryotes or prokaryotes, or integrates into the host chromosome.

[0082] As used herein in the context of introducing a nucleic acid sequence into a cell, the term "introduced" refers to any method suitable for transferring the nucleic acid sequence into the cell. Such methods for introduction include but are not limited to protoplast fusion, transfection, transformation, conjugation, and transduction (See e.g., Ferrari et al., "Genetics," in Hardwood et al, (eds.), Bacillus, Plenum Publishing Corp., pages 57-72, [1989]).

[0083] As used herein, the terms "transformed" and "stably transformed" refers to a cell that has a non-native (heterologous) polynucleotide sequence integrated into its genome or as an episomal plasmid that is maintained for at least two generations.

Modified Proteases

[0084] The present invention provides methods and compositions for the production of mature

proteases in bacterial host cells. The compositions include modified polynucleotides that encode modified proteases, which have at least one mutation in the pro region; the modified serine proteases encoded by the modified polynucleotides; expression cassettes, DNA constructs, and vectors comprising the modified polynucleotides that encode the modified proteases; and the bacterial host cells transformed with the vectors of the invention. The methods include methods for enhancing the production of mature proteases in bacterial host cells *e.g.* *Bacillus sp.* host cells. The produced proteases find use in the industrial production of enzymes, suitable for use in various industries, including but not limited to the cleaning, animal feed and textile processing industry.

[0085] The basic mechanism by which proteins are transported across membranes appears to be universal, with important features conserved between bacteria and eukaryotes. Because they can secrete certain proteins in large quantities into the growth medium, *Bacillus* species are used for the industrial production of enzymes such as alkaline serine proteases. Proteases are produced in vivo from a precursor protease known as a pre-pro-protease, which comprises a pre region, also known as signal peptide, a pro region and a mature region of the protease. Protein secretion across the *Bacillus sp.* cell envelope is a complex process that includes insertion of the precursor protein into the membrane and translocation of the protein across the membrane. The pre region serves as a signal peptide for protein secretion across the membrane and is hydrolyzed by a signal peptidase. The extracellular part of the maturation process involves folding of the pro-protease, self-processing of the pro region, and degradation of the pro-region to create the active mature form of the enzyme (Nagarajan V. Protein Secretion in "Bacillus subtilis and other Gram-Positive Bacteria" Ch.49, p 713 - 726 [1993]; Ruan et al., Biochemistry, 38:8562-8571 [2009]).

[0086] In some embodiments, the invention provides a modified polynucleotide encoding a modified protease that is generated by introducing at least one mutation in the pro polynucleotide of the precursor protease. The modified polynucleotide is generated from a precursor polynucleotide that comprises a polynucleotide encoding the pro region of the protease (pro polynucleotide), and a polynucleotide encoding the mature region of the protease (mature polynucleotide), wherein the pro polynucleotide is modified to contain at least one mutation to generate a modified polynucleotide that encodes the modified protease of the invention. The precursor polynucleotide further comprises a polynucleotide encoding a signal peptide (pre polynucleotide). The pre, pro and mature regions of the unmodified protease can be derived from a wild-type or variant parent protease of animal, vegetable or microbial origin. In some embodiments, the pro and mature regions of the unmodified precursor protease are derived from one parent protease, while the pre region is derived from a different parent protease. In other embodiments, the pre, pro and mature regions are derived from three different parent proteases. In some embodiments, the parent protease is of bacterial origin. In some embodiments, the parent protease is a protease of the subtilisin type (subtilases, subtilopeptidases, EC 3.4.21.62), which comprise catalytically active amino acids, also referred to as serine proteases. In some embodiments, the parent protease is a *Bacillus sp.* protease. Preferably, the parent protease is a serine protease derived from *Bacillus clausii*, or *Bacillus lentus*.

Precursor polynucleotides encoding precursor proteases

[0087] In some embodiments, the unmodified precursor polynucleotide encodes a full-length protease comprising the mature region of a parent protease, such as a protease derived from *Bacillus clausii* and *Bacillus lentus*, homologs and variants thereof, operably linked to a polynucleotide *e.g.* gctgaagaagcaaaagaaaaatatattaattggcttaatgagcaggaagctgtcagtgagttttagaacaagtagaggcaaatgacgaggtcgc

cattctctctgaggaagaggaagtcgaaattgaattgctcatgaattgaaacgattcctgtttatccgttgagttaagcccagaagatgtggacgcg
cttgaactcgatccagcgatttcttatattgaagaggatgcagaagtaacgacaatg (SEQ ID NO:6), that encodes the pro
region of SEQ ID NO:7

AEAAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAIS
YIEEDA EVTTM (SEQ ID NO:7). Examples of mature parent proteases include the wild-type *B. lentus*
protease

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNGHGHVAG
TIAALNNSIGVLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVN
SATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYAGLDIVAPGVNVQSTYPGS
TYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR (SEQ ID
NO:9), and variants thereof such as

the protease of SEQ ID NO:11

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNGHGHVAG
TIAALDNSIGVLGVAPSAELYAVKVLGASGSGAIISSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVN
SATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYAGLDIVAPGVNVQSTYPGS
TYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR (SEQ ID
NO:11); the wild-type

Bacillus clausii PB92 protease Maxacal (U.S. Patent 5,217,878)

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNGHGHVAG
TIAALNNSIGVLGVAPNAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAV
NSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYAGLDIVAPGVNVQSTYPG
STYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; (SEQ ID
NO:13), and variants thereof such as

the protease of SEQ ID NO:15

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNGHGHVAG
TIAALNNSIGVLGVAPNAELYAVKVLGASGSGSVSSIAQGLEWAGNNVMHVANLSLGLQAPSATLEQAVN
SATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYAGLDIVAPGVNVQSTYPGS
TYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; (SEQ
ID NO:15),

the protease of SEQ ID NO:17

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNGHGHVAG
TIAALNNSIGVLGVAPNAELYAVKVLGASGSGSVSSIAQGLEWAGNNVMHVANLSLGLQAPSATLEQAV
NSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYAGLDIVAPGVNVQSTYPG
STYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; (SEQ
ID NO:17),

the protease of SEQ ID NO:19

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNGHGHVAG
TIAALNNSIGVLGVAPNAELYAVKVLGASGGGSSNSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAV
NSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYAGLDIVAPGVNVQSTYPG
STYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; (SEQ
ID NO:19),

the protease of SEQ ID NO:21

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNGHGHVAG

TIAALDNSIGVLGVAPRAELYAVKVLGASGSGSVSSIAQGLEWAGNNRMHVANLSLGLQAPSATLEQAVN
SATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYAGLDIVAPGVNVQSTYPGS
TYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; (SEQ
ID NO:21),

the protease of SEQ ID NO:23

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNGHGHVAG
TIAALDNSIGVLGVAPRAELYAVKVLGASGSGSVSSIAQGLEWAGNNRMHVANLSLGLQAPSATLEQAVN
SATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRADFSQYAGLDIVAPGVNVQSTYPGS

TYASLNGTSMATPHVAGAAALVKQKNPSWSNRQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; (SEQ ID NO:23); and

the protease of SEQ ID NO:25

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNHGHGTHVAGTIAALDNSIGVLGVAPRAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVANLSLGLQAPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRADFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRRHKLKNTATSLGSTNLYGSGLVNAEAATR; (SEQ ID NO:25).

Examples of polynucleotides encoding the mature proteases of SEQ ID NOS:9, 11, 13, 15, 17, 19, 21, 23 and 25 are SEQ ID NOS:8, 10, 12, 14, 16, 18, 20, 22, and 24, respectively, shown in Figure 5. It is understood that a polypeptide may be coded for by more than one nucleotide sequence due to the degeneracy of the genetic code.

[0088] The mature proteases of SEQ ID NOS: 9, 11, 13, 15, 17, 19, 21, 23 and 25 differ from each other by up to 9 amino acids (Figure 1). In some embodiments, the pro polypeptide of SEQ ID NO:7 is naturally and operably linked to the mature sequences of SEQ ID NOS: 9, 11, 13, 15, 17, 19, 21, 23 and 25. Thus, in some embodiments, the precursor polynucleotides comprise polynucleotides encoding the pro region of SEQ ID NO:7 is operably linked to a mature region chosen from SEQ ID NOS: 9, 11, 13, 15, 17, 19, 21, 23 and 25, resulting in the pro-proteases of SEQ ID NOS:38-46, respectively:

SEQ ID NO:38:

AEAAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAISYIEEDA EVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNHGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; (SEQ ID NO:38);

SEQ ID NO:39:

AEAAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAISYIEEDA EVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNHGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAIISSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; (SEQ ID NO:39);

SEQ ID NO:40:

AEAAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAISYIEEDA EVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNHGHGTHVAGTIAALNNSIGVLGVAPNAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; (SEQ ID NO:40);

SEQ ID NO:41:

AEAAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAISYIEEDA EVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDGNHGHGTHVAGTIAALNNSIGVLGVAPNAELYAVKVLGASGSGSVSSIAQGLEWAGNNVMHVANLSLGLQAPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; (SEQ ID NO:41);

SEQ ID NO:42:

AEAAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAISYIEEDA EVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQD

GNGHG I HVAG I I AALNNSIGVLGVAPNAELYAVKVLGASGMGSVSSIAQGLEWAGNNVMHVNLSLGLQ
 APSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAP
 GVNQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVN
 AEAATR; SEQ ID NO:42);

SEQ ID NO:43:

AAEAKKEYLIGFNEQEAVSEFVEQVEANDEVAILSEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAIS
 YIEEDA EVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQD
 GNGHGTHVAGTIAALNNSIGVLGVAPNAELYAVKVLGASGGGSSNSIAQGLEWAGNNGMHVNLSLGS
 PPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAP
 GVNQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVN
 AEAATR; SEQ ID NO:43);

SEQ ID NO:44:

AAEAKKEYLIGFNEQEAVSEFVEQVEANDEVAILSEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAIS
 YIEEDA EVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQD
 GNGHGTHVAGTIAALDNSIGVLGVAPRAELYAVKVLGASGSGSVSSIAQGLEWAGNNRMHVNLSLGLQ
 APSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAP
 GVNQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVN
 AEAATR; SEQ ID NO:44);

SEQ ID NO:45:

AAEAKKEYLIGFNEQEAVSEFVEQVEANDEVAILSEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAIS
 YIEEDA EVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQD
 GNGHGTHVAGTIAALDNSIGVLGVAPRAELYAVKVLGASGSGSVSSIAQGLEWAGNNRMHVNLSLGLQ
 APSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRADFSQYGAGLDIVAP

GVNQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNRQIRNHLKNTATSLGSTNLYGSGLVN
 AEAATR; SEQ ID NO:45); and

SEQ ID NO:46:

AAEAKKEYLIGFNEQEAVSEFVEQVEANDEVAILSEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAIS
 YIEEDA EVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQD
 GNGHGTHVAGTIAALDNSIGVLGVAPRAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVNLSLGLQ
 APSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRADFSQYGAGLDIVAP
 GVNQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRRHKLKNTATSLGSTNLYGSGLVN
 AEAATR (SEQ ID NO:46).

[0089] Other mature parent proteases that are operably linked to the pro polypeptide of SEQ ID NO:7, comprise homologs of mature proteases from *Bacillus* sp. such as P27693_Bacillus_alkalophilus e.g. SEQ ID NO:47, P20724_Bacillus_sp_YAB e.g. SEQ ID NO:48, BAA25184_Bacillus_sp e.g. SEQ ID NO:49, YP_174261_B_clausii_KSM-K16 e.g. SEQ ID NO:50, BAA06157 Bacillus sp G-825-6 (SEQ ID NO:51) and BAF34115_A_transvaalensis e.g. SEQ ID NO:52 (Figure 3). In some embodiments, the unmodified precursor polynucleotide encodes a precursor protease comprising the mature region of a protease that is at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, at least about 99% identical to the mature region of SEQ ID NO:9, 11, 13, 15, 17, 19, 21, 23 and 25 which is operably linked to the pro polypeptide of SEQ ID NO:7.

[0090] Subjecting the amino acid sequence of the pro region of SEQ ID NO:7 to a BLAST query revealed that in addition to being identical to the pro region of the naturally-occurring pro region of the P41362 B. clausii and P27693 Bacillus alkalophilus (Figure 2), the pro region of SEQ ID NO:7 has a high degree of identity with the amino acid sequence of the pro region of proteases from as GG36 B. lentus

267048, (SEQ ID NO:53), P20724_Bacillus_sp_YAB (SEQ ID NO:54), BAA25184_Bacillus_sp (SEQ ID NO:55), YP_174261_B_clausii_KSM-K16 *e.g.* SEQ ID NO:56, BAA06157 Bacillus sp G-825-6 (SEQ ID NO:57) and BAF34115_A_transvaalensis *e.g.* SEQ ID NO:58, (Figure 2). It is expected that mutations made in the pro region of SEQ ID NOS:53-58 and corresponding to the mutations of SEQ ID NO:7 that enhance the production of the mature protease to which it is operably linked, will enhance the production of the mature protease to which the pro region of SEQ ID NOS:53-58 is operably linked. Thus, in some embodiments, the unmodified precursor polynucleotide comprises a pro polynucleotide encoding a pro polypeptide that is chosen from SEQ ID NOS:53-58 and that is operably linked to the mature protease of SEQ ID NO:9, variants and homologs thereof. For example, the pro polynucleotide encoding a pro polypeptide chosen from SEQ ID NOS:53-58 is operably linked to a variant of the mature protease of SEQ ID NO:9 *e.g.* SEQ ID NOS:11, 13, 15, 17, 18, 21, 23, and 25. Similarly, the pro polynucleotide encoding a pro polypeptide chosen from SEQ ID NOS:53-58 is operably linked to a homolog of the mature protease of SEQ ID NO:9 *e.g.* SEQ ID NOS: SEQ ID NO:47-52. In other embodiments, the unmodified precursor polynucleotide comprises a pro polynucleotide encoding a pro polypeptide that is at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97% or at least about 99% identical to that of SEQ ID NO:7 operably linked to the mature protease of SEQ ID NO:9 or any one of SEQ ID NOS: 11, 13, 15, 17, 18, 21, 23, and 25 and 47-52.

[0091] The percent identity shared by polynucleotide or polypeptide sequences is determined by direct comparison of the sequence information between the molecules by aligning the sequences and determining the identity by methods known in the art. An example of an algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul, et al., J. Mol. Biol., 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. These initial neighborhood word hits act as starting points to find longer HSPs containing them. The word hits are expanded in both directions along each of the two sequences being compared for as far as the cumulative alignment score can be increased. Extension of the word hits is stopped when: the cumulative alignment score falls off by the quantity X from a maximum achieved value; the cumulative score goes to zero or below; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (See, Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M⁵, N⁻⁴, and a comparison of both strands.

[0092] The BLAST algorithm then performs a statistical analysis of the similarity between two sequences (See *e.g.*, Karlin and Altschul, Proc. Nat'l. Acad. Sci. USA 90:5873-5787 [1993]). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a serine protease nucleic acid of this invention if the smallest sum probability in a comparison of the test nucleic acid to a serine protease nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001. Where the test nucleic acid encodes a serine protease polypeptide, it is considered similar to a specified serine protease nucleic acid if the comparison results in a smallest sum probability of less than about 0.5, and more preferably less than about 0.2.

[0093] The pro region of SEQ ID NO:7 was used to search the NCBI non-redundant protein database (version March 26, 2009). The command line BLAST program (version 2.2.17) was used with default

parameters. The obtained sequences that were found to have sequences similar to the pro region (SEQ ID NO:7) were divided into pro regions and mature regions, which were further analyzed as follows to generate the alignments shown in Figures 2 and 3.

[0094] The alignments of the amino acid sequences of the pro region (Figure 2) and the mature region (Figure 3) of various serine proteases to the pro region (SEQ ID NO:7) and mature region of GG36 (SEQ ID NO:9) were obtained using the multiple alignment programs ClustalW and MUSCLE. The alignment was first performed using the program ClustalW (version 1.83) with default parameters. The alignment was refined five times using the program MUSCLE (version 3.51) with default parameters. Only the regions corresponding to the mature region or pro region of were chosen in the alignment. The percent identity was calculated as the number of identical residues aligned between the two sequences in question divided by the number of residues aligned in the alignment. As discussed above, the alignments show that there are several pro and mature sequences that share a high degree of amino acid identity to that of the pro (SEQ ID NO:7) and mature (SEQ ID NO:9) regions of GG36.

[0095] In some embodiments, in addition to encoding the pro-protease, the unmodified precursor polynucleotide further comprises a pre polynucleotide encoding a signal peptide, which is operably linked to the pro-protease. In some embodiments, the signal peptide is the AprE signal peptide VRSKKLWISLLFALTILFTMAFSNMSAQA (SEQ ID NO: 3) encoded by the polynucleotide of gtgagaagcaaaaaattgtggatcagctgtgttgcgttaacgtaatcttacgatggcgttcagcaacatgtctgcgcaggct (SEQ ID NO:2). In other embodiments, the signal peptide is a fusion signal peptide VRSKKLWIVASTALLISVAFSSSIASA (SEQ ID NO:5) encoded by the polynucleotide of gtgagaagcaaaaaattgtggatcgtcgcgtcgaccgcactactcatttctgttgccttttagttcatcgatcgcatcggct (SEQ ID NO:4). In yet other embodiments, the precursor polynucleotide comprises the polynucleotide that encodes the signal peptide that is naturally and operably linked to the pro-protease. Any signal sequence that can effectuate efficient secretion of a modified protease in a *Bacillus* sp host cell can be operably linked to a pro-protease of the invention. Such signal peptides include signal peptides of bacterial origin that direct secretion of proteins via bacterial secretion pathways e.g. Sec pathway, TAT pathway, and eukaryotic signal sequences that are applicable for expressing proteins in prokaryotic host cells (EP1481059B1).

Modified polynucleotides encoding modified proteases

[0096] The unmodified precursor polynucleotide described above, is modified to encode a modified protease by introducing at least one mutation at any one of amino acids at positions 1- 84 of the pro polypeptide of SEQ ID NO:7, which is operably linked to a mature protease. In some embodiments, the at least one mutation is an amino acid substitution.

