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- (54) Benævnelse: **Anvendelse og fremstilling af en opbevaringsstabil neutral metalloprotease**
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FIELD OF THE INVENTION

The present invention provides a neutral metalloprotease enzyme that has improved activity or stability. In some embodiments, the neutral metalloprotease finds use in applications including, but not limited to cleaning, bleaching and disinfecting.

5 BACKGROUND OF THE INVENTION

Detergent and other cleaning compositions typically include a complex combination of active ingredients. For example, most cleaning products include a surfactant system, enzymes for cleaning, bleaching agents, builders, suds suppressors, soil-suspending agents, soil-release agents, optical brighteners, softening agents, dispersants, dye transfer inhibition compounds, abrasives, bactericides, and perfumes. Despite the complexity of current detergents, there are many stains that are difficult to completely remove. Furthermore, there is often residue build-up, which results in discoloration (*e.g.*, yellowing) and diminished aesthetics due to incomplete cleaning. These problems are compounded by the increased use of low (*e.g.*, cold water) wash temperatures and shorter washing cycles. Moreover, many stains are composed of complex mixtures of fibrous material, mainly incorporating carbohydrates and carbohydrate derivatives, fiber, and cell wall components (*e.g.*, plant material, wood, mud/clay based soil, and fruit). These stains present difficult challenges to the formulation and use of cleaning compositions.

In addition, colored garments tend to wear and show appearance losses. A portion of this color loss is due to abrasion in the laundering process, particularly in automated washing and drying machines. Moreover, tensile strength loss of fabric appears to be an unavoidable result of mechanical and chemical action due to use, wearing, and/or washing and drying. Thus, a means to efficiently and effectively wash colored garments so that these appearance losses are minimized is needed.

In sum, despite improvements in the capabilities of cleaning compositions, there remains a need in the art for detergents that remove stains, maintain fabric color and appearance, and prevent dye transfer. In addition, there remains a need for detergent and/or fabric care compositions that provide and/or restore tensile strength, as well as provide anti-wrinkle, anti-bobbling, and/or anti-shrinkage properties to fabrics, as well as provide static control, fabric softness, maintain the desired color appearance, and fabric anti-wear properties and benefits. In particular, there remains a need for the inclusion of compositions that are capable of removing the colored components of stains, which often remain attached to the fabric being laundered. In addition, there remains a need for improved methods and compositions suitable for textile bleaching.

30 SUMMARY OF THE INVENTION

The invention provides an isolated neutral metalloprotease variant having at least 70% amino acid identity with the neutral metalloprotease comprising SEQ ID NO:3 or SEQ ID NO:18, and having an amino acid sequence comprising at least one mutation equivalent to a mutation in a neutral metalloprotease comprising the amino acid sequence set forth in SEQ ID NO:18, wherein said mutations are selected from

35 T004C, T004E, T004H, T004I, T004K, T004L, T004M, T004N, T004P, T004R, T004S, T004V, T004W, T004Y, G012D, G012E, G012I, G012K, G012L, G012M, G012Q, G012T, G012V, G012W, K013C, K013D, K013E, K013F, K013G, K013H, K013I, K013L, K013M, K013N, K013Q, K013S, K013T, K013V, K013Y, T014F, T014G, T014H, T014I, T014K, T014L, T014M, T014P, T014Q, T014R, T014S, T014V, T014W, T014Y, S023A, S023D, S023F, S023I, S023K, S023L, S023M, S023N, S023P, S023Q, S023R, S023T, S023V, S023W, S023Y, G024A, G024D, G024F, G024G, G024H, G024I, G024K, G024L, G024M,