[0097] In some embodiments, the modified polynucleotide encodes an amino acid substitution at least at one amino acid position selected from positions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, and 84, of the pro polypeptide of SEQ ID NO:7, operably linked to a mature protease that is at least 60% identical to the mature protease of SEQ ID NO:9. In some embodiments, the mature protease is at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, at least about 99% identical to the mature region of SEQ ID NO:9. Mature proteases that are at least 60% identical to the mature protease of SEQ ID NO:9 (*B. lentus* protease GG36), include the

wild-type *Bacillus clausii* PB92 protease Maxacal (SEQ ID NO:13), and variants thereof such as SEQ ID NO:11, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, and SEQ ID NO:25; and homologs of SEQ ID NO:9 including homologs of mature proteases from *Bacillus sp.* such as P27693_*Bacillus alcalophilus* e.g. SEQ ID NO:47, P20724_*Bacillus sp.*_YAB e.g. SEQ ID NO:48, BAA25184_*Bacillus sp.* e.g. SEQ ID NO:49, YP_174261_*B. clausii*_KSM-K16 e.g. SEQ ID NO:50, BAA06157 *Bacillus sp.* G-825-6 (SEQ ID NO:51) and BAF34115_*A. transvaalensis* e.g. SEQ ID NO:52, (Figure 3). Preferably, the modified pro polynucleotide encodes a mutation at least at one amino acid at position chosen from positions 6, 30 and 32 of the pro polypeptide of SEQ ID NO: 7, which is operably linked to the mature protease of any one of the proteases of SEQ ID NOS: 9, 11, 13, 15, 17, 19, 21, 23, and 25. The at least one mutation is an amino acid substitution of the glutamic acid (E) at position 6 and/or 30; and/or the amino acid substitution of the alanine (A) at position 32. It is intended that any of the other 19 amino acids that substitute the glutamic acid (E) at position 6 and/or 30, and/or the alanine (A) at position 32 of the pro region of SEQ ID NO:7 may be used to encode a modified protease from which the mature form is produced at a level that is greater than that obtained from processing of the corresponding unmodified precursor protein. In some embodiments, the at least one mutation is a substitution chosen from the following substitutions:

[0098] E6A, E6R, E6C, E6Q, E6H, E6I, E6K, E6L, E6M, E6S, E6Y, E6N, E6G, E6F, E6P, E6T, E6W, E6V, E30A, E30R, E30N, E30D, E30G, E30H, E30L, E30K, E30F, E30S, E30T, E30V, E30R, E30Q, E30G, E30I, E30L, E30M, E30F, E30P, E30T, E30W, E30Y, E30C, E30M, E30F, E30V, A32K, A32T, A32Q, A32S, A32V, A32 L, and A32F of the pro polypeptide of the SEQ ID NO:7. For example, any one of the substitutions chosen from E6A, E6R, E6C, E6Q, E6H, E6I, E6K, E6M, E6S, E6Y, E30A, E30R, E30N, E30D, E30Q, E30G, E30L, E30M, E30P, E30S, E30T, E30W, E30Y, E30V, A32, A32R, A32C, A32E, A32G, A32L, A32K, A32F, A32T, A32Y, and A32V are made in the pro region of SEQ ID NO:7 to produce the mature protease of SEQ ID NO:17; any one of the substitutions chosen from E6A, E6R, E6N, E6C, E6Q, E6G, E6H, E6M, E6F, E6P, E6S, E6T, E6W, E6V, A32K, A32T, and A32V, are made in the pro region of SEQ ID NO:7 to produce the mature protease of SEQ ID NO:9; any one of the substitutions chosen from E6A, E6H, E6K, and E6R, E30A, E30R, E30N, E30D, E30G, E30H, E30L, E30K, E30F, E30S, E30T, and E30V, are made in the pro region of SEQ ID NO:7 to produce the mature protease of SEQ ID NO:19; any one of the substitutions chosen from E6A, E6R, E6Q, E6G, E6L, E6K, E6M, E6F, E6T, E6V, E30R, E30Q, E30G, E30I, E30L, E30M, E30F, E30P, E30T, E30W, E30Y, E30V, A32Q, A32S, A32T, and A32V, are made in the pro region of SEQ ID NO:7 to produce the mature protease of SEQ ID NO:11; and any one of the substitutions chosen from E30A, E30R, E30N, E30D, E30C, E30G, E30H, E30M, E30F, E30S, E30W, A32 L, A32F, and A32V, are made in the pro region of SEQ ID NO:7 to produce the mature protease of SEQ ID NO:21. The at least one substitution enhances the production of the mature protease when compared to the production of the mature protease expressed from a precursor protease that does not comprise the at least one substitution in the pro region of SEQ ID NO:7 to which it is operably linked.

[0099] In some other embodiments, the modification of the pro region of SEQ ID NO:7 includes a combination of mutations. For example, modification of the pro region of SEQ ID NO:7 includes a combination of at least two substitutions. In other embodiments, modification of the pro region of SEQ ID NO:7 includes a combination of at least three, at least four, at least five, at least six, at least seven, at least 8, at least nine, or at least 10 substitutions. Modifications of the pro region also include a combination of at least one substitution and one deletion; a combination of at least one substitution and at least one insertion; a combination of at least one insertion and one deletion, and a combination of at least one substitution, at least one deletion, and at least one insertion. Preferably, the modification of the pro region of SEQ ID NO:7 includes at least two substitutions that result in a combination of substitutions at positions 6 and 30 (i.e. E6X-E30X), 6 and 32 (i.e. E6X-A32X) or 30 and 32 (i.e. E30X-A32X). For

example, the modified polynucleotide encodes a pro region comprising a combination of substitutions chosen from E6R-A32K, E6N-A32K, E6D-A32K, E6I-A32K, E6K-A32K, E6M-A32K, E6P-A32K, E6S-A32K, E6T-A32K, E6N-A32K, E30W-A32K, E30V-A32K, E6A-E30G, E6R-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6S-E30G, E6W-E30G, E30G-A32R, E30G-A32Q, E30G-A32E, E30G-A32G, E30G-A32H, E30G-A32I, E30G-A32K, E30G-A32S, E30G-A32T, E30G-A32W, E30G-A32V, E6G-E30G-A32E, E6G-E30G-A32S, E6G-E30G-A32T, E6G-E30G-A32W, E6A-E30G, E6R-E30G, E6N-E30G, E6D-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6M-E30G, E6F-E30G, E6P-E30G, E6S-E30G, E6T-E30G, E6W-E30G, E6V-E30G, E6Y-E30G, E6A-E30S, E6G-E30S, E6L-E30S, E6K-E30S, E6F-E30S, E6P-E30S, E6Y-E30S, E6V-E30S, E30S-A32R, E30S-A32N, E30S-A32D, E30S-A32C, E30S-A32Q, E30S-A32E, E30S-A32G, E30S-A32H, E30S-A32L, E30S-A32K, E30S-A32M, E30S-A32F, E30S-A32P, E30S-A32S, E30S-A32T, E30S-A32W, E30S-A32Y, and E30S-A32V. For example, modification of the pro region of SEQ ID NO:7 includes a combination of at least two substitutions chosen from E6R-A32K, E6N-A32K, E6D-A32K, E6I-A32K, E6K-A32K, E6M-A32K, E6PA32K, E6S-A32K, E6T-A32K, E6N-A32K, E30W-A32K, and E30V-A32K to produce the mature protease of SEQ ID NO:9; modification of the pro region of SEQ ID NO:7 includes a combination of at least two substitutions chosen from E6A-E30G, E6R-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6S-E30G, E6W-E30G, E30G-A32R, E30G-A32Q, E30G-A32E, E30G-A32G, E30G-A32H, E30G-A32I, E30G-A32K, E30G-A32S, E30G-A32T, E30G-A32W, E30G-A32V to produce the mature protease of SEQ ID NO:17; modification of the pro region of SEQ ID NO:7 includes a combination of at least two substitutions chosen from E6A-E30G, E6R-E30G, E6N-E30G, E6D-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6M-E30G, E6F-E30G, E6P-E30G, E6S-E30G, E6T-E30G, E6W-E30G, E6V-E30G, E6Y-E30G to produce the mature protease of SEQ ID NO:19; and modification of the pro region of SEQ ID NO:7 includes a combination of at least two substitutions chosen from E6A-E30S, E6G-E30S, E6L-E30S, E6K-E30S, E6F-E30S, E6P-E30S, E6Y-E30S, E6V-E30S, E30S-A32R, E30S-A32N, E30S-A32D, E30S-A32C, E30S-A32Q, E30S-A32E, E30S-A32G, E30S-A32H, E30S-A32L, E30S-A32K, E30S-A32M, E30S-A32F, E30S-A32P, E30S-A32S, E30S-A32T, E30S-A32W, E30S-A32Y, and E30S-A32V to produce the mature protease of SEQ ID NO:21. Other examples of modifications of the pro region of SEQ ID NO:7 include at least three substitutions that result in a combination of substitutions at positions 6, 30 and 32 (i.e. E6X-E30X-A32X). For example, modification of the pro region of SEQ ID NO:7 includes a combination of at least three substitutions chosen from E6G-E30G-A32E, E6G-E30G-A32S, E6G-E30G-A32T, E6G-E30G-A32W to produce the mature protease of SEQ ID NO:17. The at least two or three substitutions enhance the production of the mature protease when compared to the production of the mature protease expressed from a precursor protease that does not comprise the at least two or three substitutions in the pro region of SEQ ID NO:7 to which it is operably linked.

[0100] Several methods are known in the art that are suitable for generating modified polynucleotide sequences of the present invention, including but not limited to site-saturation mutagenesis, scanning mutagenesis, insertional mutagenesis, deletion mutagenesis, random mutagenesis, site-directed mutagenesis, and directed-evolution, as well as various other recombinatorial approaches. The commonly used methods include DNA shuffling (Stemmer WP, *Proc Natl Acad Sci U S A.* 25;91(22):10747-51 [1994]), methods based on non-homologous recombination of genes e.g. ITCHY (Ostermeier et al., *Bioorg Med Chem.* 7(10):2139-44 [1999]), SCRACHY (Lutz et al. *Proc Natl Acad Sci U S A.* 98(20):11248-53 [2001]), SHIPREC (Sieber et al., *Nat Biotechnol.* 19(5):456-60 [2001]), and NRR (Bittker et al., *Nat Biotechnol.* 20(10):1024-9 [2001]; Bittker et al., *Proc Natl Acad Sci USA.* 101(18):7011-6 [2004]), and methods that rely on the use of oligonucleotides to insert random and targeted mutations, deletions and/or insertions (Ness et al., *Nat Biotechnol.* 20(12):1251-5 [2002]; Coco et al., *Nat Biotechnol.* 20(12):1246-50 [2002]; Zha et al., *Chembiochem.* 3;4(1):34-9 [2003], Glaser et al., *J Immunol.* 149(12):3903-13 [1992], Sondek and Shortle, *Proc Natl Acad Sci U S A* 89(8):3581-5 [1992], Yáñez et al., *Nucleic Acids Res.* 32(20):e158 [2004], Osuna et al., *Nucleic Acids Res.* 32(17):e136

[2004], Gaytán et al., Nucleic Acids Res. 29(3):E9 [2001], and Gaytán et al., Nucleic Acids Res. 30(16):e84 [2002]).

[0101] In addition to encoding the modified pro-protease, the modified precursor polynucleotide further comprises a pre polynucleotide encoding a signal peptide. In some embodiments, the signal peptide is the AprE signal peptide (SEQ ID NO:3) encoded by the polynucleotide of SEQ ID NO:2. For example, full-length modified precursor proteases include the proteases of SEQ ID NOS:, wherein the pro region of said precursor proteases comprises at least one mutation. In some embodiments, the at least one mutation is an amino acid substitution is made at the position equivalent to position 6, 30 or 32 of the pro region of SEQ ID NO:7. Alternatively, the signal peptide is a fusion signal peptide of SEQ ID NO:5 encoded by the polynucleotide of SEQ ID NO:4. Signal peptides that are naturally linked to the mature protease may also be used to express the full-length modified proteases described herein. Examples of full-length precursor proteases that can be modified to comprise at least one amino acid substitution at a position chosen from 6, 30 and 32 of the pro region of SEQ ID NO:7 include:

the full-length protease of SEQ ID NO:59

VRSKKLWISLLFALTLIFTMAFSNMSAQAAEEAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEEEVEI
ELLHEFETIPVLSVELSPEDVDALDPAISYIEEDAEVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVA
 VLDTGISTHPDLNIRGGASFVPGEPSTQDGNHGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGS
 GSVSSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYAN
 AMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPS
 WSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; SEQ ID NO:59);

the full-length protease of SEQ ID NO:60

VRSKKLWISLLFALTLIFTMAFSNMSAQAAEEAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEEEVEI
ELLHEFETIPVLSVELSPEDVDALDPAISYIEEDAEVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVA
 VLDTGISTHPDLNIRGGASFVPGEPSTQDGNHGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGS
 GAISSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANA
 MAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSW
 SNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; SEQ ID NO:60);

the full-length protease of SEQ ID NO:61

VRSKKLWISLLFALTLIFTMAFSNMSAQAAEEAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEEEVEI
ELLHEFETIPVLSVELSPEDVDALDPAISYIEEDAEVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVA
 VLDTGISTHPDLNIRGGASFVPGEPSTQDGNHGHGTHVAGTIAALNNSIGVLGVAPNAELYAVKVLGASGS
 GSVSSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYAN
 AMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPS
 WSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; SEQ ID NO:61);

the full-length protease of SEQ ID NO:62

VRSKKLWISLLFALTLIFTMAFSNMSAQAAEEAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEEEVEI
ELLHEFETIPVLSVELSPEDVDALDPAISYIEEDAEVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVA
 VLDTGISTHPDLNIRGGASFVPGEPSTQDGNHGHGTHVAGTIAALNNSIGVLGVAPNAELYAVKVLGASGS
 GSVSSIAQGLEWAGNVMHVANLSLGLQAPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYAN
 AMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPS
 WSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; SEQ ID NO:62);

the full-length protease of SEQ ID NO:63

VRSKKLWISLLFALTLIFTMAFSNMSAQAAEEAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEEEVEI
ELLHEFETIPVLSVELSPEDVDALDPAISYIEEDAEVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVA
 VLDTGISTHPDLNIRGGASFVPGEPSTQDGNHGHGTHVAGTIAALNNSIGVLGVAPNAELYAVKVLGASGM
 GSVSSIAQGLEWAGNVMHVANLSLGLQAPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYAN
 AMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPS
 WSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; SEQ ID NO:63);

the full-length protease of SEQ ID NO:64

VRSKKLWISLLFALTIFTMAFSNMSAQAAEEAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEEEVEI
ELLHEFETIPVLSVELSPEDVDALDPAISYIEEDAEVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVA
VLDTGISTHPDLNIRGGASFVPGEPTQDGNGHGHVAGTIAALNNSIGVLGVAPNAELYAVKVLGASGG
GSNSSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYAN
AMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPS
WSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; SEQ ID NO:64);

the full-length protease of SEQ ID NO:65

VRSKKLWISLLFALTIFTMAFSNMSAQAAEEAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEEEVEI
ELLHEFETIPVLSVELSPEDVDALDPAISYIEEDAEVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVA
VLDTGISTHPDLNIRGGASFVPGEPTQDGNGHGHVAGTIAALDNSIGVLGVAPRAELYAVKVLGASGS
GSVSSIAQGLEWAGNRMHVANLSLGLQAPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYAN
AMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPS
WSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; SEQ ID NO:65),

the full-length protease of SEQ ID NO:66

VRSKKLWISLLFALTIFTMAFSNMSAQAAEEAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEEEVEI
ELLHEFETIPVLSVELSPEDVDALDPAISYIEEDAEVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVA
VLDTGISTHPDLNIRGGASFVPGEPTQDGNGHGHVAGTIAALDNSIGVLGVAPRAELYAVKVLGASGS
GSVSSIAQGLEWAGNRMHVANLSLGLQAPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYAN
AMAVGATDQNNNRADFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPS
WSNRQIRNHLKNTATSLGSTNLYGSGLVNAEAATR; SEQ ID NO:66), and

the full-length protease of SEQ ID NO:67

VRSKKLWISLLFALTIFTMAFSNMSAQAAEEAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEEEVEI
ELLHEFETIPVLSVELSPEDVDALDPAISYIEEDAEVTTMAQSVPWGISRVQAPAAHNRGLTGSGVKVA
VLDTGISTHPDLNIRGGASFVPGEPTQDGNGHGHVAGTIAALDNSIGVLGVAPRAELYAVKVLGASGS
GSVSSIAQGLEWAGNNGMHVANLSLGLQAPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYAN
AMAVGATDQNNNRADFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPS
WSNVQIRRHKLKNTATSLGSTNLYGSGLVNAEAATR; SEQ ID NO:67).

The pre region (signal peptide; SEQ ID NO:3) is shown in bold, the pro region (SEQ ID NO:7) is underlined, and the mature region is italicized.

[0102] As previously described, in addition to being identical to the naturally-occurring pro region of the P41362 *B. clausii* and P27693 *Bacillus alkalophilus* (Figure 2), the pro region of SEQ ID NO:7 has a high degree of identity with the amino acid sequence of the pro region of proteases from as GG36 *B. lentus* 267048, (SEQ ID NO:53), P20724 *Bacillus*_sp_YAB (SEQ ID NO:54), BAA25184 *Bacillus*_sp (SEQ ID NO:55), YP_174261 *B. clausii*_KSM-K16 e.g. SEQ ID NO:56, BAA06157 *Bacillus* sp G-825-6 (SEQ ID NO:57) and BAF34115 *A. transvaalensis* e.g. SEQ ID NO:58, (Figure 2). It is expected that mutations made in the pro region of SEQ ID NOs:53-58 and corresponding to the mutations of SEQ ID NO:7 that enhance the production of the mature protease to which it is operably linked, will enhance the production of the mature protease to which the modified pro region of SEQ ID NOs: 53-58 is operably linked. For example, any one of the modified polynucleotides that encode the pro region of SEQ ID NOs: 53-58 can be modified to encode an amino acid substitution at least at one amino acid position selected from positions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, and 84, wherein the position is numbered by correspondence with the amino acid sequence of the pro polypeptide of SEQ ID NO:7. Preferably, any one of the modified polynucleotides that encode the pro region of SEQ ID NOs: 53-58 is modified to encode a mutation at least at one amino acid at position chosen from positions 6, 30 and 32. In some embodiments, the at least one mutation chosen from

positions 6, 30 and 32 is a substitution chosen from the following substitutions: E6A, E6R, E6C, E6Q, E6H, E6I, E6K, E6L, E6M, E6S, E6Y, E6N, E6G, E6F, E6P, E6T, E6W, E6V, E30A, E30R, E30N, E30D, E30G, E30H, E30L, E30K, E30F, E30S, E30T, E30V, E30R, E30Q, E30G, E30I, E30L, E30M, E30F, E30P, E30T, E30W, E30Y, E30C, E30M, E30F, E30V, A32K, A32T, A32Q, A32S, A32V, A32 L, and A32F, wherein the positions are numbered by correspondence with the amino acid sequence of the pro polypeptide of the SEQ ID NO:7. In other embodiments, the modified polynucleotide encodes a pro region comprising a combination of substitutions chosen from a combination of substitutions made at positions 6 and 32 (i.e. E6X-E30X), at positions 30 and 32 (i.e. E30X-A32X). For example, the modified polynucleotide encodes a pro region comprising a combination of substitutions chosen from E6R-A32K, E6N-A32K, E6D-A32K, E6I-A32K, E6K-A32K, E6M-A32K, E6P-A32K, E6S-A32K, E6T-A32K, E6N-A32K, E30W-A32K, E30V-A32K, E6A-E30G, E6R-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6S-E30G, E6W-E30G, E30G-A32R, E30G-A32Q, E30G-A32E, E30G-A32G, E30G-A32H, E30G-A32I, E30G-A32K, E30G-A32S, E30G-A32T, E30G-A32W, E30G-A32V, E6G-E30G-A32E, E6G-E30G-A32S, E6G-E30G-A32T, E6G-E30G-A32W, E6A-E30G, E6R-E30G, E6N-E30G, E6D-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6M-E30G, E6F-E30G, E6P-E30G, E6S-E30G, E6T-E30G, E6W-E30G, E6V-E30G, E6Y-E30G, E6A-E30S, E6G-E30S, E6L-E30S, E6K-E30S, E6F-E30S, E6P-E30S, E6Y-E30S, E6V-E30S, E30S-A32R, E30S-A32N, E30S-A32D, E30S-A32C, E30S-A32Q, E30S-A32E, E30S-A32G, E30S-A32H, E30S-A32L, E30S-A32K, E30S-A32M, E30S-A32F, E30S-A32P, E30S-A32S, E30S-A32T, E30S-A32W, E30S-A32Y, and E30S-A32V, wherein the positions are numbered by correspondence with the amino acid sequence of the pro polypeptide of the SEQ ID NO:7. In yet other embodiments, the modified polynucleotide encodes a pro region comprising a combination of substitutions chosen from E6G-E30G-A32E, E6G-E30G-A32S, E6G-E30G-A32T, E6G-E30G-A32W, wherein the positions are numbered by correspondence with the amino acid sequence of the pro polypeptide of the SEQ ID NO:7. Any one of the pro regions of SEQ ID NOS:7 and 53-58 that is modified to contain the at least one two or three substitutions as described above, is operably linked to a mature protease that is at least 60% identical to the mature protease of SEQ ID NO:9. Mature proteases that are at least 60% identical to the mature protease of SEQ ID NO:9 (*B. lentus* protease GG36), include the wild-type *Bacillus clausii* PB92 protease Maxacal (SEQ ID NO:13), and variants thereof such as SEQ ID NO:11, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, and SEQ ID NO:25; and homologs of SEQ ID NO:9 including homologs of mature proteases from *Bacillus* sp. such as P27693_*Bacillus alcalophilus* e.g. SEQ ID NO:47, P20724_*Bacillus* sp_YAB e.g. SEQ ID NO:48, BAA25184_*Bacillus* sp e.g. SEQ ID NO:49, YP_174261_*B. clausii*_KSM_K16 e.g. SEQ ID NO:50, BAA06157 *Bacillus* sp G-825-6 (SEQ ID NO:51) and BAF34115_*A. transvaalensis* e.g. SEQ ID NO:52, (Figure 3).

[0103] Any one of the pro regions of SEQ ID NOS: 7 and 53-58 that is modified to contain the at least one substitution described above, and that is operably linked to a mature protease that is at least 60% identical to the mature protease of SEQ ID NO:9, is further operably linked to a signal peptide. Preferably, the signal peptide is the AprE signal peptide (SEQ ID NO:3) encoded by the polynucleotide of SEQ ID NO:2. Alternatively, the signal peptide is a fusion signal peptide VRSKKLWIVASTALLISVAFSSSIASA (SEQ ID NO:5) encoded by the polynucleotide of SEQ ID NO:4 gtgagaagcaaaaaattgtggatcgctgcgtcgaccgcactactcattctgtgtcttttagttcatcgatcgcatcggt (SEQ ID NO:4). Any signal sequence that can effectuate efficient secretion of a modified protease in a *Bacillus* sp host cell can be operably linked to a pro-protease of the invention. Such signal peptides include signal peptides of bacterial origin that direct secretion of proteins via bacterial secretion pathways e.g. Sec pathway, TAT pathway, and eukaryotic signal sequences that are applicable for expressing proteins in prokaryotic host cells (EP1481059B1).

[0104] The at least one amino acid substitution at position 6, 30, and/or 32 made in the pro region of

SEQ ID NO:7 can be introduced at equivalent amino acid positions in the pro regions of a pre-protease to enhance the production of the mature enzyme, wherein the signal peptide can be chosen from the signal peptides of SEQ ID NOS: 3, and 5, signal peptides that are naturally and operably linked to the pro-protease, and any signal sequence that can effectuate efficient secretion of a modified protease in a *Bacillus* sp host cell *e.g.* *Bacillus subtilis*.

[0105] As indicated above, in some embodiments, the present invention provides vectors comprising the aforementioned modified polynucleotides. In some embodiments, the vector is an expression vector in which the modified polynucleotide sequence encoding the modified protease of the invention is operably linked to additional segments required for efficient gene expression (e.g., a promoter operably linked to the gene of interest). In some embodiments, these necessary elements are supplied as the gene's own homologous promoter if it is recognized, (i.e., transcribed by the host), and a transcription terminator that is exogenous or is supplied by the endogenous terminator region of the protease gene. In some embodiments, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antimicrobial-containing media is also included.

[0106] In some embodiments, the expression vector is derived from plasmid or viral DNA, or in alternative embodiments, contains elements of both. Exemplary vectors include, but are not limited to pXX, pC194, pJH101, pE194, pHP13 (Harwood and Cutting (eds), Molecular Biological Methods for Bacillus, John Wiley & Sons, [1990], in particular, chapter 3; suitable replicating plasmids for *B. subtilis* include those listed on page 92; Perego, M. (1993) Integrational Vectors for Genetic Manipulations in Bacillus subtilis, p. 615-624; A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), Bacillus subtilis and other Gram-positive bacteria: biochemistry, physiology and molecular genetics, American Society for Microbiology, Washington, D.C.).

[0107] For expression and production of protein(s) of interest e.g. a protease, in a cell, at least one expression vector comprising at least one copy of a polynucleotide encoding the modified protease, and preferably comprising multiple copies, is transformed into the cell under conditions suitable for expression of the protease. In some particularly embodiments, the sequences encoding the proteases (as well as other sequences included in the vector) are integrated into the genome of the host cell, while in other embodiments, the plasmids remain as autonomous extra-chromosomal elements within the cell. Thus, the present invention provides both extrachromosomal elements as well as incoming sequences that are integrated into the host cell genome. It is intended that each of the vectors described herein will find use in the present invention. In some embodiments, the polynucleotide construct encoding the modified protease is present on an integrating vector (e.g., pJH-GG36; Figure 4) that enables the integration and optionally the amplification of the modified polynucleotide into the bacterial chromosome. Examples of sites for integration include, but are not limited to the *aprE*, the *amyE*, the *veg* or the *pps* regions. Indeed, it is contemplated that other sites known to those skilled in the art will find use in the present invention. In some embodiments, transcription of the polynucleotides encoding the modified proteases is effectuated by a promoter that is the wild-type promoter for the selected precursor protease. In some other embodiments, the promoter is heterologous to the precursor protease, but is functional in the host cell. Specifically, examples of suitable promoters for use in bacterial host cells include but are not limited to the *amyE*, *amyQ*, *amyL*, *pstS*, *sacB*, *pSPAC*, *pAprE*, *pVeg*, *pHpall* promoters, the promoter of the *B. stearothermophilus* maltogenic amylase gene, the *B. amyloliquefaciens* (BAN) amylase gene, the *B. subtilis* alkaline protease gene, the *B. clausii* alkaline protease gene the *B. pumilis* xylosidase gene, the *B. thuringiensis* cryIIIA, and the *B. licheniformis* alpha-amylase gene. In some embodiments, the promoter is the AprE promoter having the sequence:

[illegible]

attctatccctctctgtaaggaattttcagaataccttcatcctatgctgtaaaaaatacagataataatccatgctcaggaagcacaacgagcagc

tcatttgaacgaatttttcgacaggaatttgcgggactcaggagcatttaacctaaaaagcatgacatttcagcataatgaacatttactcatgtcta
 ttctgtctttctgtatgaaaaatagttatttcgagtcctacggaatagcgagatgatatacctaaatagagataaaatcatctcaaaaaatgggt
 ctactaaaatatttcatctattacaataaattcacagaatagctctttaagtaagtcctactgaattttttaaaggagagggttaaaga (SEQ ID

NO: 1).

Additional promoters include, but are not limited to the A4 promoter, as well as phage Lambda P_R or P_L promoters, and the E. coli lac, trp or tac promoters.

[0108] Precursor and modified proteases are produced in host cells of any suitable Gram-positive microorganism, including bacteria and fungi. For example, in some embodiments, the modified protease is produced in host cells of fungal and/or bacterial origin. In some embodiments, the host cells are *Bacillus sp.*, *Streptomyces sp.*, *Escherichia sp.* or *Aspergillus sp.*. In some embodiments, the modified proteases are produced by *Bacillus sp.* host cells. Examples of *Bacillus sp.* host cells that find use in the production of the modified proteins of the present invention include, but are not limited to *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilis*, *B. thuringiensis*, *B. clausii*, and *B. megaterium*, as well as other organisms within the genus *Bacillus*. In some embodiments, *B. subtilis* host cells find use. U.S. Patents 5,264,366 and 4,760,025 (RE 34,606) describe various *Bacillus* host strains that find use in the present invention, although other suitable strains find use in the present invention.

[0109] Several industrial strains that find use in the present invention include non-recombinant (*i.e.*, wild-type) *Bacillus sp.* strains, as well as variants of naturally occurring strains and/or recombinant strains. In some embodiments, the host strain is a recombinant strain, wherein a polynucleotide encoding a polypeptide of interest has been introduced into the host. In some embodiments, the host strain is a *B. subtilis* host strain and particularly a recombinant *Bacillus subtilis* host strain. Numerous *B. subtilis* strains are known, including but not limited to 1A6 (ATCC 39085), 168 (1A01), SB19, W23, Ts85, B637, PB1753 through PB1758, PB3360, JH642, 1A243 (ATCC 39,087), ATCC 21332, ATCC 6051, MI113, DE100 (ATCC 39,094), GX4931, PBT 110, and PEP 211 strain (See *e.g.*, Hoch et al., Genetics, 73:215-228 [1973]) (See also, U.S. Patent No. 4,450,235; U.S. Patent No. 4,302,544; and EP 0134048; each of which is incorporated by reference in its entirety). The use of *B. subtilis* as an expression host well known in the art (See *e.g.*, See, Palva et al., Gene 19:81-87 [1982]; Fahnestock and Fischer, J. Bacteriol., 165:796-804 [1986]; and Wang et al., Gene 69:39-47 [1988]).

[0110] In some embodiments, the *Bacillus* host is a *Bacillus sp.* that includes a mutation or deletion in at least one of the following genes, *degU*, *degS*, *degR* and *degQ*. Preferably the mutation is in a *degU* gene, and more preferably the mutation is *degU(Hy)32*. (See *e.g.*, Msadek et al., J. Bacteriol., 172:824-834 [1990]; and Olmos et al., Mol. Gen. Genet., 253:562-567 [1997]). A preferred host strain is a *Bacillus subtilis* carrying a *degU32(Hy)* mutation. In some further embodiments, the *Bacillus* host comprises a mutation or deletion in *scoC4*, (See, *e.g.*, Caldwell et al., J. Bacteriol., 183:7329-7340 [2001]); *spoIIIE* (See, Arigoni et al., Mol. Microbiol., 31:1407-1415 [1999]); and/or *oppA* or other genes of the *opp* operon (See *e.g.*, Perego et al., Mol. Microbiol., 5:173-185 [1991]). Indeed, it is contemplated that any mutation in the *opp* operon that causes the same phenotype as a mutation in the *oppA* gene will find use in some embodiments of the altered *Bacillus* strain of the present invention. In some embodiments, these mutations occur alone, while in other embodiments, combinations of mutations are present. In some embodiments, an altered *Bacillus* that can be used to produce the modified proteases of the invention is a *Bacillus* host strain that already includes a mutation in one or more of the above-mentioned genes. In addition, *Bacillus sp.* host cells that comprise mutation(s) and/or deletions of endogenous protease genes find use. In some embodiments, the *Bacillus* host cell comprises a deletion of the *aprE* and the *nprE* genes. In other embodiments, the *Bacillus sp.* host cell comprises a deletion of 5 protease genes

(US20050202535), while in other embodiments, the *Bacillus* sp. host cell comprises a deletion of 9 protease genes (US20050202535).

[0111] Host cells are transformed with modified polynucleotides encoding the modified proteases of the present invention using any suitable method known in the art. Whether the modified polynucleotide is incorporated into a vector or is used without the presence of plasmid DNA, it is introduced into a microorganism, in some embodiments, preferably an *E. coli* cell or a competent *Bacillus* cell. Methods for introducing DNA into *Bacillus* cells involving plasmid constructs and transformation of plasmids into *E. coli* are well known. In some embodiments, the plasmids are subsequently isolated from *E. coli* and transformed into *Bacillus*. However, it is not essential to use intervening microorganisms such as *E. coli*, and in some embodiments, a DNA construct or vector is directly introduced into a *Bacillus* host.

[0112] Those of skill in the art are well aware of suitable methods for introducing polynucleotide sequences into *Bacillus* cells (See e.g., Ferrari et al., "Genetics," in Harwood et al. (ed.), *Bacillus*, Plenum Publishing Corp. [1989], pages 57-72; Saunders et al., *J. Bacteriol.*, 157:718-726 [1984]; Hoch et al., *J. Bacteriol.*, 93:1925-1937 [1967]; Mann et al., *Current Microbiol.*, 13:131-135 [1986]; and Holubova, *Folia Microbiol.*, 30:97 [1985]; Chang et al., *Mol. Gen. Genet.*, 168:11-115 [1979]; Vorobjeva et al., *FEMS Microbiol. Lett.*, 7:261-263 [1980]; Smith et al., *Appl. Env. Microbiol.*, 51:634 [1986]; Fisher et al., *Arch. Microbiol.*, 139:213-217 [1981]; and McDonald, *J. Gen. Microbiol.*, 130:203 [1984]). Indeed, such methods as transformation, including protoplast transformation and conjugation, transduction, and protoplast fusion are known and suited for use in the present invention. Methods of transformation are used to introduce a DNA construct provided by the present invention into a host cell. Methods known in the art to transform *Bacillus*, include such methods as plasmid marker rescue transformation, which involves the uptake of a donor plasmid by competent cells carrying a partially homologous resident plasmid (Contente et al., *Plasmid* 2:555-571 [1979]; Haima et al., *Mol. Gen. Genet.*, 223:185-191 [1990]; Weinrauch et al., *J. Bacteriol.*, 154:1077-1087 [1983]; and Weinrauch et al., *J. Bacteriol.*, 169:1205-1211 [1987]). In this method, the incoming donor plasmid recombines with the homologous region of the resident "helper" plasmid in a process that mimics chromosomal transformation.

[0113] In addition to commonly used methods, in some embodiments, host cells are directly transformed (i.e., an intermediate cell is not used to amplify, or otherwise process, the DNA construct prior to introduction into the host cell). Introduction of the DNA construct into the host cell includes those physical and chemical methods known in the art to introduce DNA into a host cell without insertion into a plasmid or vector. Such methods include, but are not limited to calcium chloride precipitation, electroporation, naked DNA, liposomes and the like. In additional embodiments, DNA constructs are co-transformed with a plasmid, without being inserted into the plasmid. In further embodiments, a selective marker is deleted from the altered *Bacillus* strain by methods known in the art (See, Stahl et al., *J. Bacteriol.*, 158:411-418 [1984]; and Palmeros et al., *Gene* 247:255-264 [2000]).

[0114] In some embodiments, the transformed cells of the present invention are cultured in conventional nutrient media. The suitable specific culture conditions, such as temperature, pH and the like are known to those skilled in the art. In addition, some culture conditions may be found in the scientific literature such as Hopwood (2000) *Practical Streptomyces Genetics*, John Innes Foundation, Norwich UK; Hardwood et al., (1990) *Molecular Biological Methods for Bacillus*, John Wiley and from the American Type Culture Collection (ATCC).

[0115] In some embodiments, host cells transformed with polynucleotide sequences encoding modified proteases are cultured in a suitable nutrient medium under conditions permitting the expression of the present protease, after which the resulting protease is recovered from the culture. The medium used to

culture the cells comprises any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g., in catalogues of the American Type Culture Collection). In some embodiments, the protease produced by the cells is recovered from the culture medium by conventional procedures, including, but not limited to separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt (e.g., ammonium sulfate), chromatographic purification (e.g., ion exchange, gel filtration, affinity, etc.). Thus, any method suitable for recovering the protease(s) of the present invention finds use in the present invention. Indeed, it is not intended that the present invention be limited to any particular purification method.

[0116] The protein produced by a recombinant host cell comprising a modified protease of the present invention is secreted into the culture media. In some embodiments, other recombinant constructions join the heterologous or homologous polynucleotide sequences to nucleotide sequence encoding a protease polypeptide domain which facilitates purification of the soluble proteins (Kroll DJ et al (1993) DNA Cell Biol 12:441-53). Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals (Porath J (1992) Protein Expr Purif 3:263-281), protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen, San Diego CA) between the purification domain and the heterologous protein also find use to facilitate purification.

[0117] As indicated above, the invention provides for modified full-length polynucleotides that encode modified full-length proteases that are processed by a *Bacillus* host cell to produce the mature form at a level that is greater than that of the same mature protease when processed from an unmodified full-length enzyme by a *Bacillus* host cell grown under the same conditions. The level of production is determined by the level of activity of the secreted enzyme.

[0118] One measure of production can be determined as relative activity, which is expressed as a percent of the ratio of the value of the enzymatic activity of the mature form when processed from the modified protease to the value of the enzymatic activity of the mature form when processed from the unmodified precursor protease. A relative activity equal or greater than 100% indicates that the mature form a protease that is processed from a modified precursor is produced at a level that is equal or greater than the level at which the same mature protease is produced but when processed from an unmodified precursor. Thus, in some embodiments, the relative activity of a mature protease processed from the modified protease is at least about 100%, at least about 110%, at least about 120%, at least about 130%, at least about 140%, at least about 150%, at least about 160%, at least about 170%, at least about 180%, at least about 190%, at least about 200%, at least about 225%, at least about 250%, at least about 275%, at least about 300%, at least about 325%, at least about 350%, at least about 375%, at least about 400%, at least about 425%, at least about 450%, at least about 475%, at least about 500%, at least about 525%, at least about 550%, at least about 575%, at least about 600%, at least about 625%, at least about 650%, at least about 675%, at least about 700%, at least about 725%, at least about 750%, at least about 800%, at least about 825%, at least about 850%, at least about 875%, at least about 850%, at least about 875%, at least about 900%, and up to at least about 1000% or more when compared to the corresponding production of the mature form of the protease that was processed from the unmodified precursor protease. Alternatively, the relative activity is expressed as the ratio of production which is determined by dividing the value of the activity of the protease processed from a modified precursor by the value of the activity of the same protease when processed from an

unmodified precursor. Thus, in some embodiments, the ratio of production of a mature protease processed from a modified precursor is at least about 1, at least about 1.1, at least about 1.2, at least about 1.3 at least about, 1.4, at least about 1.5, at least about 1.6, at least about 1.7, at least about 1.8, at least about 1.9, at least about 2, at least about 2.25, at least about 2.5, at least about 2.75, at least about 3, at least about 3.25, at least about 3.5, at least about 3.75, at least about, at least about 4.25, at least about 4.5, at least about 4.75, at least about 5, at least about 5.25, at least about 5.5, at least about 5.75, at least about 6, at least about 6.25, at least about 6.5, at least about 6.75, at least about 7, at least about 7.25, at least about 7.5, at least about 8, at least about 8.25, at least about 8.5, at least about 8.75, at least about 9, and up to at least about 10.