40

G024N, G024P, G024R, G024S, G024T, G024V, G024W, G024Y, K033H, Q045C, Q045D, Q045E, Q045F, Q045H, Q045I, Q045K, Q045L, Q045M, Q045N, Q045P, Q045R, Q045T, Q045W, N046A, N046C, N046E, N046F, N046G, N046H, N046I, N046K, N046L, N046M, N046P, N046Q, N046R, N046S, N046T, N046V, N046W, N046Y, R047E, R047K, R047L, R047M, R47Q, R047S, R047T, Y049A, Y049C, Y049D, Y049E, Y049F, Y049H, Y049I, Y049K, Y049L, Y049N, Y049R, Y049T, Y049V, Y049W, N050D, N050F, N050G, N050H, N050I, N050K, N050L, N050M, N050P, N050Q, N050W, N050Y, T054C, T054D, T054E, T054F, T054G, T054H, T054I, T054K, T054L, T054M, T054N, T054P, T054Q, T054R, T054S, T054V, T054W, T054Y, S058D, S058H, S058I, S058L, S058N, S058P, S058Q, T059A, T059C, T059E, T059G, T059H, T059I, T059K, T059L, T059M, T059N, T059P, T059Q, T059R, T059S, T059V, T059W, T060D, T060F, T060I, T060K, T060L, T060N, T060Q, T060R, T060V, T060W, T060Y, T065C, T065E, T065F, T065H, T065I, T065K, T065L, T065M, T065P, T065Q, T065R, T065V, T065Y, S066C, S066D, S066E, S066F, S066H, S066I, S066K, S066L, S066N, S066P, S066Q, S066R, S066T, S066V, S066W, S066Y, Q087A, Q087D, Q087E, Q087H, Q087I, Q087K, Q087L, Q087M, Q087N, Q087R, Q087T, Q087V, Q087W, N090C, N090D, N090E, N090F, N090G, N090H, N090L, N090R, N090T, N096G, N096H, N096K, N096R, K097H, K097Q, K097W, K100A, K100D, K100E, K100F, K100H, K100N, K100P, K100Q, K100R, K100S, K100V, K100Y, R110A, R110C, R110E, R110H, R110K, R110L, R110M, R110N, R110S, R110Y, D119E, D119H, D119I, D119L, D119Q, D119R, D119S, D119T, D119V, D119W, G128C, G128F, G128H, G128K, G128L, G128M, G128N, G128Q, G128R, G128W, G128Y, S129A, S129C, S129D, S129F, S129G, S129H, S129I, S129K, S129L, S129M, S129Q, S129R, S129T, S129V, S129W, S129Y, F130I, F130K, F130L, F130M, F130Q, F130R, F130T, F130V, F130Y, S135P, G136I, G136L, G136P, G136V, G136W, G136Y, S137A, M138I, M138K, M138Q, M138V, D139A, D139C, D139E, D139G, D139H, D139I, D139K, D139L, D139M, D139P, D139R, D139S, D139V, D139W, D139Y, V140C, Q151I, E152A, E152C, E152D, E152F, E152G, E152H, E152L, E152M, E152N, E152R, E152S, E152W, N155D, N155K, N155Q, N155R, D178A, D178C, D178G, D178H, D178K, D178L, D178M, D178N, D178P, D178Q, D178R, D178S, D178T, D178V, D178W, D178Y, T179A, T179F, T179H, T179I, T179K, T179L, T179M, T179N, T179P, T179Q, T179R, T179S, T179V, T179W, T179Y, E186A, E186C, E186D, E186G, E186H, E186K, E186L, E186M, E186N, E186P, E186Q, E186R, E186S, E186T, E186V, E186W, E186Y, V190H, V190I, V190K, V190L, V190Q, V190R, S191F, S191G, S191H, S191I, S191K, S191L, S191N, S191Q, S191R, S191W, L198M, L198V, S199C, S199D, S199E, S199F, S199I, S199K, S199L, S199N, S199Q, S199R, S199V, Y204H, Y204T, G205F, G205H, G205L, G205M, G205R, G205S, G205Y, K211C, K211D, K211G, K211M, K211N, K211Q, K211R, K211S, K211T, K211V, K214A, K214C, K214E, K214I, K214L, K214M, K214N, K214Q, K214S, K214V, L216A, L216C, L216F, L216H, L216Q, L216R, L216S, L216Y, N218K, N218P, T219D, D220A, D220E, D220H, D220K, D220N, D220P, A221D, A221F, A221I, A221K, A221L, A221M, A221N, A221S, A221V, A221Y, G222C, G222H, G222N, G222R, Y224F, Y224H, Y224N, Y224R, T243C, T243G, T243H, T243I, T243K, T243L, T243Q, T243R, T243W, T243Y, K244A, K244C, K244D, K244E, K244F, K244G, K244L, K244M, K244N, K244Q, K244S, K244T, K244V, K244W, K244Y, V260A, V260D, V260E, V260G, V260H, V260I, V260K, V260L, V260M, V260P, V260Q, V260R, V260S, V260T, V260W, V260Y, Y261C, Y261F, Y261I, Y261L, T263E, T263F, T263H, T263I, T263L, T263M, T263Q, T263V, T263W, T263Y, S265A, S265C, S265D, S265E, S265K, S265N, S265P, S265Q, S265R, S265T, S265V, S265W, K269E, K269F, K269G, K269H, K269I, K269L, K269M, K269N, K269P,

K269Q, K269S, K269T, K269V, K269W, K269Y, A273C, A273D, A273H, A273I, A273K, A273L, A273N, A273Q, A273R, A273Y, R280A, R280C, R280D, R280E, R280F, R280G, R280H, R280K, R280L, R280M, R280S, R280T, R280V, R280W, R280Y, L282F, L282G, L282H, L282I, L282K, L282M, L282N, L282Q, L282R, L282V, L282Y, S285A, S285C, S285D, S285E, S285K, S285P, S285Q, S285R, S285W, Q286A, 5 Q286D, Q286E, Q286K, Q286P, Q286R, A289C, A289D, A289E, A289K, A289L, A289R, A293C, A293R, N296C, N296D, N296E, N296K, N296R, N296V, A297C, A297K, A297N, A297Q, A297R, and G299N;

wherein said amino acid sequence identity with SEQ ID NO: 3 or SEQ ID NO: 18 is determined using BLAST with default parameters;

wherein said neutral metalloprotease variant has improved thermostability as compared to wild-type 10 *B. amyloliquefaciens* neutral metalloprotease having the sequence of SEQ ID NO: 18;

improved performance under lower or higher pH conditions, as compared to wild-type *B. amyloliquefaciens* neutral metalloprotease having the sequence of SEQ ID NO: 18; or improved autolytic stability, as compared to wild-type *B. amyloliquefaciens* neutral metalloprotease having the sequence of SEQ ID NO: 18.