[0119] There are various assays known to those of ordinary skill in the art for detecting and measuring activity of proteases. In particular, assays are available for measuring protease activity that are based on the release of acid-soluble peptides from casein or hemoglobin, measured as absorbance at 280 nm or colorimetrically using the Folin method (See e.g., Bergmeyer et al., "Methods of Enzymatic Analysis" vol. 5, Peptidase, Proteinases and their Inhibitors, Verlag Chemie, Weinheim [1984]). Some other assays involve the solubilization of chromogenic substrates (See e.g., Ward, "Proteinases," in Fogarty (ed.), Microbial Enzymes and Biotechnology, Applied Science, London, [1983], pp 251-317). Other exemplary assays include, but are not limited to succinyl-Ala-Ala-Pro-Phe-para nitroanilide assay (SAAPFpNA) and the 2,4,6-trinitrobenzene sulfonate sodium salt assay (TNBS assay). Numerous additional references known to those in the art provide suitable methods (See e.g., Wells et al., Nucleic Acids Res. 11:7911-7925 [1983]; Christianson et al., Anal. Biochem., 223:119 -129 [1994]; and Hsia et al., Anal Biochem., 242:221-227 [1999]). It is not intended that the present invention be limited to any particular assay method(s).

[0120] Other means for determining the levels of production of a mature protease in a host cell include, but are not limited to methods that use either polyclonal or monoclonal antibodies specific for the protein. Examples include, but are not limited to enzyme-linked immunosorbent assays (ELISA), radioimmunoassays (RIA), fluorescent immunoassays (FIA), and fluorescent activated cell sorting (FACS). These and other assays are well known in the art (See e.g., Maddox et al., J. Exp. Med., 158:1211 [1983]).

[0121] Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as should not be unduly limited to such specific embodiments.

EXPERIMENTAL

[0122] In the experimental disclosure which follows, the following abbreviations apply: ppm (parts per million); M (molar); mM (millimolar); μ M (micromolar); nM (nanomolar); mol (moles); mmol (millimoles); μ mol (micromoles); nmol (nanomoles); gm (grams); mg (milligrams); μ g (micrograms); pg (picograms); L (liters); ml and mL (milliliters); μ l and μ L (microliters); cm (centimeters); mm (millimeters); μ m (micrometers); nm (nanometers); U (units); V (volts); MW (molecular weight); sec (seconds); min(s) (minute/minutes); h(s) and hr(s) (hour/hours); °C (degrees Centigrade); QS (quantity sufficient); QC (QuikChange), ND (not done); NA (not applicable); rpm (revolutions per minute); w/v (weight to volume); v/v (volume to volume); g (gravity); OD (optical density); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); suc-AAPF-pNA (succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenyl-alanyl-para-

nitroanilide); DMSO (dimethyl sulfoxide); cDNA (copy or complementary DNA); DNA (deoxyribonucleic acid); ssDNA (single stranded DNA); dsDNA (double stranded DNA); dNTP (deoxyribonucleotide triphosphate); DTT (1,4-dithio-DL-threitol); H₂O (water); dH₂O (deionized water); HCl (hydrochloric acid); MgCl₂ (magnesium chloride); MOPS (3-[N-morpholino]propanesulfonic acid); NaCl (sodium chloride); PAGE (polyacrylamide gel electrophoresis); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); PEG (polyethylene glycol); PCR (polymerase chain reaction); PMSF (phenylmethylsulfonyl fluoride); RNA (ribonucleic acid); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl) aminomethane); SOC (2% Bacto-Tryptone, 0.5% Bacto Yeast Extract, 10 mM NaCl, 2.5 mM KCl); Terrific Broth (TB; 12 g/l Bacto Tryptone, 24 g/l glycerol, 2.31 g/l KH₂PO₄, and 12.54 g/l K₂HPO₄); OD₂₈₀ (optical density at 280 nm); OD₆₀₀ (optical density at 600 nm); A₄₀₅ (absorbance at 405 nm); V_{max} (the maximum initial velocity of an enzyme catalyzed reaction); HEPES (N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid]); Tris-HCl (tris[Hydroxymethyl]aminomethane-hydrochloride); TCA (trichloroacetic acid); HPLC (high pressure liquid chromatography); RP-HPLC (reverse phase high pressure liquid chromatography); TLC (thin layer chromatography); EDTA (ethylenediaminetetracetic acid); EtOH (ethanol); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); TAED (N,N,N',N'-tetraacetythylenediamine).

[0123] The following examples are provided in order to demonstrate and further illustrate certain embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

[0124] To determine the effect of amino acid substitutions in the alkaline protease pro region on the production of the mature form of the protease to which the pro region is operably linked, one, two and three amino acid substitutions were introduced at amino acids at positions 6, 30 and 32 of the pro region of SEQ ID NO:7 when operably linked to the mature proteases of SEQ ID NOS:9, 11, 17, 19, and 21 as described in Examples 1-5, respectively.

EXAMPLE 1

The effect of mutations in the pro region of SEQ ID NO:7 on the production of the mature alkaline protease of SEQ ID NO:9.

[0125] (a) Site -saturation mutagenesis of amino acids at positions 6, 30 or 32 of the pro region
Site-saturation mutagenesis of the pro region on the production of the mature protease of SEQ ID NO:9 was performed using the QuikChange® site-directed mutagenesis kit (QC; Stratagene) according to the directions of the manufacturer. A DNA cassette comprising the AprE promoter, and the polynucleotide that encodes the full-length protease of SEQ ID NO:59 was cloned into the EcoRI and HindIII restriction sites of the pJH101 vector (Ferrari et al. J. Bacteriol. 154:1513-1515 [1983]) pJH-Pn (Figure 4A) to generate the pJH-P9 plasmid. (Pn refers to the SEQ ID NO of the mature protease that is expressed from the pJH-Pn plasmid). The DNA cassette comprised the *B. subtilis* aprE promoter
gaattcctccattttctctgctatcaaaataacagactcgtgattttcaaacgagctttcaaaaagcctctgcccttgcaaatcggatgctgtctat
aaaattcccgatattgggttaaacagcggcgcaatggcgccgcatctgatgtcttggcggaatgttcatcttatttctcctcctcaataatttttc
attctatccctttctgtaaagtatttttcagaatactttatcatcatgctttgaaaaatatcacgataataatccattgttctcaggaagcacacgcagg
tcatttgaacgaatttttcgacaggaatttgcgggactcaggagcatttaacctaaaaagcatgacatttcagcataatgaacatttactcatgtcta
ttttgctttctgtatgaaaatagttatttcgagtcctacgaaatagcgagagatgatatacctaaatagagataaaatcatctcaaaaaatgggt
ctactaaaatattatcatctattacaataaattcacagaatagcttttaagtaagtcactctgaattttttaaaggagagggttaaaga (SEQ ID
NO:1),

the polynucleotide sequence

gtgagaagcaaaaaattgtggatcagctgtgtttgctgaacgttaattcttacgatggcgttcagcaacatgtctgcgaggct (SEQ ID NO:2), which encodes the AprE signal peptide VRSKKLWISLLFALTLIFTMAFSNMSAQA (SEQ ID NO:3), the polynucleotide sequence

gctgaagaagcaaaagaaaaattttaattggcttaattgagcaggaagctgtcagtgagttttagaacaagtagaggcaaatgacgaggtcgc cattctctctgaggaagaggaagtcgaaattgaattgcttcatgaattgaaacgattcctgtttatccgttgagtaagcccagaagatgtggacgcg cttgaactcgatccagcgatttcttatattgaagaggatgcagaagtaacgacaatg (SEQ ID NO:6), which encodes the unmodified pro region

AEAAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAIS YIEEDA EVTTM (SEQ ID NO:7), and the polynucleotide sequence

gcgcaatcagtgccatggggaattagccgtgtgcaagccccagctgccataaccgtggattgacaggttctggtgtaaaagtgtctcctcgata cagggtatttccactcatccagacttaaatattcgtgtggtgtagcttaccaggggaaccatccactcaagatgggaatgggcatggcagcat gtggccgggacgattgtgctttaacaattcagttggcgttctgtagcgcgagcgcggaactatacgtgttaagtattaggggagagcg

gttcagggtcgtcagctcagttgcccaaggattggaatgggaggggaacaatggcatgcacgttgctaattgagtttaggaagccctcgccaagt gccacacttgagcaagcgtttaatagcgcgacttctagaggcgttctgttagcggcatctggaaattcagggtcagggtcaatcagctatccggc ccgttatgcgaacgcaatggcagtcggagctactgacaaaacaacaaccgagcgtttcacagtatggcgcagggcttgacattgtgcac cagggtgaaacgtgcagagcacataccaggttcaacgtatgccagcttaaacggtacatcgatggctactcctcatgttcagggtgcagcagccc ttgttaacaaaagaacccatctgttccaatgtacaatccgcaatcatcagaataacggaacgagcttaggaagcagcaacttgatggaa ggggactgtcaatgcagaagctgcaactcgt (SEQ ID NO:8),

which encodes the mature region of protease 9 (P9).

AQSVPWGISRVQAPAAHNRGLTGSVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDNGHGHVAG TIAALNNSIGVLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVN SATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYAGLDIVAPGVNVQSTYPGS TYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR (SEQ ID NO:9).

[0126] Each of the 3 codons in the pro region of SEQ ID NO:7, exemplified by NNG/C, comprised in the full-length protease of SEQ ID NO:59, were mutated to be substituted by the 32 possible nucleotide triplets that encode the 20 naturally occurring amino acids to generate three libraries as follows. An aliquot of plasmid pJH-P9 DNA comprising the sequence encoding the full-length protease was mutated to generate a first library of clones encoding all possible substitutions of glutamic acid (E) at position 6 (E6X) of the pro region (SEQ ID NO:7); a second aliquot was mutated to generate a second library of clones encoding all possible substitutions of glutamic acid (E) at position 30 (E30X) of the pro region (SEQ ID NO:7); and a third aliquot was mutated to generate a third library of clones encoding all possible substitutions of arginine (A) at position 32 (A32X) of the pro region (SEQ ID NO:7). Complementary overlapping primers were designed for mutating the codons of interest with about 18 bases flanking the NNS codon. The polynucleotide sequences of the forward and reverse primers used to mutate the amino acids at positions 6, 30 and 32 are given in Table 1.

TABLE 1

Primer Name*	Primer Sequence	Bases Left**	Bases Right***
6F	GCTGCTGAAGAAGCAAAANNSAAATATTTAA TTGGCTTTAATG (SEQ ID NO:26)	18	22
6R	CATTAAAGCCAATTAAATATTTSNNTTTTGCT TCTTCAGCAGC (SEQ ID NO:27)	22	18
30F	CAAGTAGAGGCAAAATGACNNSTCGCCATT		

Primer Name*	Primer Sequence	Bases Left**	Bases Right***
	CTCTCTGAG (SEQ ID NO:28)	18	18
30R	CTCAGAGAGAATGGCGACSNNGTCATTTGC CTCTACTTG (SEQ ID NO:29)	18	18
32F	GAGGCAAATGACGAGGTCNNSATTCTCTCT GAGGAAGAG (SEQ ID NO:30)	18	18
32 R	CTCTTCCTCAGAGAGAATSNNGACCTCGTC ATTGCGCTC (SEQ ID NO:31)	18	18
*The primer names provided indicate the amino acid position at which the substitution is made; "R" indicates that the primer is the reverse primer and "F" indicates that the primer is a forward primer. For example, 6F is the forward primer that was used in the substitution of amino acid at position 6 of the pro sequence set forth in SEQ ID NO:7. ***"Bases left" and ****"Bases Right" indicate the number of bases to the left and to the right of the mutating codon ("NNS") that are present in the primer. These bases are complementary to the bases of the template precursor polynucleotide bases.			

[0127] pJH-P9 DNA was used as template in the QuikChange (QC) mutagenesis reaction as follows. Two microliters of pJH-P9 miniprep DNA (50ng) were added to 40. μ L of sterile distilled H₂O, 1 μ L of PfuTurbo, 5 μ L 10x Pfu buffer, 1 μ L dNTPs (Roche), 0.5 μ L of forward primer (5 μ M), and 0.5 μ L reverse primer (5 μ M), for a total of 50 μ L. The DNA amplification reaction (PCR) was performed under the following cycling conditions: 95°C for 1min, once, followed by 19-20 cycles of 95°C for 1min., 55°C for 1 min, and 68°C for 12 min. Five microliters of the PCR reaction were analyzed by electrophoresis using a 1.2% E-gel (Invitrogen). Subsequently, the mutated amplified DNA was digested twice, using 1 μ L *DpnI* at 37°C for 2 to 8 hours. A negative control was generated under similar conditions, but in the absence of primers. One microliter of each of the *DpnI*-digested reaction products was used to transform fifty microliters of one-shot TOP10 chemically competent cells (Invitrogen) using the manufacturer's protocol. The transformed cells were grown in Luria's Broth (LB) with shaking at 37 C for 1 hour, then streaked on Luria Agar (LA) plates containing 50 ppm carbenicillin, and allowed to grow at 37°C overnight. Following the overnight incubation, individual colonies were picked, used to inoculate 150 μ L of LB containing 50 ppm carbenicillin, and grown overnight at 37°C in 96-well microtiter plates. An aliquot of the culture grown in the micro titer plates was transferred to LA plates containing 50 ppm carbenicillin, and the plates were sent to Quintara Inc. for isolation and sequence analysis of the mutated DNA. Glycerol was added to a final concentration of 20% to the cultures remaining in the microtiter plates, which were then frozen at -80°C and stored.

(b) Generation of *B. subtilis* strains expressing modified Pn proteases.

[0128] Aliquots of the E. coli microtiter cell cultures harboring the mutated pro sequences were used to inoculate 5 ml of LB+50ppm carbenicillin. Plasmid DNA was prepared using a Qiagen kit (Qiagen), and a portion of each plasmid DNA was used to transform *B. subtilis* host cells. Ten microliters of the plasmid

DNA (pJH-P9) were used to transform 100ul of *B. subtilis comK* competent cells (genotype: $\Delta aprE$, $\Delta nprE$, *degUHy32*, *oppA*, *DspoIIE3501*, *amyE::xylRPxylAcomK-phleo*). A control plasmid containing the P9 construct comprising the unmutated pro sequence (unmutated SEQ ID NO:7) was also transformed to *B. subtilis comK* cells. The transformed cells were incubated at 37°C for 45 minutes while shaking at 250 rpm. Cells from the transformation mixture were plated onto LA plates containing 1.6% skim milk and 5 ppm chloramphenicol (CMP) and incubated overnight in at 37°C. One colony, from each of the transformations, was picked and re-streaked on the LA plates containing 5ppm CMP+1.6% skim milk.

[0129] Bacterial colonies harboring the control plasmid or a plasmid encoding a modified protease were used to inoculate 150 uL of Luria Broth containing 5ppm CMP in wells of a microtiter plate. The microtiter plates were then incubated for four hours at 37°C while rotating at 250 rpm. 10ul of each of the cultures were transferred to a new micro-titer plate containing 140ul of Grants II media, pH 7.3, and the cultures were grown in a shaking incubator at 37°C, 250 rpm for 40 hours. (Grants II media was prepared as follows: Solution I: 10g of Soytone were dissolved in 500 ml water and autoclaved for 20-25 minutes; Solution II: 3 ml of 1M K₂HPO₄, 75 g glucose, 3.6g urea, 100ml Grant's 10X MOPS were diluted into 400ml water. Solutions I and II were mixed and the pH adjusted to pH7.3 with HCl/NaOH. The final volume was adjusted to 1 L, and the final solution was sterilized through 0.22-um PES filter.) Following the incubation, the microtiter plates were centrifuged and the supernatant of each of the cultures was assayed for protease activity using the AAPF assay described below.

(c) Measurement of Modified Protease Production: AAPF Assay of Protease Activity

[0130] Each of the *B. subtilis* cultures obtained as described in Example 1(b), was assayed for the production of the modified proteases. The enzymes produced were assayed for activity against the substrate, succinyl -L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanalide (AAPF). The assay measured the production of modified protease as the increase in absorbance at 405 nm/min resulting from the hydrolysis and release of p-nitroaniline (Estell et al., J Biol Chem., 260:6518-6521 (1985)). The measurements were made using the Softmax Pro software, and the specified conditions were set as: Type: Kinetic; Reduction: Vmax Points (Read best 15/28 points); Lm1: 405 nm; Time: 5 minutes; and Interval: 11 Seconds. Ten microliters of each of the *B. subtilis* cultures were diluted to 100ul of Tris Buffer, containing 10 mM Tris + 0.005% TWEEN®-80, pH 8.6; and 25ul of 100 mg/ml AAPF substrate to assay for protease activity. The relative activity of each of the modified proteases was calculated, and the effect of each amino acid substitution on the production of the corresponding modified protease was determined as a ratio of the activity of the mature protease processed from each modified protease to the activity of the mature protease processed from the unmodified protease precursor protease. Once the DNA construct was stably integrated into a competent *Bacillus subtilis* strain, the activity of the modified proteases was measured in microtiter assays and the activity was compared to the activity of the corresponding protease processed from the unmodified precursor.

[0131] Ten microliters of overnight Grant II Media cell cultures were diluted to 100ul of Tris Buffer, containing 10 mM Tris + 0.005% TWEEN®-80 pH 8.6; and 25ul of 100 mg/ml AAPF substrate were used to assay for protease activity. Assays were done in microtiter plates and the Softmax Pro Software was used.

[0132] The results given in Tables 2, 3, and 4, showed that all but one of the amino acid substitutions of amino acids at positions 6 of the pro region (SEQ ID NO:7) within the precursor protease (SEQ ID NO:59) lead to an enhanced production of the mature form of the protease of SEQ ID NO:9, whereas all

but one of the amino acid substitutions at positions 30 or 32 showed similar or diminished protease production when compared to the production of the mature protease when processed from an unmodified pro region. In addition, site saturation of each of the substituted amino acids showed that each amino acid can be substituted by two or more amino acids at the same position to increase the production of the mature form relative to that obtained from the precursor protease having unmodified pro region.