15 The isolated variant neutral metalloprotease may comprise multiple mutations selected from S023W/G024M, T004V/S023W/G024W, S023W/G024Y/A288V, T004L/S023W/G024Y, N046Q/N050F/T054L, N050Y/T059R/S129Q, S023W/G024W, A273H/S285P/E292G, S023Y/G024Y, S023Y/G024W, T004S/S023Y/G024W, N046Q/T054K, S023W/G024Y, T004V/S023W, T059K/S066N, N046Q/N050W/T054H/T153A, T004V/S023W/G024Y, L282M/Q286P/A289R, 20 N046Q/R047K/N050Y/T054K, L044Q/T263W/S285R, T004L/S023W/G024W, R047K/N050F/T054K, A273H/S285R, N050Y/T059K/S066Q, T054K/Q192K, N046Q/N050W, L282M/Q286K, T059K/S066Q, T004S/S023W, L282M/Q286R/A289R/K011N, L282M/A289R, N046Q/N050W/T054H, T059K/S129Q, T004S/S023N/G024Y/F210L, T004V/S023W/G024M/A289V, L282M/Q286K/A289R/S132T, N050W/T054H, L282M/Q286R, L282F/Q286K/A289R, T059R/S066Q, R047K/N050W/T054H, 25 S265P/L282M/Q286K/A289R, L282M/Q286R/T229S, L282F/Q286K, T263W/S285R, S265P/L282M/Q286K, T263H/A273H/S285R, T059R/S129V, S032T/T263H/A273H/S285R, T059R/S066Q/S129Q, T004S/G024W, T004V/S023W/G024M, T059K/S066Q/S129Q, L282M/Q286K/A289R/I253V, T004V/S023Y/G024W, T059R/S066N/S129Q, N050F/T054L, T004S/S023N/G024W, T059R/S066N, T059R/S066N/S129V, Q286R/A289R, 30 N046Q/R047K/N050F/T054K, S265P/L282M/Q286P/A289R, S265P/L282M/Q286R/A289R Q062K/S066Q/S129I, S023N/G024W, N046Q/R047K/N050W/T054H, R047K/T054K, T004L/G024W, T014M/T059R/S129V, T059R/S066Q/N092S/S129I, R047K/N050W/T054K, T004V/G024W, N047K/N050F/T054K, S265P/L282F/Q286K/N061Y, L282F/Q286K/E159V, T004V/S023Y/G024M, S265P/L282F/A289R/T065S, T059K/F063L/S066N/S129V, T004L/S023W, N050F/T054H, 35 T059R/S066Q/S129V, V190I/D220E/S265W/L282F, T004S/S023Y/G024M, T004L/S023N/G024Y, T059K/S066N/S129I, T059R/S066N/S129I, L282M/Q286R/A289R/P162S, N046Q/N050F/T179N, T059K/Y082C/S129V, T059K/S129I, N050Y/T054K, T059K/S066Q/V102A/S129Q, T059R/S066Q/S129I, T059W/S066N/S129V/S290R, T059R/S129I, T059K/S066Q/S129I, T059K/S066Q/S129V, S265P/L282M/Q286R/A289R/T202S/K203N, T004V/S023N/G024W, S265P/Q286K, S265P/L282F/A289R, 40 D220P/S265W, L055F/T059W/S129V, T059R/S129Q/S191R, N050W/T054K, T004S/S023W/G024M,

R047K/N050F/T054H, T059K/S066N/K088E, T059K/S066Q/S129I/V291L, L282M/Q286R/A289R,
T059R/S066N/F085S/S129I, L282F/Q286P/A289R, L282F/Q286R/A289R,
G099D/S265P/L282F/Q286K/A289R, N046Q/N050F, N050Y/T059W/S066N/S129V, T009I/D220P/S265N,
V190F/D220P/S265W, N157Y/T263W/A273H/S285R, T263W/A273H/S285R, T263W/S285W,
5 T004V/S023Y, N046Q/R047K/N050W, N050W/T054L, N200Y/S265P/L282F/Q286P/A289R,
T059R/S066Q/P264Q, T004V/G024Y, T004L/G024Y, N050Y/S191I, N050Y/T054L,
T004L/S023W/G024Y/N155K, F169I/L282F/Q286R/A289R, L282M/Q286K/A289R,
F130L/M138L/E152W/D183N, N046Q/R047K/N050Y/T054H, T004V/G024M,
N050Y/T059W/S066Q/S129V, S023N/G024Y, T054H/P162Q, T004S/S023W/G024Y, N050Y/T054H,
10 L282F/Q286R/A289R/F169I, R047K/N050W, V190F/D220P, L282M/F173Y, T004L/S023Y,
N050W/A288D, V190I/D220P/S265Q, S265P/L282F/Q286P/A289R, S265P/L282F/Q286R/A289R,
N046Q/N050Y/T054K, T059W/S066Q, T263W/A273H/S285W T263W/A273H/S285P, S023Y/G024M,
T004L/S023N/G024W, T004V/S023N/G024Y, T059W/S066N/S129Q, T004S/S023Y,
T004S/S023N/G024M, T059W/S066N/A070T, T059W/S066Q/S129Q, T263W/A273H, A273H/S285P,
15 N046Q/R047K/N050Y/T054L, N046Q/R047K/N050Y, R047K/N050Y, T263H/S285W, R047K/N050F,
N046Q/R047K/N050F/T054H, S023N/G024M, T004S/G024Y, R047K/N050Y/T054H,
T059W/S066N/S129i, R047K/T054L, T004S/S023W/G024W, M138L/E152F/T146S, D220P/S265N,
T004S/G024M, T004V/S023N, N046Q/N050F/T054K, N046Q/N050Y/T054H, Q062H/S066Q/S129Q,
T059W/S129Q, T059W/S129V, N050F/T054K, R047K/N050F/T054L, V190I/D220P/S265W,
20 N112I/T263H/A273H/S285R, T059W/S066N/S129V, T059W/S066Q/S129I, T059W/S129I, T263W/S285P,
V190I/D220P, A289V/T263H/A273H, T263H/A273H/S285P, N90S/A273H/S285P, R047K/N050Y/T054L,
T004S/S023N, T059R/S129Q, N046Q/R047K/T054H, T059W/S066Q/S129V, E152W/T179P,
N050Y/S066Q/S129V, T202S/T263W/A273H, T263W/A273H/S285P, M138L/E152W/T179P,
N046Q/R047K, N046Q/T054H/F176L, T004L/G024M, T004S/L282M, T263H/A273H,
25 T263H/A273H/S285W, T004L/S023Y/G024M, L282F/Q286P, T004V/S023Y/G024Y, V190F/S265W,
M138L/E152F, V190F/D220E/S265W, N046Q/N050F/T054H, N157Y/S285W, T004F/S023Y/G024M,
T004V/S023N/G024M, L198I/D220E/S265Q, N046Q/N050Y/T054K/A154T, S016L/D220E/S265W,
D220E/S265W, D220E/A237S/S265W, S066Q/S129Q, V190F/D220E/S265Q/T267I, L282M/F173Y/T219S,
E152F/T179P, V190I/S265W, M138L/S066Q, M138L/E152W, T059W/S066Q/A070T/S129I, V
30 190F/D220E/S265N, V190F/S265N, N046Q/N050Y, and M138L/E152F/T179P.

The isolated variant neutral metalloprotease may comprise multiple mutations selected from
V190I/D220P, V190I/D220P/S265Q, V190L/D220E, V190I/D220E/S265Q, V190I/D220E/S265W/L282F,
V190L/D220E/S265Q, V190I/D220E/S265W, V190L/D220E/S265N, T059R/S066Q/S129I,
V190I/D220E/S265N, V190L/D220E/S265W, V190I/D220E, T059W/S066N/S129V, T059K/S066Q/S129V,
35 T059K/Y082C/S129V, T059R/S066N/S129I, S066Q/S129V, T059R/S066Q/S129V, T059R/S129I,
N050Y/T059W/S066N/S129V, D220P/S265N, S066Q/S129I, T059W/S066Q/S129V, T059K/S066Q/S129I,
T059R/S129V, N050Y/S066Q/S129V, T059W/S066Q/S129I, N050Y/T059W/S066Q/S129V, T059K/S129I,
D220P/S265W, F130L/M138L/T179P, S066N/S129I, T059R/S066N/S129V, F130I/M138L/T179P,
T059R/S066Q/N092S/S129I, S066N/S129V, D220E/S265Q, F130L/M138L/E152W/T179P, T059W/S129V,
40 S265P/L282M/Q286R/A289R, S265P/L282F/Q286R/A289R, T059W/S066N/S129I, V190I/D220P/S265W,