TABLE 2

Effect of amino acid substitution at position 6 of the pro region on the production of the mature protease of SEQ ID NO:9	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E6 (control)	100
E6A	119
E6R	236
E6N	377
E6C	425
E6Q	455
E6G	458
E6H	117
E6M	280
E6F	411
E6P	529
E6S	512
E6T	480
E6W	277
E6Y	7
E6V	550

TABLE 3

Effect of amino acid substitution at position 30 of the pro region on the production of the mature protease of SEQ ID NO:9	
Mutation (Substitution) at position in pro region	Percent activity relative to the unmodified precursor
E30 (control)	100
E30A	76
E30R	73
E30N	82
E30D	80
E30G	47
E30H	78
E30I	61
E30L	66
E30M	75

Effect of amino acid substitution at position 30 of the pro region on the production of the mature protease of SEQ ID NO:9	
Mutation (Substitution) at position in pro region	Percent activity relative to the unmodified precursor
E30F	70
E30P	69
E30S	73
E30T	83
E30W	62
E30V	60

TABLE 4

Effect of amino acid substitution at position 32 of the pro region on the production of the mature protease of SEQ ID NO:9	
Mutation (Substitution) at positions in pro region	Percent activity relative to activity from the unmodified precursor
A32 (control)	100
A32R	64
A32N	76
A32Q	75
A32E	46
A32G	78
A32H	60
A32I	67
A32L	76
A32K	121
A32F	32
A32P	37
A32S	74
A32T	99
A32W	80
A32V	98

(d) Site -saturation mutagenesis: generation of combinations of substitutions in the pro region.

[0133] The plasmid expressing the A32K substitution in the pro region (SEQ ID NO:7) comprised in the full-length protease of SEQ ID NO:59 was subjected to a second round of site-saturation mutagenesis of the codon at position 6 to create a first library of polynucleotides that encode a full-length protease containing a substitution of amino acid 6 in combination with the A32K substitution of the pro region of the protease. The mutation at position 6 was created using the QuikChange® site-directed mutagenesis kit (QC; Stratagene) according to the directions provided by the manufacturer using forward and reverse primers of SEQ ID NOS:26 and 27, respectively. Similarly, a second library of polynucleotides was

created to encode a full-length protease containing a substitution of amino acid 30 in combination with the A32K substitution in the pro region of the protease was created. The complementary overlapping forward (CAAGTAGAGGCAAATGACNNSGTCAAATTCTCTCTGAG; SEQ ID NO:32) and reverse primers (CTCAGAGAGAATTTTGACSNNGTCATTTGCCTCTACTTG; SEQ ID NO:33) were used for mutating the position 30.

[0134] The QC reaction, amplification of the plasmid DNA, and transformation of *E. coli* cells were performed as described in Example 1 (a). The subsequent transformation of *Bacillus subtilis* competent cells was also performed as described in Example 1 (b). Supernatants from *Bacillus* cultures expressing proteases from modified or unmodified precursor were analyzed for protease activity using the AAPF assay as described in Example 1(c).

[0135] Results shown in Tables 5 and 6 indicate that most substitutions of the amino acid at position 6 (Table 5) of the pro region when in combination with the substitution A32K further enhance the production of the mature form of the protease expressed from a polynucleotide encoding an unmodified pro region or a pro region containing the A32K substitution. However, the combination of amino acid substitutions at position 30 when in combination with the A32K substitution did not enhance the production of the mature protease of SEQ ID NO:9 (Table 6).

TABLE 5

Effect of the combination of amino acid substitution A32K with substitutions of amino acid at position 6 of the pro region on the production of mature protease of SEQ ID NO:9	
Mutation (Substitution) at positions in pro region	Percent activity relative to activity from the modified precursor E6-A32K
E6-A32K (control, modified)	100
E6R-A32K	160
E6N-A32K	120
E6D-A32K	170
E6C-A32K	71
E6G-A32K	79
E6I-A32K	103
E6L-A32K	50
E6K-A32K	132
E6M-A32K	127
E6F-A32K	71
E6P-A32K	161
E6S-A32K	82
E6T-A32K	134
E6W-A32K	60
E6Y-A32K	95
E6V-A32K	63

TABLE 6

Eff ect of the combination of amino acid substitution A32K with substitutions of amino acid at position 30 of the pro region on the production of mature protease of SEQ ID NO:9	
Mutation (substitution) at positions in pro region	Percent activity relative to activity from the modified precursor E30-A32K
E30-A32K (control, modified)	100
E30A-A32K	38
E30R-A32K	33
E30N-A32K	33
E30D-A32K	34
E30C-A32K	33
E30Q-A32K	33
E30G-A32K	38
E30H-A32K	40
E30I-A32K	30
E30L-A32K	33
E30K-A32K	47
E30M-A32K	45
E30F-A32K	36
E30P-A32K	72
E30S-A32K	67
E30T-A32K	26
E30W-A32K	85
E30Y-A32K	96
E30V-A32K	65

(e) Effect of amino acid substitution(s) in the pro region of the precursor protease on the production of the mature protease of SEQ ID NO:9 in shake flask cultures.

[0136] To test the effect of amino acid substitutions in the pro region on the production of the mature protease of SEQ ID NO:9 in shake flask cultures, several of the *Bacillus subtilis* strains grown in the microtiter plates as described above were grown as follows. *Bacillus subtilis* strains expressing the modified precursor comprising a single substitution at one of positions 6, 30 and 32 of the pro region, or comprising a combination of two substitutions of amino acids at positions 6 and 32, or 30 and 32 of the pro region and that were previously grown in microtiter plates, were first plated on Luria Agar plates containing 5ppm chloramphenicol and 1.6% skim milk. A single colony was used to inoculate 5 ml of Luria Broth containing 5 ppm chloramphenicol. Each 5 ml culture was grown for 5 hours at 37°C while shaking at 250 rpm. A 250 ml shake flask containing 25 ml of Grant's II media was inoculated with 1 ml of the 5 ml culture of strains comprising a single substitution, and the 250 ml culture was incubated for 40, at 37°C while shaking at 250 rpm. Strains comprising double substitutions were grown for 40 and/or for 48 hours, as shown by the data given in Tables 9 and 10. Supernatant from the shake flask cultures was

assayed for AAPF activity as described in Example 1 (c). The results for the activity in strains comprising a single substitution are shown in Tables 7 and 8, and the results for the activity in strains comprising a substitution at position 6 or 32 in combination with the A32K substitution are shown in Tables 9 and 10, respectively.

[0137] The results show that the enhancement of protease production obtained from modified precursor proteases in microtiter cultures is mimicked in most shake flask cultures. Eight of 22 strains did not mirror the production of protease in shake flask that was seen in microtiter plates. Four of the eight strains, which produced more protease than their respective controls when grown in microtiter plates produced less protease than the respective controls when grown in shake flasks (E6C, E6F, E6D-A32K and E6K-A32K). The remaining four strains, which produced less protease than their respective controls when grown in microtiter plates produced more protease than the respective controls when grown in shake flasks (A32T, A32V, E6S-A32K, E30V-A32K, and E30W-A32K). It is likely that the production of protease by these strains is affected by the different growth conditions imposed in microtiter versus shake flask cultures. One skilled in the art would know how to optimize growth conditions.

TABLE 7

Effect of amino acid substitution at position 6 of the pro region on the production of the mature protease of SEQ ID NO:9 in shake flask cultures	
Mutation (substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor (48 hours)
E6 (control)	100
E6N	111
E6C	84
E6Q	149
E6G	140
E6F	91
E6P	110
E6S	114
E6T	113
E6V	145

TABLE 8

Effect of amino acid substitution at position 32 of the pro region of the mature protease of SEQ ID NO:9 in shake flask cultures	
Mutation (substitution) at positions in pro region	Percent activity relative to activity from the unmodified precursor (48 hours)
A32 (control)	100
A32K	140
A32T	260
A32V	190

TABLE 9

Effect of the combination of amino acid substitution A32K with substitutions of amino acid at position 6 of the pro region of the mature protease of SEQ ID NO:9 in shake flask cultures		
Mutation (substitution) at positions in pro region	Percent activity relative to activity from the modified precursor A32K	
	40 hours	48 hours
E6-A32K (control, modified)	100	100
E6R-A32K	108	126
E6N-A32K	91	110
E6D-A32K	64	80
E6K-A32K	59	65
E6M-A32K	126	179
E6S-A32K	77	106

TABLE 10

Effect of the combination of amino acid substitution A32K with substitutions of amino acid at position 30 of the pro region of the mature protease of SEQ ID NO:9 in shake flask cultures		
Mutation (substitution) at positions in pro region of GG36 precursor	Percent activity relative to activity from the modified precursor A32K	
	40 hours	48 hours
E30-A32K (control, modified)	100	100
E30P-A32K	73	86
E30W-A32K	130	93
E30Y-A32K	90	99
E30V-A32K	92	132

EXAMPLE 2

The effect of mutations in the pro region of SEQ ID NO:7 on the production of the mature alkaline protease of SEQ ID NO:11.

Site -saturation mutagenesis of amino acids at positions 6, 30 or 32 of the pro region

[0138] Site-saturation mutagenesis of the pro region on the production of the mature protease of SEQ ID NO:11 was performed using the QuikChange® site-directed mutagenesis kit (QC; Stratagene) according to the directions of the manufacturer. A DNA cassette comprising the AprE promoter, and the polynucleotide that encodes the full-length protease of SEQ ID NO:60 was cloned into the EcoRI and HindIII restriction sites of the pJH101 vector (Ferrari et al. J. Bacteriol. 154:1513-1515 [1983]) pJH-Pn (Figure 4A) to generate the pJH-P11 plasmid. (Pn refers to the SEQ ID NO of the mature protease that is expressed from the pJH-Pn plasmid). The DNA cassette comprised the *B. subtilis* aprE promoter

aaattcctcattttctctcctatcaaaataacacactcotoattttccaaacaaactttcaaaaaaacctctcccttcaaatcgaatccctctctat

 aaaattccgatattggttaaacagcggcgcaatggcgccgcatctgctgttctgctggcgaaattcatcttattcttccctcctaataatttttc
 attctatcccttttctgaaagttattttcagaatactttatcatcatgctttgaaaaatatcacgataataccattgtctcacggaagcacacgcagg
 tcatitgaacgaatttttcgacaggaatttgcgggactcaggagcatttaacctaaaaagcatgacatttcagcataatgaacatttactcatgtcta
 ttttctgttcttctgtatgaaaaagttatttcgagtcctacggaatagcgagagatataacctaataagagataaaatcatctcaaaaaatgggt
 ctactaaaaatatttccatctattacaataaaattcacagaatagctctttaagtaagtcactctgaattttttaaaggagagggtaaaga (SEQ ID
 NO:1),

the polynucleotide sequence

gtgagaagcaaaaaattgtgatcagctgtgtttgctgtaacgttaatctttacgatggcggtcagcaacatgtctgcgcaggct (SEQ ID
 NO:2), which encodes the AprE signal peptide VRSKKLWISLLFALTIFTMAFSNMSAQA (SEQ ID NO:3),

the polynucleotide sequence

gctgaagagcaaaagaaaaatatttaattggcttaafgagcaggaagctgtcagtgagtttgaacaagtagaggcaaatgacgaggtcgc
 cattctctcaggaagaggaagtcgaattgaattgctcatgaatttgaacgattcctgtttatccgttgagttaagcccagaagatgtggacgcg
 ctggaactcgcagcagattcttatattgaaggagatgcagaagtaacgacaatg (SEQ ID NO:6)

which encodes the unmodified pro region

AEEAKEKYLI GFNEQEAVSE FVEQVEANDE VAILSEEEEV EIELLHEFET IPVLSVELSP EDVDALELDP
 AISYIEEDAE VTTM (SEQ ID NO:7), and the polynucleotide sequence

gcgcaatcagtgccatggggaattagccgtgtgcaagcccgagctgcccataaccgtggattgacagggttcgtgtgtaaaagtgtgtcctcgata
 cagggtattccactcatccagacttaaatattcgtgggtggcgtagctttgtaccaggggaacctccactcaagatgggaatgggcatggcacgcgat
 gtggccgggacgattgtcgtctagacaattcgaattggcgttctggcgtagcgccgagcgcggaactatacgtgttaaagtattggggcgagcg
 gttcaggcgccatcagctcagttgcccaaggattggaatgggcagggacaacatggcatgcacgttgctaattgagtttaggaagcccttcgccc
 gtgccacacttgagcaagctgttaatagcgcgactctagaggcgttctgttagcgccatctggaaattcagggtcaggctcaatcagctatccgg
 ccggttatgcgaacgcaatggcagtcggagctactgacaaaacaacaaccgcgcagctttcacaglatggcgagggttgacattgtcgcga
 ccagggtgtaaacgtgcagagcacataccagggttcaacgtatgccagcttaaacggtacatcgatggctactcctcatgttcaggtgcagcagcc
 ctgttaaaaaaagaaccatctgttccaatgtacaaatccgcaatcatctaaagaatacggcaacgagcttaggaagcacgaactgtatgga
 agcgggaactgtcaatgcagaagctgcaactcgt

(SEQ ID NO:10), which encodes the mature region of protease P11 (SEQ ID NO:11)

AQSVPWGISRVQAPAAHNRLTGSGVKVAVLDTGISTHPDLNIRGGASFPVGPSTQDGNHGHGTHVAG
 TIAALDNSIGVLGVAPSAELYAVKVLGASGSGAIISSIAQGLEWAGNNGMHVANLSLGSPPSATLEQAVN
 SATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGS
 TYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR (SEQ ID
 NO:11).

[0139] Each of the 3 codons in the pro region of SEQ ID NO:7, exemplified by NNG/C, comprised in the full-length protease of SEQ ID NO:60, were mutated to be substituted by the 32 possible nucleotide triplets that encode the 20 naturally occurring amino acids to generate three libraries as follows. An aliquot of plasmid pJH-P11 DNA comprising the sequence encoding the full-length protease was mutated to generate a first library of clones encoding all possible substitutions of glutamic acid (E) at position 6 (E6X) of the pro region (SEQ ID NO:7); a second aliquot was mutated to generate a second library of clones encoding all possible substitutions of glutamic acid (E) at position 30 (E30X) of the pro region (SEQ ID NO:7); and a third aliquot was mutated to generate a third library of clones encoding all possible substitutions of arginine (A) at position 32 (A32X) of the pro region (SEQ ID NO:7). Complementary overlapping primers were designed for mutating the codons of interest with about 18 bases flanking the NNS codon. The polynucleotide sequences of the forward and reverse primers used to mutate the amino acids at positions 6, 30 and 32 are given in Table 1.

[0140] The QC reaction, amplification of the plasmid DNA, and transformation of *E. coli* cells were performed as described in Example 1(a). The subsequent transformation of *Bacillus subtilis* competent cells was also performed as described in Example 1(b). Supernatants from *Bacillus* cultures expressing proteases from modified or unmodified precursor were analyzed for protease activity using the AAPF assay as described in Example 1(c).

[0141] The results given in Tables 11, 12, and 13, show that most amino acid substitutions of amino acids at positions 6, 30 and 32 of the pro region (SEQ ID NO:7) in the precursor protease (SEQ ID NO:60) lead to an enhanced production of the mature form of the protease. In addition, site saturation of each of the substituted amino acids showed that each amino acid can be substituted by two or more amino acids at the same position to increase the production of the mature form relative to that obtained from the precursor protease having unmodified pro region.

TABLE 11

Effect of amino acid substitution at position 6 of the pro region on the production of the mature protease of SEQ ID NO:11	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E6 (control)	100
E6A	125
E6R	120
E6Q	161
E6G	187
E6L	177
E6K	160
E6M	165
E6F	131
E6P	72
E6S	56
E6T	103
E6W	52
E6V	265

TABLE 12

Effect of amino acid substitution at position 30 of the pro region on the production of the mature protease of SEQ ID NO:11	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E30 (control)	100
E30A	90
E30R	112
E30N	97
E30Q	126
E30G	111
E30I	132
E30L	128
E30M	152
E30F	102
E30P	132

Effect of amino acid substitution at position 30 of the pro region on the production of the mature protease of SEQ ID NO:11	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E30T	125
E30W	105
E30Y	125
E30V	142

TABLE 13

Effect of amino acid substitution at position 32 of the pro region on the production of the mature protease of SEQ ID NO:11	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
A30 (control)	100
A32R	99
A32D	98
A32C	100
A32Q	11
A32G	96
A32H	98
A32L	98
A32M	98
A32F	93
A32P	93
A32S	117
A32T	129
A32V	124

Effect of amino acid substitution(s) in the pro region of the precursor protease on the production of the mature protease of SEQ ID NO:11 in shake flask cultures.

[0142] To test the effect of amino acid substitutions in the pro region on the production of the mature protease of SEQ ID NO:11 in shake flask cultures, several of the *Bacillus subtilis* strains comprising a substitution at position 6 of the pro region in the precursor protease as described above were grown for 48 hours as described in Example 1 (e). Supernatant from the shake flask cultures was assayed for AAPF activity as described in Example 1(c).

[0143] The results shown in Table 14 indicate that substitutions made at position 6 of the P11 precursor sequence which were shown to enhance the production of the mature protease (SEQ ID NO:11) in cultures grown in microtiter plates also increase the production of the protease in cultures grown in shake flasks.

TABLE 14

Effect of amino acid substitutions at position 6 of the pro region on the production of the mature protease of SEQ ID NO:11 in shake flask cultures	
Mutation (substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor (48 hours)
E6 (control)	100
E6A	157
E6R	137
E6Q	82
E6G	88
E6L	130
E6K	121
E6M	99
E6F	43
E6V	116

EXAMPLE 3

The effect of mutations in the pro region of SEQ ID NO:7 on the production of the mature alkaline protease of SEQ ID NO:19.