- F130L/E152W/T179P, F130L/M138L/E152F/T179P, Q062K/S066Q/S129I, T059K/S066N/S129I, E152H/T179P, S265P/L282M/Q286K/A289R, F130L/M138L/E152H/T179P, T263W/A273H/S285R, D220E/S265N, F130I/M138L/E152H/T179P, F130V/M138L/E152W/T179P, F130I/M138L/E152W/T179P, T059W/S129I, D220E/S265W, F130V/M138L/T179P, F130L/E152V/T179P, T059R/S129Q,
- 5 T263W/S285P, F130I/M138L/E152F/T179P, E152W/T179P, V190L/S265Q, F130L/E152F/T179P, L282M/Q286R/A289R/PI62S, D220P/S265Q, M138L/E152F/T179P, F130I/E152H/T179P, M138L/E152W/T179P, F130L/T179P, F130L/M138L/E152W/T179P/Q286H, F130L/M138L/E152H, T263W/A273H/S285W, S265P/Q286K, T059W/S066Q/S129Q, T263W/S285R, T059W/S066N/S129Q, T263W/S285W, T059R/S066N/S129Q, S265P/L282M/Q286R/A289R/T202S/K203N, T059W/S129Q,
- 10 Q062H/S066Q/S129Q, L282M/Q286R/A289R, V190L/D220E/S265NN291I, V 190L/S265N, F 130L/M138L/E152W, N050Y/T059R/S129Q, F130I/T179P, T059K/S066Q/S129Q, T059K/S129Q, S265P/L282M/Q286P/A289R, S265P/L282F/Q286P/A289R, T263W/A273H/S285P, S265P/L282NVQ286K, S016L/D220E/S265W, S066Q/S129Q, S265P/L282M/Q286P, L282F/Q286R/A289R, F130V/E152W/T179P, L044Q/T263W/S285R L055F/T059W/S129V,
- 15 V190L/S265W, Q286R/A289R, G99D/S265P/L282F/Q286K/A289R, F130L/M138L/E152F, T059R/S066Q/S129Q, F130L/E152H, S066N/S129Q, T004S/S023N/G024M/K269N, S265P/L282M, E152F/T179P, T059W/S066N/S129V/S290R, L282F/Q286K/A289R, F130L/M138L, F130I/M138L/E152W, S265P/L282F, F130I/M138L/E152H, F130V/M138L/E152H, V190I/S265Q, M138L/E152M, S265P/L282F/Q286P, M138L/E152H, T059K/S066N/K088E, V190I/S265W, F130L/E152W,
- 20 L282M/Q286K/A289R, L282M/Q286K/A289R/I253V, T263W/A273H, V190I/S265N, M138L/E152W, A273H/S285R, F130I/M138L, F130L/E152F, F130V/M138L/E152W, T059K/S066Q/V102A/S129Q, F130V/E152H/T179P, F130I/M138L/E152F, F130V/M138L/E152F, M138L/E152F, L282M/Q286R, F130I/E152H, S265P/L282F/A289R/T065S, T263H/A273H/S285R, F130V/M138L, T014M/T059R/S129V, L282M/Q286R/A289R/K11N, A273H/S285P, L282M/Q286K/A289R/S132T, T263H/A273H/S285W,
- 25 F130V/E152W, S265P/L282F/Q286K/N061Y, F130I/E152W, L198I/D220E/S265Q, V190I/S265L, T263H/S285W, S265P/L282F/A289R, M138L/S066Q, F130I/E152F, N90S/A273H/S285P, S032T/T263H/A273H/S285R, L282F/Q286P/A289R, N157Y/T263W/A273H/S285R, V105A/S129V, T263H/A273H/S285P, S129Q/L282H, T059W/S066Q, F130V/E152H, S023W/G024Y, T004V/S023N, T059R/S066Q, N050W/T054L, L282M/Q286P/A289R, A115V/V190L/S265W, L282M/Q286K,
- 30 T059R/S066N, L282F/Q286P, T004V/S023W/G024M, S265P/L282F/Q286R/L78H, L282F/Q286K, T004V/S023W/G024Y, S023W/G024M, T059R/R256S, F130V/E152F, T004V/G024W, N050W/T054K, S023Y/G024M, T004V/S023Y, T004V/S023Y/G024M, N050Y/T054H, S023W/G024W, T004V/S023Y/G024Y, T004V/S023N/G024W, F130L/M138L/E152F/T179P/V291I, N050Y/T059K/S066Q, T004V/S023Y/G024W, T059K/S066N, T004V/S023N/G024Y, S023Y/G024W, N050F/T054L,
- 35 R047K/T054K, S023N/G024W, L282M/A289R, S023Y/G024Y, T004V/G024M, R047K/N050F/T054K, N050F/T054K, T059K/S066Q, S023N/G024M, S023N/G024Y, T004L/S023N, R047K/N050W/T054H, T004L/S023W/G024Y, T004S/S023W, N046Q/N050W/T054H/AI42T, T004L/S023Y, T004V/S023W, N050W/T054H, T004S/S023N, T004S/L282M, T004L/S023W, N050F/T054H, N050Y/T054L, and R047K/N050W/T054K.

The isolated variant neutral metalloprotease may comprise multiple mutations selected from S066Q/S129V, S066Q/S129I, N050Y/S066Q/S129V, S066N/S129I; T059K/S066Q/S129V, S066N/S129V, F130L/E152W/T179P, S265P/L282M/Q286R/A289R, F130L/E152V/T179P, T059K/S066Q/S129I, T263W/S285P, T059K/S066N/S129I, T263W/A273H/S285P, S265P/L282F/Q286R/A289R,

5 F130V/E152W/T179P, T263W/A273H/S285R, V190I/D220P/S265W, F130L/E152H, S066N/S129Q, S265P/L282M/Q286K/A289R, V190I/D220E, T059R/S066N/S129I, V190I/D220E/S265W, T059K/S129I, T059R/S066Q/S129I, F130I/M138L/E152H/T179P, F130I/T179P, T263W/A273H/S285W, S016L/D220E/S265W, S066Q/S129Q, V190I/D220E/S265Q, T059R/S066Q/S129V, D220E/S265N, V190L/D220E, D220E/S265W, V190I/D220P, V190L/D220E/S265N, L044Q/T263W/S285R,

10 S265P/L282M/Q286P/A289R, F130L/M138L/E152H/T179P, T263W/S285R, L282M/Q286R/A289R, T263W/S285W, F130I/E152H/T179P, V190I/D220E/S265N, V190L/D220E/S265W, V190I/D220P/S265Q, T059R/S066N/S129V, V190L/D220E/S265Q, E152H/T179P, F130L/M138L/E152F/T179P, Q062H/S066Q/S129Q, T059R/S129V, V190I/D220E/S265W/L282F, V190I/S265Q, F130L/E152F/T179P, D220E/S265Q, E152W/T179P, T059K/S066Q/S129Q, F130L/M138L/T179P, F130I/M138L/E152F/T179P,

15 F130L/M138L/E152W/T179P, N050Y/T059W/S066Q/S129V, S265P/L282M/Q286K, T059R/S129I, F130V/E152H/T179P, D220P/S265N, S265P/L282M/Q286P, F130I/E152H, T059R/S066Q/N092S/S129I, F130L/T179P, G99D/S265P/L282F/Q286K/A289R, T263W/A273H, V190I/S265N, D220P/S265W, F130L/E152W, F130L/M138L/E152H, S265P/L282M, V190I/S265Q, F130L/E152F, T059K/S129Q, Q286R/A289R, M138L/E152W/T179P, F130I/M138L/E152H, D220P/S265Q, V190L/S265N,

20 F130I/M138L/E152W, S265P/Q286K, V190L/S265Q, V190I/S265W, F130L/M138L/E152F, F130V/E152H, E152F/T179P, N050Y/T059W/S066N/S129V, T059R/S066N/S129Q, F130I/E152W, F130V/E152W, T059R/S066Q/S129Q, T263H/A273H/S285P, N90S/A273H/S285P, V190L/D220E/S265N/V291I, T059R/S129Q, A273H/S285P, F130I/M138L/E152W/T179P, F130V/M138L/E152F, N050Y/T059R/S129Q, T059W/S066Q/S129I, F130V/M138L/T179P,

25 F130V/M138L/E152W/T179P, V190L/S265W, F130V/M138L/E152W, T059W/S066Q/S129V, V190I/S265Q, F130V/M138L/E152H, F130I/E152F, N157Y/T263W/A273H/S285R, T263H/S285W, M138L/E152F/T179P, A115V/V190L/S265W, M138L/E152M, T263H/A273H/S285W, F130L/M138L/E152W, T059K/S066N/K088E, F130I/M138L/E152F, F130I/M138L/T179P, T004V/S023N, T059K/S066Q/N102A/S129Q, F130L/M138L, N047K/N050F/T054K, T263H/A273H/S285R,

30 F130L/M138L/E152W/T179P/Q286H, M138L/E152H, M138L/S066Q, L282M/Q286R/A289R/P162S, L282F/Q286R/A289R, Q062K/S066Q/S129I, A273H/S285R, S265P/L282F/Q286P, S265P/L282F/Q286P/A289R, S265P/L282M/Q286R/A289R/T202S/K203N, T059W/S066N/S129I, V190I/S265L, T059W/S066N/S129V, F130I/M138L, L282M/Q286K/A289R/I253V, R047K/N050F/T054K, M138L/E152F, N050W/T054K, L198I/D220E/S265Q, L282F/Q286K/A289R, N050F/T054K,

35 L282M/Q286R, M138L/E152W, S265P/L282F, F130V/E152F, T059W/S066N/S129Q, F130V/M138L, T263H/A273H, L282M/Q286K/A289R, N046Q/N050W/T054H/A142T, T059W/S066Q/S129Q, S265P/L282F/A289R/T065S, N050F/T054H, S129Q/L282H, L282M/Q286K/A289R/S132T, L282M/Q286R/A289R/K11N, T059K/S066N, R047K/N050W/T054K, T059K/S066Q, T004V/S023Y, T059W/S066N/S129V/S290R, N050Y/T059K/S066Q, and R047K/N050Y.

The invention further provides a polynucleotide encoding the neutral metalloprotease of the invention, an expression vector comprising such a polynucleotide, and a host cell comprising such an expression vector.

Also provided is a method for producing an enzyme having neutral metalloprotease activity,
5 comprising cultivating the host cell of the invention under conditions suitable for the production of said neutral metalloprotease variant.

The method may further comprise the step of harvesting said produced neutral metalloprotease variant.

The host cell may be a *Bacillus* species.

10 The invention also provides compositions comprising the neutral metalloprotease variant of the invention, including cleaning compositions, animal feed compositions, textile processing compositions, and leather processing compositions.