(a) Site -saturation mutagenesis of amino acids at positions 6, 30 or 32 of the pro region

[0144] Site-saturation mutagenesis of the pro region on the production of the mature protease of SEQ ID NO:19 was performed using the QuikChange® site-directed mutagenesis kit (QC; Stratagene) according to the directions of the manufacturer. A DNA cassette comprising the AprE promoter, and the polynucleotide that encodes the full-length protease of SEQ ID NO:64 was cloned into the EcoRI and HindIII restriction sites of the pJH101 vector (Ferrari et al. J. Bacteriol. 154:1513-1515 [1983]) pJH-Pn (Figure 4A) to generate the pJH-P19 plasmid. (Pn refers to the SEQ ID NO of the mature protease that is expressed from the pJH-Pn plasmid). The DNA cassette comprised the *B. subtilis* aprE promoter

gaattcctccattttcttctgctatcaaaataacagactcgtgatttccaaacgagctttcaaaaaagcctctgccccttgc aaatcggatgcctgtctat
 aaaattcccgatattggtaaacagcggcgcaatggcggcgcatctgatgtcttggctggcgaaatgttcaatctatttcttccctcctcaataatttttc
 attctatcccttttctgtaaaagtatttttcagaatactttatcatcatgctttgaaaaatatcacgataataatccattgttctcaoggaagcacacgcagg
 tcaattgaacgaatttttcgacaggaattgcccgggactcaggagcatttaacctaaaaagcatgacatttcagcataatgaacattactcatgtcta
 tttcgtctttctgtatgaaaatagttatttcgagctctacggaatagcgagagatgatacctaataatagagataaaatcatctcaaaaaatgggt
 ctactaaaatatttccatctattacaataaaattcacagaatagcttttaagtaagtctactctgaattttttaaaggagagggttaaaga (SEQ ID
 NO:1),

the polynucleotide sequence
 gtgagaagcaaaaaattgtggatcagctgtgtttgctgtaacgttaactttacgatggcggtcagcaacatgtctgcgcaggct (SEQ ID
 NO:2), which encodes the AprE signal peptide VRSKLLWISLLFALTILFTMAFSNMSAQA (SEQ ID NO:3),
 the polynucleotide sequence
 gctgaagaagcaaaagaaaaatatttaattggcttaatgagcaggaagctgtcagtgagttttagaacaagtagaggcaaatgacgagggtcgc
 catctctctgaggaagaggaagtcgaattgaattgcttcatgaattgaaacgattcctgtttatccgttgagtttaagccagaagatgtggacgog
 cttaactcaatccaacatttcttatatfoaaacaaatocaaaataacaaacaata (SEQ ID NO:6).

which encodes the unmodified pro region

AEEAKEKYLI GFNEQEAVSE FVEQVEANDE VAILSEEEV EIELLHEFET IPVLSVELSP EDVDALELDP AISYIEEDAE VTTM (SEQ ID NO:7), and the polynucleotide sequence

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gcgcaatcggtaccatggggaattagccgtgtgcaagccccagctgccataaccgtggattgacagggtctggtgtaaagttgctgcctcgata
caggatattccactcatccagactaaatattcgtggtggcgtagttttgtaccaggggaaccatccactcaagatgggaatgggcatggcagcat
gtggctgggacgattgctcttaacaattcgtggtggtgtagcaccgaacgcggaaactatacgtgttaaagtattagggcgagcgg
tggcgttogaacagctcgattgccaaggattggaatgggcagggaacaatggcatgcacgtgctaattgagtttaggaagcccttcgccaagt
gccacactgagcaagctgttaatagcgcgactttagaggcgttctgtttagcggcatctggcaattcagggtcaggctcaatcagctatccggc
ccgttatgcgaacgcgaatggcagtcggagctactgacaaaacaacaaccgcgccagctttcacagtatggcgagggttgacattgtcgcac
cagggtgtaaacgtgcagagcacataccagggtcaacgtatgccagcttaaacggtacatcgatggctactcctcatgttcagggtgcagcagccc
ttgttaacaaaagaaccatcttggccaatgtacaatccgcaatcatctaaagaatacggcaacgagcttaggaagcacgaactgtatggaa
gcggactgtcaatgcagaagcggcaacacgt
```

(SEQ ID NO:18) encoding mature region of protease 19 (P19) (SEQ ID NO:19)

```
AQSVPWGISRVQAPAAHNRLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDGNHGHGTHVAG
TIALNNSIGVLGVAPNAELYAVKVLGASGGGSSNSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAV
NSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYAGLDIVAPGVNVQSTYPG
STYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSLVNAEAATR (SEQ
ID NO:19).
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[0145] Two codons in the pro region of SEQ ID NO:7, exemplified by NNG/C, comprised in the full-length protease of SEQ ID NO:64, were mutated to be substituted by the 32 possible nucleotide triplets that encode the 20 naturally occurring amino acids to generate two libraries as follows. An aliquot of plasmid pJH-P19 DNA comprising the sequence encoding the full-length protease was mutated to generate a first library of clones encoding all possible substitutions of glutamic acid (E) at position 6 (E6X) of the pro region (SEQ ID NO:7); and a second aliquot was mutated to generate a second library of clones encoding all possible substitutions of arginine (A) at position 32 (A32X) of the pro region (SEQ ID NO:7). Complementary overlapping primers were designed for mutating the codons of interest with about 18 bases flanking the NNS codon. The polynucleotide sequences of the forward and reverse primers used to mutate the amino acids at positions 6 and 32 are given in Table 1.

[0146] The QC reaction, amplification of the plasmid DNA, and transformation of *E. coli* cells were performed as described in Example 1 (a). The subsequent transformation of *Bacillus subtilis* competent cells was also performed as described in Example 1 (b). Supernatants from *Bacillus* cultures expressing proteases from modified or unmodified precursor were analyzed for protease activity using the AAPF assay as described in Example 1(c).

[0147] The results given in Tables 15 and 16 show that amino acid substitution of most of the amino acids of the precursor protease lead to an enhanced production of the mature form of the protease. In addition, site saturation of each of the substituted amino acids showed that each amino acid can be substituted by two or more amino acids at the same position to increase the production of the mature form relative to that obtained from the precursor protease having unmodified pro region.

TABLE 15

Effect of amino acid substitution at position 6 of the pro region on the production of the mature protease of SEQ ID NO:19	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E6 (control)	100
E6A	128

Effect of amino acid substitution at position 6 of the pro region on the production of the mature protease of SEQ ID NO:19	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E6C	25
E6D	53
E6G	95
E6H	133
E6I	36
E6K	111
E6L	50.
E6N	35
E6P	28
E6Q	45
E6R	100
E6S	71
E6W	31
E6T	75

TABLE 16

Effect of amino acid substitution at position 30 of the pro region on the production of the mature protease of SEQ ID NO:19	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E30 (control)	100
E30A	488
E30R	384
E30N	405
E30D	241
E30G	374
E30H	371
E30I	51
E30L	211
E30K	265
E30F	168
E30P	66
E30S	601
E30T	351
E30V	254

(b) Site -saturation mutagenesis: generation of combinations of substitutions in the pro region.

[0148] The plasmid expressing the E30G substitution in the pro region (SEQ ID NO:7) comprised in the full-length protease of SEQ ID NO:64 was subjected to a second round of site-saturation mutagenesis of the codon at position 6 to create a library of polynucleotides that encode a full-length protease containing a substitution of amino acid 6 in combination with the E30G substitution of the pro region of the protease. The mutation at position 6 was created using the QuikChange® site-directed mutagenesis kit (QC; Stratagene) according to the directions provided by the manufacturer using forward and reverse primers of SEQ ID NOS:26 and 27, respectively.

[0149] The QC reaction, amplification of the plasmid DNA, and transformation of *E. coli* cells were performed as described in Example 1 (a). The subsequent transformation of *Bacillus subtilis* competent cells was also performed as described in Example 1 (b). Supernatants from *Bacillus* cultures expressing proteases from modified or unmodified precursor were analyzed for protease activity using the AAPF assay as described in Example 1(c).

[0150] Results shown in Table 17 indicate that most substitutions of amino acid at position 6 of the pro region when in combination with the substitution E30G at amino acid position 30 lead to an enhanced production of the mature form of the protease.

TABLE 17

Effect of the combination of amino acid substitution E30G with substitutions of amino acid at position 6 of the pro region on the production of mature protease of SEQ ID NO:19	
Mutation (Substitution) at positions in pro region of precursor E6X-E30G	Percent activity relative to activity from the unmodified precursor E6-E30
E6-E30 (control, unmodified)	100
E6A-E30G	188
E6R-E30G	126
E6N-E30G	158
E6D-E30G	126
E6C-E30G	220
E6Q-E30G	147
E6G-E30G	107
E6H-E30G	144
E6L-E30G	96
E6K-E30G	117
E6M-E30G	114
E6F-E30G	152
E6P-E30G	108
E6S-E30G	108
E6T-E30G	100
E6W-E30G	104
E6V-E30G	185
E6Y-E30G	148

(c) Effect of amino acid substitution(s) in the pro region of the precursor protease on the production of the mature protease of SEQ ID NO:19 in shake flask cultures.

[0151] To test the effect of amino acid substitutions in the pro region on the production of the mature protease of SEQ ID NO:19 in shake flask cultures, several of the *Bacillus subtilis* strains grown in the microtiter plates as described above and containing the combinations of substitutions E6A-E30G, E6C-E30G, and E6V-E30G in the pro region were grown for 48 hours as described in Example 1(e). Supernatant from the shake flask cultures was assayed for AAPF activity as described in Example 1(c).

[0152] The results shown in Table 18 indicate that the combination of mutations E6A and E30G in the pro region of the protease precursor leads to enhanced production of the mature protease (SEQ ID NO:19) compared to the production of the protease processed from the precursor containing the single mutation E30G.

TABLE 18

Effect of the combination of amino acid substitution E30G with substitutions of amino acid at position 6 of the pro region of the mature protease of SEQ ID NO:19 in shake flask cultures	
Mutation (substitution) at positions in modified pro region	Percent activity relative to activity from the modified precursor E30G (48 hours)
E6-E30G	100
E6A-E30G	178
E6C-E30G	97
E6V-E30G	92

EXAMPLE 4

The effect of mutations in the pro region of SEQ ID NO:7 on the production of the mature alkaline protease of SEQ ID NO:17.

[0153] **Site -saturation mutagenesis of amino acids at positions 6, 30 or 32 of the pro region** Site-saturation mutagenesis of the pro region on the production of the mature protease of SEQ ID NO:17 was performed using the QuikChange® site-directed mutagenesis kit (QC; Stratagene) according to the directions of the manufacturer. A DNA cassette comprising the AprE promoter, and the polynucleotide that encodes the full-length protease of SEQ ID NO:63 was cloned into the EcoRI and HindIII restriction sites of the pJH101 vector (Ferrari et al. J. Bacteriol. 154:1513-1515 [1983]) pJH-Pn (Figure 4A) to generate the pJH-P17 plasmid. (Pn refers to the SEQ ID NO of the mature protease that is expressed from the pJH-Pn plasmid). The DNA cassette comprised the *B. subtilis* aprE promoter

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gaattcctccatttctctgctatcaaaataacagactcgtgattttccaaacgagctttcaaaaagcctctgccccttgcaaatcggatgcctgtctat
aaaattcccgatatttggttaaacacggcgcgaatggcggcgcgcacatctgatgtcttctgttggcgaatgttcattcttctcctcctcaataatttttc
attctatcccttttctgtaaaagttattttcagaatactttatcatcatgctttgaaaaataatcacgataataatccattgttctacggaagcacacgcagg
tcatttgaacgaatttttgcagcaggaatttgcgggactcaggagcatttaacctaaaaaagcatgacatttcagcataatgaacatttactcatgtcta
ttttggttctttctgtatgaaaaatagttatttcgagtcctacggaataagcgagatgatatacctaaatagatataaaatcatctcaaaaaatgggt

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ctactaaaaatatttccatctattacaataaattcacagaatagcttttaagtaagtctactctgaattttttaaaaggagagggtaaaga (SEQ ID

NO:1),

the polynucleotide sequence

gtgagaagcaaaaaattgtggatcagctgtgtgttcgtaacgttaattcttacgatggcggtcagcaacatgtctgcgcaggct (SEQ ID NO:2), which encodes the AprE signal peptide VRSKKLWISLLFALTLIFTMAFSNMSAQA (SEQ ID NO:3),

the polynucleotide sequence

gctgaagaagcaaaagaaaaataatttgcttaattgagcaggaagctgtcagtgagttttagaacaagtagaggcaaatgacgagggtcgc cattctctctgaggaagaggaagtgcgaattgaattgcttcatgaattgaaacgattcctgttttatccgttgagtaagcccagaagatgtggacgcg cttgaactcgatccagcgatttcttatattgaagaggatgcagaagtaacgacaatg (SEQ ID NO:6), which encodes the unmodified pro region

AEEAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAIS YIEEDA EVTTM (SEQ ID NO:7), and the polynucleotide sequence

GCGCAATCGGTACCATGCGGAATTAGCCGTGTGCAAGCCCCAGCTGCCATAACCGTGGAATTGACA GGTCTCGGTGTAAAGTTGCTGTCTCGATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGG TGGCGTAGCTTTGTACAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCAGCATGTGG CTGGGACGATTGCTGCTTTAAACAATTGATTGGCGTTCTTGGCGTAGCACCGAACGCGGAACATA CGCTGTTAAAGTATTAGGGGCGAGCGGTATGGGTTCCGTCAGCTCGATTGCCAAGGATTGGAATG GGCAGGGAACAATGTTATGCACGTTGCTAATTTGAGTTTAGGACTGCAGGCACCAAGTGCCACACTT GAGCAAGCTGTTAATAGCGCGACTTCTAGAGGCGTTCTTGTGTAGCGGCATCTGGCAATTCAGGT GCAGGCTCAATCAGCTATCCGGCCCCGTTATGCGAACGCAATGGCAGTCGGAGCTACTGACCAAAAC AACACCGCGCCAGCTTTTACAGTATGGCGCAGGGCTTGACATTGTGCGACCAGGTGTAAACGTG CAGAGCACATACCCAGGTTCAACGTATGCCAGCTTAAACGGTACATCGATGGCTACTCCTCATGTTG CAGGTGCAGCAGCCCTTGTTAAACAAAAGAACCCATCTTGGTCCAATGTACAAATCCGCAATCATCT AAAGAATACGGCAACGAGCTTAGGAAGCACGAACCTGTATGGAAGCGGACTTGTCAATGCAGAAGC GGCAACACGT (SEQ ID NO:16),

which encodes the mature region of protease 17 (P17)

AQSVPWGISRVQAPAAHNRLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPTQDNGHGHVHAG TIAALNNSIGVLGVAPNAELYAVKVLGASGMGVSISIAQGLEWAGNNVMHVNLSLGLQAPSATLEQAV NSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYAGLDIVAPGVNVQSTYPG STYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSLVNAEAATR (SEQ ID NO:17).

[0154] Each of the 3 codons in the pro region of SEQ ID NO:7, exemplified by NNG/C, comprised in the full-length protease of SEQ ID NO:63, were mutated to be substituted by the 32 possible nucleotide triplets that encode the 20 naturally occurring amino acids to generate three libraries as follows. An aliquot of plasmid pJH-P17 DNA comprising the sequence encoding the full-length protease was mutated to generate a first library of clones encoding all possible substitutions of glutamic acid (E) at position 6 (E6X) of the pro region (SEQ ID NO:7); a second aliquot was mutated to generate a second library of clones encoding all possible substitutions of glutamic acid (E) at position 30 (E30X) of the pro region (SEQ ID NO:7); and a third aliquot was mutated to generate a third library of clones encoding all possible substitutions of arginine (A) at position 32 (A32X) of the pro region (SEQ ID NO:7). Complementary overlapping primers were designed for mutating the codons of interest with about 18 bases flanking the NNS codon. The polynucleotide sequences of the forward and reverse primers used to mutate the amino acids at positions 6, 30 and 32 are given in Table 1.

[0155] The QC reaction, amplification of the plasmid DNA, and transformation of *E. coli* cells were performed as described in Example 1(a). The subsequent transformation of *Bacillus subtilis* competent cells was also performed as described in Example 1(b). Supernatants from *Bacillus* cultures expressing proteases from modified or unmodified precursor were analyzed for protease activity using the AAPF assay as described in Example 1(c).

[0156] The results given in Tables 19, 20 and 21 show that most of the amino acid substitutions of amino acids at positions 6, 30 or 32 of the pro region of the P17 precursor protease lead to an enhanced production of the mature form of the protease (SEQ ID NO:17). In addition, site saturation of each of the substituted amino acids showed that each amino acid can be substituted by two or more amino acids at the same position to increase the production of the mature form relative to that obtained from the precursor protease having unmodified pro region.

TABLE 19

Effect of amino acid substitution at position 6 of the pro region on the production of the mature protease of SEQ ID NO:17	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E6 (control)	100
E6A	152
E6R	231
E6C	172
E6Q	229
E6G	95
E6H	144
E6I	106
E6L	76
E6K	269
E6M	124
E6F	55
E6P	20
E6S	154
E6T	34
E6W	48
E6Y	114
E6V	55

TABLE 20

Effect of amino acid substitution at position 30 of the pro region on the production of the mature protease of SEQ ID NO:17	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E30 (control)	100
E30A	190
E30R	152
E30N	122
E30D	106
E30C	89
E30Q	128
E30G	223

Effect of amino acid substitution at position 30 of the pro region on the production of the mature protease of SEQ ID NO:17	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E30H	83
E30I	78
E30L	112
E30M	137
E30P	129
E30S	159
E30T	176
E30W	243
E30Y	130
E30V	131

TABLE 21

Effect of amino acid substitution at position 32 of the pro region on the production of the mature protease of SEQ ID NO:17	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
A32 (control)	100
A32R	142
A32D	81
A32C	170
A32Q	53
A32E	100
A32G	139
A32L	145
A32K	216
A32F	137
A32P	50
A32S	81
A32T	154
A32Y	277
A32V	146

(b) Site -saturation mutagenesis: generation of combinations of two substitutions in the pro region.

[0157] The plasmid expressing the E30G substitution in the pro region (SEQ ID NO:7) comprised in the full-length protease of SEQ ID NO:63 was subjected to a second round of site-saturation mutagenesis of

the codon at position 6 to create a first library of polynucleotides that encode a full-length protease containing a substitution of amino acid 6 in combination with the E30G substitution of the pro region of the protease. The mutation at position 6 was created using the QuikChange® site-directed mutagenesis kit (QC; Stratagene) according to the directions provided by the manufacturer using forward and reverse primers of SEQ ID NOS:26 and 27, respectively. Similarly, a second library of polynucleotides was created to encode a full-length protease containing a substitution of amino acid 32 in combination with the E30G substitution in the pro region of the protease was created. The complementary overlapping forward

GAGGCAAATGACGGCGTCNNSATTCTCTCTGAGGAAGAG (SEQ ID NO:34) and

reverse primers CTCTTCCTCAGAGAGAATSNNAGCGCCGTCATTTGCCTC (SEQ ID NO:35), were used for mutating the position 32.

[0158] The QC reaction, amplification of the plasmid DNA, and transformation of *E. coli* cells were performed as described in Example 1(a). The subsequent transformation of *Bacillus subtilis* competent cells was also performed as described in Example 1(b). Supernatants from *Bacillus* cultures expressing proteases from modified or unmodified precursor were analyzed for protease activity using the AAPF assay as described in Example 1(c).

[0159] Results shown in Tables 22 and 23 indicate that most substitutions of the amino acid at position 6 (Table 22) of the pro region of P17 when in combination with the substitution E30G further enhance the production of the mature form of the protease expressed from a polynucleotide encoding an unmodified pro region or a pro region containing the E30G substitution. Similarly, the results shown in Table 23 show that the combination of the E30G substitution with several substitutions at position 32 also further enhanced the production of the mature form of the protease expressed from a polynucleotide encoding an unmodified pro region or a pro region containing the E30G substitution.

TABLE 22

Effect of the combination of amino acid substitution E30G with substitutions of amino acid at position 6 of the pro region on the production of mature protease of SEQ ID NO:17	
Mutation (Substitution) at positions in pro region	Percent activity relative to activity from the modified precursor E30G
E30G (control)	100
E6A-E30G	106
E6R-E30G	136
E6C-E30G	157
E6Q-E30G	107
E6G-E30G	180
E6H-E30G	134
E6L-E30G	50
E6K-E30G	153
E6M-E30G	78
E6P-E30G	74
E6S-E30G	182
E6T-E30G	92
E6W-E30G	148

Effect of the combination of amino acid substitution E30G with substitutions of amino acid at position 6 of the pro region on the production of mature protease of SEQ ID NO:17	
Mutation (Substitution) at positions in pro region	Percent activity relative to activity from the modified precursor E30G
E6Y-E30G	33
E6V-E30G	69

TABLE 23

Effect of the combination of amino acid substitution E30G with substitutions of amino acid at position 32 of the pro region on the production of mature protease of SEQ ID NO:17	
Mutation (Substitution) at positions in pro region	Percent activity relative to activity from the modified precursor E30G-A32
E30G (control)	100
E30G-A32R	188
E30G-A32N	94
E30G-A32D	69
E30G-A32C	38
E30G-A32Q	133
E30G-A32E	109
E30G-A32G	105
E30G-A32H	115
E30G-A32I	150
E30G-A32L	38
E30G-A32K	189
E30G-A32P	69
E30G-A32S	122
E30G-A32T	116
E30G-A32W	161
E30G-A32Y	13
E30G-A32V	110

(c) Site -saturation mutagenesis: generation of combinations of three substitutions in the pro region.

[0160] The plasmid expressing the combination of to substitutions E6G-E30G in the pro region comprised in the full-length protease of SEQ ID NO:63 was subjected to another round of site-saturation mutagenesis of the codon at position 32 to create a library of polynucleotides that encode a full-length protease containing a substitution of amino acid 32 in combination with the E6G-E30G combination of substitutions in the pro region of the protease. The mutation at position 32 was created using the QuikChange® site-directed mutagenesis kit (QC; Stratagene) according to the directions provided by the

manufacturer using forward and reverse primers of SEQ ID NOS:34 and 35, respectively.

[0161] The QC reaction, amplification of the plasmid DNA, and transformation of *E. coli* cells were performed as described in Example 1 (a). The subsequent transformation of *Bacillus subtilis* competent cells was also performed as described in Example 1 (b). Supernatants from *Bacillus* cultures expressing proteases from modified or unmodified precursor were analyzed for protease activity using the AAPF assay as described in Example 1(c).

[0162] Results shown in Table 24 show that a triple substitution of amino acids at positions 6, 30 and 32 further enhance the production of the mature protease when processed from a pro region containing the two substitutions at positions 6 and 30. In particular, the A32E, A32S, A32T, and A32W when in combination with the E6G-E30G double substitution increase the production of the mature protease (SEQ ID NO:17) by about 73%, 3%, 33%, and 23%, respectively, relative to the level produced by the doubly mutated precursor comprising the E6G-E30G combination of substitutions. Considering that the E6G-E30G combination produces about 80% more mature than the single E30G substitution alone, the triple mutations E6G-E30G-A32E, E6G-E30G-A32S, E6G-E30G-A32T, and E6G-E30G-A32W can be calculated to produce 311%, 185%, 239% and 221%, respectively, of the level processed from the pro region containing the single E30G substitution.

TABLE 24

Effect of the combination of amino acid substitution E6G-E30G with substitution of amino acid at position 32 of the pro region on the production of mature protease of SEQ ID NO:17	
Mutation (substitution) at positions in pro region	Percent activity E6G-E30G-A32X relative to activity from the modified precursor E6G-E30G
E6G-E30G (control)	100
E6G-E30G-A32R	47
E6G-E30G-A32N	34
E6G-E30G-A32D	72
E6G-E30G-A32C	43
E6G-E30G-A32Q	87
E6G-E30G-A32E	173
E6G-E30G-A32G	58
E6G-E30G-A32H	88
E6G-E30G-A32I	21
E6G-E30G-A32L	34
E6G-E30G-A32K	69
E6G-E30G-A32M	88
E6G-E30G-A32F	90
E6G-E30G-A32P	100
E6G-E30G-A32S	102
E6G-E30G-A32T	133
E6G-E30G-A32W	123
E6G-E30G-A32Y	42

Effect of the combination of amino acid substitution E6G-E30G with substitution of amino acid at position 32 of the pro region on the production of mature protease of SEQ ID NO:17	
Mutation (substitution) at positions in pro region	Percent activity E6G-E30G-A32X relative to activity from the modified precursor E6G-E30G
E6G-E30G-A32V	50

(d) Effect of amino acid substitution(s) in the pro region of the precursor protease on the production of the mature protease of SEQ ID NO:17 in shake flask cultures.

[0163] To test the effect of amino acid substitutions in the pro region on the production of the mature protease of SEQ ID NO:17 in shake flask cultures, several of the *Bacillus subtilis* strains grown in the microtiter plates as described above and containing a substitution at position 30 in combination with a second substitution at position 6 or 32, and strains containing the combination of three substitutions in the pro region were grown for 48 hours as described in Example 1(e). Supernatant from the shake flask cultures was assayed for AAPF activity as described in Example 1 (c).

[0164] The results for the activity in strains comprising the combination of two E6-E30G or E30G-A32, and three amino acid substitutions E6G-E30G-A32X are shown in Tables 25, 26 and 27, respectively. The results show that the enhancement of protease production obtained from modified precursor proteases in microtiter cultures is mimicked in shake flask cultures.

TABLE 25

Effect of the combination of amino acid substitution E30G with substitutions of amino acid at position 6 of the pro region of the mature protease of SEQ ID NO:17 in shake flask cultures	
Mutation (substitution) at positions in pro region	Percent activity relative to activity of the modified precursor E30G
E6-E30G (control)	100
E6A-E30G	115
E6R-E30G	131
E6C-E30G	91
E6G-E30G	142
E6H-E30G	150
E6K-E30G	81
E6S-E30G	111
E6W-E30G	145

TABLE 26

Effect of the combination of amino acid substitution E30G with substitutions of amino acid at position 32 of the pro region of the mature protease of SEQ ID NO:17 in shake flask cultures	
Mutation (substitution) at positions in pro region	Percent activity relative to activity of the modified precursor E30G
E30G-A32 (control)	100

Effect of the combination of amino acid substitution E30G with substitutions of amino acid at position 32 of the pro region of the mature protease of SEQ ID NO:17 in shake flask cultures

Mutation (substitution) at positions in pro region	Percent activity relative to activity of the modified precursor E30G
E30G-A32	101
E30G-A32	121
E30G-A32	157
E30G-A32	165
E30G-A32	158
E30G-A32	108

TABLE 27

Effect of the combination of amino acid substitution E6G-E30G with substitutions at position 32 of the pro region of the mature protease of SEQ ID NO:17 in shake flask cultures

Mutation (substitution) at positions in pro region	Percent protease activity relative to activity from the modified precursor E6G-E30G
E6G-E30G-A32 (control precursor)	100
E6G-E30G-A32E	142
E6G-E30G-A32P	76
E6G-E30G-A32S	98
E6G-E30G-A32T	106
E6G-E30G-A32W	135

EXAMPLE 5

The effect of mutations in the pro region of SEQ ID NO:7 on the production of the mature alkaline protease of SEQ ID NO:21.

(a) Site -saturation mutagenesis of amino acids at positions 6, 30 or 32 of the pro region.

[0165] Site-saturation mutagenesis of the pro region on the production of the mature protease of SEQ ID NO:21 was performed using the QuikChange® site-directed mutagenesis kit (QC; Stratagene) according to the directions of the manufacturer. A DNA cassette comprising the AprE promoter, and the polynucleotide that encodes the full-length protease of SEQ ID NO:21 was cloned into the EcoRI and HindIII restriction sites of the pBN3 vector (Babe et al., Appl. Biochem. 27: 117-124 [1998]). pBN3 (Figure 4B) to generate the pBN3-P21 plasmid. The P21 DNA cassette comprised the *B. subtilis* aprE promoter

gaattcctccattttctctgctatcaaaataacagactcgtgattttcaaacgagctttcaaaaaagcctctgccccctgcaaatcggaatgcctgtctat
 aaaattcccgatattggttaaacacgcgcgcaatggcggcgcatctgatgtcttcttgccgaatgttcatttttctctccctcctaataatttttc
 attctatcccttttctgtaaagttaattttcagaatactttatcatcatgctttgaaaaaatatcacgataatatccattgttctcacggaagcacacgcagg
 tcaattcaacgaatttttcaacaggaatttccggagactcagagcaattacctaagaaagcatgacattcaacataatgaacattactcatctc

ttttgtcttttctgtatgaaaaagttatttcgagtcctacggaatagcgagatgatatcctaataagagataaaatcatctcaaaaaatgggt
 ctactaaaatatttccatctattacaataaattcacagaatagcttttaagtaagtctactctgaatttttaaaaggagagggttaaaga (SEQ ID
 NO:1),

the polynucleotide sequence

gtgagaagcaaaaaattgtggatcagctgtgtttgctgtaacgttaatctttacgatggcggtcagcaacatgtctgcgaggct (SEQ ID
 NO:2), which encodes the AprE signal peptide VRSKKLWISLLFALTLLIFTMAFSNMSAQA (SEQ ID NO:3),

the polynucleotide sequence

gctgaagaagcaaaagaaaaatatttaattggcttaatgagcaggaagctgtcagtgagttttagaacaagtagaggcaaatgacgaggctgc
 cattctctctgaggaagaggaagtcgaaattgaattgcttcatgaattgaaacgattcctgtttatccgttgagtgtaagcccagaagatgtggacgcg
 cttgaactcgatccagcgatttcttatattaagaggatgcagaagtaacgacaatg (SEQ ID NO:6), which encodes the
 unmodified pro region

AEEAKEKYLIGFNEQEAVSEFVEQVEANDEVAILSEEEVEIELLHEFETIPVLSVELSPEDVDALELDPAIS

YIEEDA EVTTM (SEQ ID NO:7), and the polynucleotide sequence

GCGCAATCAGTGCCATGGGGAATTAGCCGTGTGCAAGCCCCAGCTGCCATAACCGTGGATTGACA
 GGTTCTGGTGTAAGTTGCTGTCTCGATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGG
 TGGCGCTAGCTTTGTACAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCAGCATGTGG
 CCGGGACGATTGCTGCTTTAGACAATTCGATTGGCGTTCTTGGCGTAGCGCCGAGAGCGGAACTAT
 ACGCTGTAAAGTATTAGGGGCGAGCGGTTTCAGGTTTCGGTCAGCTCGATTGCCAAGGATTGGAAT
 GGGCAGGGAACAATCGTATGCACGTTGCTAATTTGAGTTTAGGACTGCAGGCACCAAGTGCCACAC
 TTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGCGGTTCTTGTGTAGCGGCATCTGGAATTCAGG
 TGCAGGCTCAATCAGCTATCCGGCCCGTTATGCGAACGCAATGGCAGTCGGAGCTACTGACCAAAA
 CAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGGCTTGACATTGTGCGACCAGGTGTAAACGT
 GCAGAGCACATACCCAGGTTCAACGTATGCCAGCTTAAACGGTACATCGATGGCTACTCCTCATGTT
 GCAGGTGCAGCAGCCCTTGTTAAACAAAAGAACCCATCTTGGTCCAATGTACAAATCCGCAATCATC
 TAAAGAATACGGCAACGAGCTTAGGAAGCACGAACCTTGATGGAAGCGGACTTGTCATGCAGAAG
 CTGCAACTCGT (SEQ ID NO:20),

which encodes the mature region of protease 21 (P21).

AQSVPWGISRVQAPAAHNRLTGSGVKVAVLDTGISTHPDLNIRGGASFVGPSTQDGNHGHGTHVAG
 TIAALDNSIGVLGVAPRAELYAVKVLGASGSGSVSSIAQGLEWAGNNRMHVNLSLGLQAPSATLEQAVN
 SATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFQYAGLDIVAPGVNVQSTYPGS
 TYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR (SEQ ID
 NO:21).

[0166] Each of the 3 codons in the pro region of SEQ ID NO:7, exemplified by NNG/C, comprised in the full-length protease of SEQ ID NO:65, were mutated to be substituted by the 32 possible nucleotide triplets that encode the 20 naturally occurring amino acids to generate three libraries as follows. An aliquot of plasmid pJH-P21 DNA comprising the sequence encoding the full-length protease was mutated to generate a first library of clones encoding all possible substitutions of glutamic acid (E) at position 6 (E6X) of the pro region (SEQ ID NO:7); a second aliquot was mutated to generate a second library of clones encoding all possible substitutions of glutamic acid (E) at position 30 (E30X) of the pro region (SEQ ID NO:7); and a third aliquot was mutated to generate a third library of clones encoding all possible substitutions of arginine (A) at position 32 (A32X) of the pro region (SEQ ID NO:7). Complementary overlapping primers were designed for mutating the codons of interest with about 18 bases flanking the NNS codon. The polynucleotide sequences of the forward and reverse primers used to mutate the amino acids at positions 6, 30 and 32 are given in Table 1.

[0167] The QC reaction, amplification of the plasmid DNA, and transformation of *E. coli* cells were performed as described in Example 1 (a). The subsequent transformation of *Bacillus subtilis* competent cells was also performed as described in Example 1 (b). Supernatants from *Bacillus* cultures expressing proteases from modified or unmodified precursor were analyzed for protease activity using the AAPF

assay as described in Example 1(c).

[0168] The results given in Tables 28, 29, and 30 showed that all but one of the amino acid substitutions of amino acids at positions 6 of the pro region of the precursor protease lead to an enhanced production of the mature form of the protease of SEQ ID NO:21, whereas all but one of the amino acid substitutions at positions 30 or 32 showed similar or diminished protease production when compared to the production of the mature protease when processed from an unmodified pro region. In addition, site saturation of each of the substituted amino acids showed that each amino acid can be substituted by two or more amino acids at the same position to increase the production of the mature form relative to that obtained from the precursor protease having unmodified pro region.

TABLE 28

Effect of amino acid substitution at position 6 of the pro region on the production of the mature protease of SEQ ID NO:21	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E6 (control)	100
E6A	84
E6R	85
E6D	57
E6C	90
E6Q	93
E6G	96
E6H	86
E6I	76
E6L	85
E6K	84
E6M	73
E6P	63
E6S	93
E6T	94
E6W	40
E6Y	74
E6V	94

TABLE 29

Effect of amino acid substitution at position 30 of the pro region on the production of the mature protease of SEQ ID NO:21	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E30 (control)	100
E30A	110
E30R	102
E30N	129
E30D	115

Effect of amino acid substitution at position 30 of the pro region on the production of the mature protease of SEQ ID NO:21	
Mutation (Substitution) at position in pro region	Percent activity relative to activity from the unmodified precursor
E30C	108
E30Q	87
E30G	130
E30H	123
E30I	47
E30L	83
E30M	129
E30F	116
E30P	50
E30S	134
E30T	94
E30W	125
E30V	74

TABLE 30

Effect of amino acid substitution at position 32 of the pro region on the production of the mature protease of SEQ ID NO:21	
Mutation (Substitution) at positions in pro region	Percent activity relative to activity from the unmodified precursor
A32 (unmodified pro; control)	100
A32R	95
A32N	70
A32D	63
A32C	85
A32Q	67
A32G	70
A32H	76
A32L	116
A32M	97
A32F	125
A32P	64
A32S	68
A32T	61
A32V	116

Site -saturation mutagenesis: generation of combinations of substitutions in the pro region of SEQ ID NO:21.

[0169] The plasmid expressing the E30S substitution in the pro region (SEQ ID NO:7) second round of site-saturation mutagenesis of the codon at position 6 to create a first library of polynucleotides that encode a full-length protease containing a substitution of amino acid 6 in combination with the E30S substitution of the pro region of the protease. The mutation at position 6 was created using the QuikChange® site-directed mutagenesis kit (QC; Stratagene) according to the directions provided by the manufacturer using forward and reverse primers of SEQ ID NOS:26 and 27, respectively (Table 1). Similarly, a second library of polynucleotides was created to encode a full-length protease containing a substitution of amino acid 32 in combination with the E30S substitution in the pro region of the protease was created. The complementary overlapping forward primers used to create the library of mutated polynucleotides comprising the A32X mutation in combination with the E30S substitution, were the complementary forward GAGGCAAATGACTCGGTCNNSATTCTCTCTGAGGAAGAG:SEQ ID NO:36, and reverse primer CTCTTCCTCAGAGAGAATSNNGACCGAGTCATTTGCCTC SEQ ID NO:37.

[0170] The QC reaction, amplification of the plasmid DNA, and transformation of *E. coli* cells were performed as described in Example 1 (a). The subsequent transformation of *Bacillus subtilis* competent cells was also performed as described in Example 1 (b). Supernatants from *Bacillus* cultures expressing proteases from modified or unmodified precursor were analyzed for protease activity using the AAPF assay as described in Example 1(c).

[0171] Results shown in Tables 31 and 32 indicate that most substitutions of the amino acid at position 6 (Table 31) of the pro region when in combination with the substitution E30S further enhance the production of the mature form of the protease expressed from a polynucleotide encoding an unmodified pro region or a pro region containing the single E30S substitution. Similarly, most substitutions of amino acid at position 32 of the pro region when in combination with the substitution E30S at site 30, also lead to a further enhancement of production of the mature form of the protease.