The present invention provides cleaning compositions comprising (e.g.) at least 0.0001 weight percent of the neutral metalloprotease of the present invention, and optionally, an adjunct ingredient. In some
15 embodiments, the composition comprises an adjunct ingredient. In some preferred embodiments, the composition comprises a sufficient amount of a pH modifier to provide the composition with a neat pH of from about 3 to about 5, the composition being essentially free of materials that hydrolyze at a pH of from about 3 to about 5. In some particularly preferred embodiments, the materials that hydrolyze comprise a surfactant material. In additional embodiments, the cleaning composition is a liquid composition, while in
20 other embodiments, the cleaning composition is a solid composition and in still further embodiments, the cleaning composition is a gel. Indeed, it is not intended that the present invention be limited to any particular formulation and/or composition, as various formulations and/or compositions find use in the present invention. In further embodiments, the surfactant material comprises a sodium alkyl sulfate surfactant that comprises an ethylene oxide moiety.

25 The present invention additionally provides cleaning compositions that in addition to at least one neutral metalloprotease of the present invention, further comprise at least one acid stable enzyme, the cleaning composition comprising a sufficient amount of a pH modifier to provide the composition with a neat pH of from about 3 to about 5, the composition being essentially free of materials that hydrolyze at a pH of from about 3 to about 5. In further embodiments, the materials that hydrolyze comprise a surfactant material.
30 In some preferred embodiments, the cleaning composition being a liquid composition. In yet additional embodiments, the surfactant material comprises a sodium alkyl sulfate surfactant that comprises an ethylene oxide moiety. In some embodiments, the cleaning composition comprises a suitable adjunct ingredient. In some additional embodiments, the composition comprises a suitable adjunct ingredient. In some preferred embodiments, the composition comprises from about 0.001 to about 0.5 weight % of neutral metalloprotease.

35 In some alternatively preferred embodiments, the composition comprises from about 0.01 to about 0.1 weight percent of neutral metalloprotease.

The present invention also provides methods of cleaning, the comprising the steps of:

a) contacting a surface and/or an article comprising a fabric with the cleaning composition comprising the neutral metalloprotease of the present invention at an appropriate concentration; and b)

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optionally washing and/or rinsing the surface or material. In alternative embodiments, any suitable composition provided herein finds use in these methods. In some embodiments, the fabric comprises at least one grass stain. In some particularly preferred embodiments, the cleaning compositions of the present invention find use in removing grass and other stains from fabrics.

5 DESCRIPTION OF THE FIGURES

Figure 1 provides a graph showing the results from the determination of the affinity constants of purified MULTIFECT® neutral binding protein for zinc and calcium cations using the fluorescent dyes Fluo-Zn3 and Fluo-3, respectively.

Figure 2 provides a graph showing inhibition of protease activity of 0.36 mg/ml formulated
10 recombinant *B. amyloliquefaciens* nprE by Linear Alkylbenzene Sulfonate (LAS) assayed using the QuantiCleave™ protease assay.

Figure 3 provides a sequence alignment of various metalloprotease homologues (SEQ ID NOS:173-181) that find use in the present invention..

Figure 4 provides a sequence alignment of various metalloprotease homologues (SEQ ID NOS:182-
15 191) that find use in the present invention. In this Figure, the numbering is for thermolysin (*B. thermoproteolyticus*). As in Figure 3, the "*" indicates conserved residues, ":" indicates conservatively replaced residues, and "." indicates similar residues.

Figure 5 provides a sequence alignment of various metalloprotease homologues (SEQ ID NOS:192-195) identified through homology modeling.

20 Figure 6 provides a map of plasmid pJ4:GO1905.

Figure 7 provides a map of plasmid pJ4: G01906.

Figure 8 provides a map of plasmid pJ4:G01907.

Figure 9 provides a map of plasmid pJ4:G01908.

Figure 10 provides a map of plasmid pJ4:G01909.

25 Figure 11 provides a map of plasmid pJ4:G01938.

Figure 12 provides a map of plasmid pJHT.

Figure 13 provides a map of plasmid pAC.

Figure 14 provides a map of pUBnprE.

Figure 15 provides a schematic showing the amplification of the aprE promoter and *B. subtilis* nprE
30 gene fragments.

Figure 16 provides a map of plasmid pEL501.

Figure 17 provides a schematic showing the amplification of the aprE promoter and *B. subtilis* nprB gene fragments.

Figure 18 provides a map of plasmid pEL508.

35 Figure 19 provides a schematic showing the amplification of the aprE promoter and *B. stearothermophilus* nprT gene fragments, used in the production of strain EL560.

Figure 20 provides a diagram showing the construction of strain EL560.

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Figure 21 provides a schematic showing the amplification of the aprE promoter and *B. caldolyticus* npr gene fragments, used in the production of strain EL561.

Figure 22 provides a diagram showing the construction of strain EL561.

Figure 23 provides a schematic showing the amplification of the aprE promoter and *B. thuringiensis* nprB gene fragments.

Figure 24 provides a map of plasmid pEL568.

Figure 25 provides a graph showing results from experiments designed to determine the long-term storage of 0.36 mg/ml UF concentrate of neutral metalloprotease (nprE) in TIDE® 2005 base in the presence of zinc and calcium ions at 32 °C. For comparative purposes, results obtained for testing without salt and excess calcium are provided.