TABLE 31

Eff ect of the combination of amino acid substitution E30S with substitutions of amino acid at position 6 of the pro region on the production of mature protease of SEQ ID NO:21	
Mutation (Substitution) at positions in pro region	Percent activity relative to activity from the modified precursor E6-E30S
E6-E30S (control, modified)	100
E6A-E30S	116
E6N-E30S	64
E6Q-E30S	82
E6G-E30S	119
E6H-E30S	86
E6I-E30S	88
E6L-E30S	162
E6K-E30S	107
E6F-E30S	156
E6P-E30S	150
E6S-E30S	99
E6T-E30S	74

Effect of the combination of amino acid substitution E30S with substitutions of amino acid at position 6 of the pro region on the production of mature protease of SEQ ID NO:21	
Mutation (Substitution) at positions in pro region	Percent activity relative to activity from the modified precursor E6-E30S
E6W-E30S	88
E6Y-E30S	162
E6V-E30S	101

TABLE 32

Effect of the combination of amino acid substitution E30S with substitutions of amino acid at position 32 of the pro region on the production of mature protease of SEQ ID NO:21	
Mutation (substitution) at positions in pro region	Percent activity relative to activity from the modified precursor E30S-A32
E30S, A32 (control, modified)	100
E30S-A32R	113
E30S-A32N	177
E30S-A32D	229
E30S-A32C	112
E30S-A32Q	195
E30S-A32E	148
E30S-A32G	194
E30S-A32H	204
E30S-A32L	223
E30S-A32K	180
E30S-A32M	181
E30S-A32F	171
E30S-A32P	250
E30S-A32S	205
E30S-A32T	166
E30S-A32W	202
E30S-A32Y	116
E30S-A32V	141

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PROTEASER MED MODIFICEREDE PRO-OMRÅDER

PATENTKRAV

1. Anvendelse af et modificeret polynukleotid, der koder for en modificeret fuldlængde-protease, hvilket modificeret polynukleotid omfatter:
 - 5 et første polynukleotid, der koder for et signalpeptid, hvor det første polynukleotid er operativt bundet til et andet polynukleotid, der koder for pro-området ifølge SEQ ID NO: 7, hvor pro-området omfatter en kombination af substitutioner af mindst to aminosyrer ved positioner, der er udvalgt fra positionerne 6, 30 og 32 af pro-området, hvor kombinationen af substitutioner er udvalgt fra E6R-A32K, E6N-A32K, E6D-A32K, E6I-A32K, E6K-A32K, E6M-A32K, E6P-A32K, E6T-A32K, E6N-A32K, E6A-
 - 10 E30G, E6R-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6S-E30G, E6W-E30G, E30G-A32R, E30G-A32Q, E30G-A32E, E30G-A32G, E30G-A32H, E30G-A32I, E30G-A32S, E30G-A32T, E30G-A32W, E30G-A32V, E6G-E30G-A32E, E6G-E30G-A32S, E6G-E30G-A32T, E6G-E30G-A32W, E6A-E30G, E6R-E30G, E6N-E30G, E6D-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6M-E30G, E6F-E30G, E6P-E30G, E6S-E30G, E6T-E30G, E6W-E30G, E6V-E30G, E6Y-
 - 15 E30G, E6A-E30S, E6G-E30S, E6L-E30S, E6K-E30S, E6F-E30S, E6P-E30S, E6Y-E30S, E6V-E30S, E30S-A32R, E30S-A32N, E30S-A32D, E30S-A32C, E30S-A32Q, E30S-A32E, E30S-A32G, E30S-A32H, E30S-A32L, E30S-A32M, E30S-A32F, E30S-A32P, E30S-A32S, E30S-A32T, E30S-A32W, E30S-A32Y og E30S-A32V, og hvor positionerne er nummereret i overensstemmelse med aminosyresekvensen for pro-polypeptidet ifølge SEQ ID NO: 7;
 - 20 hvor det andet polynukleotid er operativt bundet til et tredje polynukleotid, der koder for det modne område af en protease, der er mindst ca. 60 % identisk med den modne protease ifølge SEQ ID NO: 9, for at tilvejebringe en forbedret produktion af en moden protease, der er oparbejdet fra den modificerede, fuldlængde-protease, sammenlignet med produktionsniveauet af den samme modne protease, når den er oparbejdet fra fuldlængde-proteasen uden substitutionerne i pro-området ifølge SEQ ID NO: 7.
 - 25 2. Anvendelse ifølge krav 1, hvor den modne protease er en alkalisk vildtype serinprotease eller serinproteasevariant, der er afledt af *Bacillus clausii* eller *Bacillus lentus*.
 3. Anvendelse ifølge krav 1 eller krav 2, hvor den modne protease har en aminosyresekvens, der er udvalgt fra SEQ ID NO: 9, 11, 13, 15, 17, 19 og 21.
 4. Anvendelse ifølge et hvilket som helst af kravene 1-3, hvor signalpeptidet har en
 - 30 aminosyresekvens, der er udvalgt fra SEQ ID NO: 3 og 5.
 5. Anvendelse ifølge et hvilket som helst af kravene 1-4, hvor substitutionerne forbedrer produktionen af den modne protease ved hjælp af en *Bacillus sp.* værtscelle.
 6. Anvendelse ifølge krav 5, hvor *Bacillus sp.* værtscellen er en *Bacillus subtilis*-værtscelle.
 7. Fremgangsmåde til forbedring af produktion af en moden protease i en *Bacillus sp.* værtscelle,
 - 35 hvilken fremgangsmåde omfatter:
 - a) tilvejebringelse af en ekspressionsvektor, der omfatter det modificerede polynukleotid som defineret i et hvilket som helst af kravene 1 til 4;
 - b) transformering af en *Bacillus sp.* værtscelle med ekspressionsvektoren; og

c) dyrkning af værtscellen under egnede forhold, således at den modne protease produceres af værtscellen;

hvor produktion af den modne protease, der er oparbejdet fra den modificerede, fuldlængde-protease, er forbedret sammenlignet med produktionsniveauet af den samme modne protease, når den er oparbejdet fra fuldlængde-proteasen uden substitutionerne i pro-området ifølge SEQ ID NO: 7;

eventuelt hvor *Bacillus sp.* værtscellen er en *Bacillus subtilis*-værtscelle.

8. Fremgangsmåde ifølge krav 7, hvor ekspressionsvektoren endvidere omfatter en AprE-promoter.

9. Fremgangsmåde ifølge krav 7 eller 8, hvor den modne protease er en alkalisk vildtype-*Bacillus clausii*- eller en *Bacillus lentus*-serin protease, variant eller homolog deraf.

10. Fremgangsmåde ifølge et hvilket som helst af kravene 7 til 9, hvor det første polynukleotid koder for signalpeptidet ifølge SEQ ID NO: 3, hvor:

a) det andet polynukleotid koder for pro-området omfatter en kombination af substitutioner, der er udvalgt fra: E6R-A32K, E6N-A32K, E6D-A32K, E6I-A32K, E6K-A32K, E6M-A32K, E6P-A32K, E6T-A32K og E6N-A32K, og hvor det tredje polynukleotid koder for en moden protease, der er udvalgt fra SEQ ID NO: 9; eller

b) det andet polynukleotid, der koder for pro-området, omfatter en kombination af substitutioner, der er udvalgt fra E6A-E30G, E6R-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6S-E30G, E6W-E30G, E30G-A32R, E30G-A32Q, E30G-A32E, E30G-A32G, E30G-A32H, E30G-A32I, E30G-A32S, E30G-A32T, E30G-A32W, E30G-A32V, E6G-E30G-A32E, E6G-E30G-A32S, E6G-E30G-A32T og E6G-E30G-A32W, og hvor det tredje polynukleotid koder for en moden protease, der er udvalgt fra SEQ ID NO: 17; eller

c) det andet polynukleotid, der koder for pro-området, omfatter en kombination af substitutioner, der er udvalgt fra E6A-E30G, E6R-E30G, E6N-E30G, E6D-E30G, E6C-E30G, E6Q-E30G, E6G-E30G, E6H-E30G, E6K-E30G, E6M-E30G, E6F-E30G, E6P-E30G, E6S-E30G, E6T-E30G, E6W-E30G, E6V-E30G og E6Y-E30G, og hvor det tredje polynukleotid koder for en moden protease, der er udvalgt fra SEQ ID NO: 19; eller

d) det andet polynukleotid, der koder for pro-området, omfatter en kombination af substitutioner, der er udvalgt fra E6A-E30S, E6G-E30S, E6L-E30S, E6K-E30S, E6F-E30S, E6P-E30S, E6Y-E30S, E6V-E30S, E30S-A32R, E30S-A32N, E30S-A32D, E30S-A32C, E30S-A32Q, E30S-A32E, E30S-A32G, E30S-A32H, E30S-A32L, E30S-A32M, E30S-A32F, E30S-A32P, E30S-A32S, E30S-A32T, E30S-A32W, E30S-A32Y og E30S-A32V, og hvor det tredje polynukleotid koder for en moden protease, der er udvalgt fra SEQ ID NO: 21.

11. Anvendelse af et polynukleotid, der koder for en modificeret fuldlængde-protease, hvor det isolerede, modificerede polynukleotid omfatter et første polynukleotid, der koder for signalpeptidet ifølge SEQ ID NO: 3, hvor det første polynukleotid er operativt bundet til et andet polynukleotid, der koder for pro-området ifølge SEQ ID NO: 7, hvor:

a) pro-området omfatter en substitution af en aminosyre, der er udvalgt fra E6A, E6C, E6Y, E30A, E30L, E30P, E30T, E30Y, E30V, A32C, A32E, A32G, A32K og A32T, hvor det andet polynukleotid er

- 3 -

operativt bundet til et tredje polynukleotid, der koder for det modne område af proteasen ifølge SEQ ID NO: 17; eller

- b) pro-området omfatter en substitution af en aminosyre, der er udvalgt fra E6A, E6C, E6F, E6P, E6T, E6W, E6V, A32K og A32T, hvor det andet polynukleotid er operativt bundet til et tredje polynukleotid, der koder for det modne område af proteasen ifølge SEQ ID NO: 9; eller

c) pro-området omfatter en substitution af en aminosyre, der er udvalgt fra E6A, E30A, E30L, E30T og E30V, hvor det andet polynukleotid er operativt bundet til et tredje polynukleotid, der koder for det modne område af proteasen ifølge SEQ ID NO: 19; eller

- d) pro-området omfatter en substitution af en aminosyre, der er udvalgt fra E6A, E6L, E6F, E6T, E6V, E30I, E30L, E30P, E30T, E30Y, E30V, A32Q, A32S og A32T, hvor det andet polynukleotid er operativt bundet til et tredje polynukleotid, der koder for det modne område af proteasen ifølge SEQ ID NO: 11; eller

- e) pro-området omfatter the aminosyre substitution E30A, hvor det andet polynukleotid er operativt bundet til et tredje polynukleotid, der koder for det modne område af proteasen ifølge SEQ ID NO: 21,

for at tilvejebringe forbedret produktion af en moden protease, der er oparbejdet fra den modificerede, fuldlængde-protease, sammenlignet med produktionsniveauet af den samme modne protease, når den er oparbejdet fra fuldlængde-proteasen uden substitutionerne i pro-området ifølge SEQ ID NO: 7.

12. Fremgangsmåde til forbedring af produktion af en moden protease i en *Bacillus subtilis*-værtscelle, hvilken fremgangsmåde omfatter

- a) tilvejebringelse af en ekspressionsvektor, der omfatter polynukleotidet som defineret i krav 11;
b) transformering af *Bacillus subtilis*-værtscellen med ekspressionsvektoren;

og

- c) dyrkning af værtscellen under egnede forhold, således at den modne protease produceres af værtscellen;

hvor produktion af den modne protease, der er oparbejdet fra den modificerede, fuldlængde-protease er forbedret, sammenlignet med produktionsniveauet af den samme modne protease, når den er oparbejdet fra fuldlængde-proteasen uden substitutionerne i pro-området ifølge SEQ ID NO: 7.

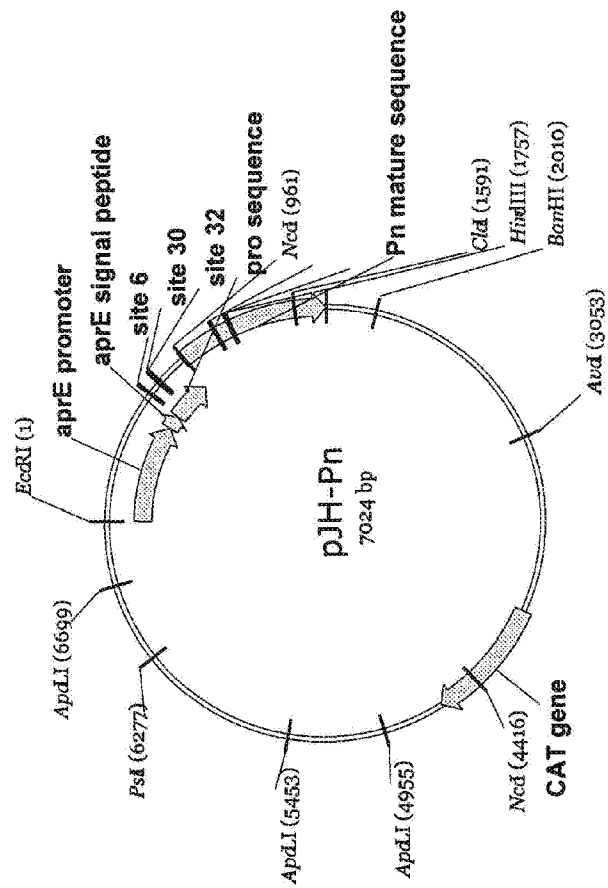


FIGURE 4A

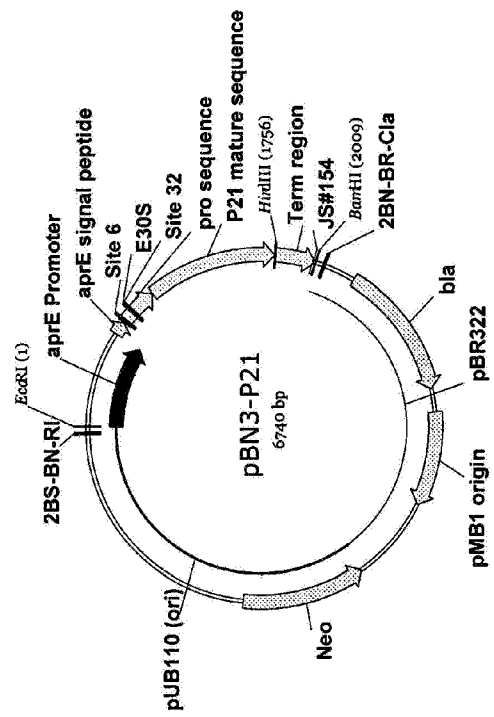


FIGURE 4B

[illegible]

FIGURE 5A

550 600
 (501) CGCAATGGCGAGTCGGAGCTACTGACCCAAACAAACAAACCGCGCCAGCTTTTCAAGTATGGCGCGAGGCTTGACATTGTCCACACAGGTGTAAACGTGCGAG
 (501) CGCAATGGCGAGTCGGAGCTACTGACCCAAACAAACAAACCGCGCCAGCTTTTCAAGTATGGCGCGAGGCTTGACATTGTCCACACAGGTGTAAACGTGCGAG
 (501) CGCAATGGCGAGTCGGAGCTACTGACCCAAACAAACAAACCGCGCCAGCTTTTCAAGTATGGCGCGAGGCTTGACATTGTCCACACAGGTGTAAACGTGCGAG
 (501) CGCAATGGCGAGTCGGAGCTACTGACCCAAACAAACAAACCGCGCCAGCTTTTCAAGTATGGCGCGAGGCTTGACATTGTCCACACAGGTGTAAACGTGCGAG
 (501) CGCAATGGCGAGTCGGAGCTACTGACCCAAACAAACAAACCGCGCCAGCTTTTCAAGTATGGCGCGAGGCTTGACATTGTCCACACAGGTGTAAACGTGCGAG
 (501) CGCAATGGCGAGTCGGAGCTACTGACCCAAACAAACAAACCGCGCCAGCTTTTCAAGTATGGCGCGAGGCTTGACATTGTCCACACAGGTGTAAACGTGCGAG
 (501) CGCAATGGCGAGTCGGAGCTACTGACCCAAACAAACAAACCGCGCCAGCTTTTCAAGTATGGCGCGAGGCTTGACATTGTCCACACAGGTGTAAACGTGCGAG
 (501) CGCAATGGCGAGTCGGAGCTACTGACCCAAACAAACAAACCGCGCCAGCTTTTCAAGTATGGCGCGAGGCTTGACATTGTCCACACAGGTGTAAACGTGCGAG
 (501) CGCAATGGCGAGTCGGAGCTACTGACCCAAACAAACAAACCGCGCCAGCTTTTCAAGTATGGCGCGAGGCTTGACATTGTCCACACAGGTGTAAACGTGCGAG
 601 650 700
 (601) AGCAGTATCCAGGTTCAACGATATGCCAGCTTAAACGGGTACATCCATGGCTACTCTCTCATGTTGCAGGTGCAGCGGCCCTTGTTTAAACAAAGAACCCCAT
 (601) AGCAGTATCCAGGTTCAACGATATGCCAGCTTAAACGGGTACATCCATGGCTACTCTCTCATGTTGCAGGTGCAGCGGCCCTTGTTTAAACAAAGAACCCCAT
 (601) AGCAGTATCCAGGTTCAACGATATGCCAGCTTAAACGGGTACATCCATGGCTACTCTCTCATGTTGCAGGTGCAGCGGCCCTTGTTTAAACAAAGAACCCCAT
 (601) AGCAGTATCCAGGTTCAACGATATGCCAGCTTAAACGGGTACATCCATGGCTACTCTCTCATGTTGCAGGTGCAGCGGCCCTTGTTTAAACAAAGAACCCCAT
 (601) AGCAGTATCCAGGTTCAACGATATGCCAGCTTAAACGGGTACATCCATGGCTACTCTCTCATGTTGCAGGTGCAGCGGCCCTTGTTTAAACAAAGAACCCCAT
 (601) AGCAGTATCCAGGTTCAACGATATGCCAGCTTAAACGGGTACATCCATGGCTACTCTCTCATGTTGCAGGTGCAGCGGCCCTTGTTTAAACAAAGAACCCCAT
 (601) AGCAGTATCCAGGTTCAACGATATGCCAGCTTAAACGGGTACATCCATGGCTACTCTCTCATGTTGCAGGTGCAGCGGCCCTTGTTTAAACAAAGAACCCCAT
 (601) AGCAGTATCCAGGTTCAACGATATGCCAGCTTAAACGGGTACATCCATGGCTACTCTCTCATGTTGCAGGTGCAGCGGCCCTTGTTTAAACAAAGAACCCCAT
 701 750 800
 (701) CTTCGTCCAAATGTACAAATCCGCAATCATCTAAGAAATACGGCAACGAGCTTTAGGAAGCAGGAACTTGTATGGAGCGGACTTGTCAATGCAAGAGCTGC
 (701) CTTCGTCCAAATGTACAAATCCGCAATCATCTAAGAAATACGGCAACGAGCTTTAGGAAGCAGGAACTTGTATGGAGCGGACTTGTCAATGCAAGAGCTGC
 (701) CTTCGTCCAAATGTACAAATCCGCAATCATCTAAGAAATACGGCAACGAGCTTTAGGAAGCAGGAACTTGTATGGAGCGGACTTGTCAATGCAAGAGCTGC
 (701) CTTCGTCCAAATGTACAAATCCGCAATCATCTAAGAAATACGGCAACGAGCTTTAGGAAGCAGGAACTTGTATGGAGCGGACTTGTCAATGCAAGAGCTGC
 (701) CTTCGTCCAAATGTACAAATCCGCAATCATCTAAGAAATACGGCAACGAGCTTTAGGAAGCAGGAACTTGTATGGAGCGGACTTGTCAATGCAAGAGCTGC
 (701) CTTCGTCCAAATGTACAAATCCGCAATCATCTAAGAAATACGGCAACGAGCTTTAGGAAGCAGGAACTTGTATGGAGCGGACTTGTCAATGCAAGAGCTGC
 (701) CTTCGTCCAAATGTACAAATCCGCAATCATCTAAGAAATACGGCAACGAGCTTTAGGAAGCAGGAACTTGTATGGAGCGGACTTGTCAATGCAAGAGCTGC
 (701) CTTCGTCCAAATGTACAAATCCGCAATCATCTAAGAAATACGGCAACGAGCTTTAGGAAGCAGGAACTTGTATGGAGCGGACTTGTCAATGCAAGAGCTGC
 801
 (801) AACTCGT (SEQ ID NO: 8)
 (801) AACTCGT (SEQ ID NO: 10)
 (801) AACACGC (SEQ ID NO: 12)
 (801) AACACGT (SEQ ID NO: 14)
 (801) AACACGT (SEQ ID NO: 16)
 (801) AACACGT (SEQ ID NO: 18)
 (801) AACTCGT (SEQ ID NO: 20)
 (801) AACTCGT (SEQ ID NO: 22)
 (801) AACTCGT (SEQ ID NO: 24)

FIGURE 5B