Figure 26 provides wash performance test data using Terg-O-Tometer (TOM) and varying soiled substrates. Panel A provides results showing the delta soil removal (%) of subtilisin (BPN' Y217L) and purified MULTIFECT® Neutral on EMPA 116 (fixed and unfixed on cotton) after washing at 15°C in TIDE®-2005 detergent liquid. Panel B provides results showing the delta soil removal (%) of subtilisin (BPN' Y217L) and purified MULTIFECT® Neutral on Equest® grass medium soiled on cotton after washing at 15°C in TIDE®-2005 detergent liquid. Panel C provides results showing the delta soil removal (%) of subtilisin (BPN' Y217L) and purified MULTIFECT® Neutral on CFT C-10 (pigment, oil, milk on cotton) after washing at 15°C in TIDE®-2005 detergent liquid.

Figure 27 provides a graph showing the results of DSC scans for 440 ppm NprE and variants obtained using the VP-Cap DSC (MicroCal™).

Figure 28 provides a graph showing the results for DSC scans for 440 ppm NprE and variants in the presence of 130 mM citrate were obtained using the VP-Cap DSC (MicroCal™).

Figure 29 provides a graph showing the thermal melting points for 440 ppm NprE in the presence of various additives and obtained using the VP-Cap DSC (MicroCal™). In this Figure, the horizontal line represents the Tm for wild-type NprE with no additives.

Figure 30 provides a graph showing the remaining activity of nprE and nprE homologs in 25% TIDE® at 25°C, after 90 minutes.

Figure 31 provides a graph showing the BMI wash performance of nprE and nprE homologs.

Figure 32 provides a graph showing the results of NprE stability measurements in various formulation mixes.

Figure 33 provides graphs (Panels A, B and C) showing the rate of NprE inactivation with different % DTPA concentrations at a fixed calcium formate concentration.

Figure 34 provides graphs (Panels A, B and C) showing the DOE analysis software generated prediction profiles of a DTPA and calcium formate composition based on response goal (decay rate).

Figure 35 provides the amino acid sequences (SEQ ID NOS:222-226) for the citrate-induced autolytic fragments of NprE highlighting the autolysis sites. Fragment 1 and 2 are the first clip, Fragment 3-5 represent the second clip. The italicized letters represent the sequenced N-termini and bold letters highlight the peptides that were identified from the in-gel digestion of the respective fragments.

DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions comprising at least one neutral metalloprotease enzyme that has improved storage stability. In some embodiments, the neutral metalloprotease finds use in cleaning and other applications. In some particularly preferred embodiments, the present invention provides methods and compositions comprising neutral metalloprotease(s) obtained from *Bacillus sp.* In some more particularly preferred embodiments, the neutral metalloprotease is obtained from *B. amyloliquefaciens*. In still further preferred embodiments, the neutral metalloprotease is a variant of the *B. amyloliquefaciens* neutral metalloprotease. In yet additional embodiments, the neutral metalloprotease is a homolog of the *B. amyloliquefaciens* neutral metalloprotease. The present invention finds particular use in applications including, but not limited to cleaning, bleaching and disinfecting.

Unless otherwise indicated, the practice of the present invention involves conventional techniques commonly used in molecular biology, microbiology, protein purification, protein engineering, protein and DNA sequencing, and recombinant DNA fields, which are within the skill of the art. Such techniques are known to those of skill in the art and are described in numerous texts and reference works (*See e.g.*, Sambrook et al., "Molecular Cloning: A Laboratory Manual", Second Edition (Cold Spring Harbor,); and Ausubel et al., "Current Protocols in Molecular Biology").

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

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. For example, Singleton and Sainsbury, Dictionary of Microbiology and Molecular Biology, 2d Ed., John Wiley and Sons, NY (1994); and Hale and Marham, The Harper Collins Dictionary of Biology, Harper Perennial, NY (1991) provide those of skill in the art with a general dictionaries of many of the terms used herein. 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 terms "a," "an," and "the" include the plural reference unless the context clearly indicates otherwise. 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.

It is intended that every maximum numerical limitation given throughout this specification includes 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.

The citation of any document is not to be construed as an admission that it is prior art with respect to the present invention.

As used herein, the term "bleaching" refers to the treatment of a material (*e.g.*, fabric, laundry, pulp, etc.) or surface for a sufficient length of time and under appropriate pH and temperature conditions to effect a brightening (*i.e.*, whitening) and/or cleaning of the material. Examples of chemicals suitable for bleaching include but are not limited to ClO_2 , H_2O_2 , peracids, NO_2 , etc.

As used herein, the term "disinfecting" refers to the removal of contaminants from the surfaces, as well as the inhibition or killing of microbes on the surfaces of items. It is not intended that the present invention be limited to any particular surface, item, or contaminant(s) or microbes to be removed.

As used herein, the term "multimer" refers to two or more proteins or peptides that are covalently or non-covalently associated and exist as a complex in solution. A "dimer" is a multimer that contains two proteins or peptides; a "trimer" contains three proteins or peptides, etc. As used herein, "octamer" refers to a multimer of eight proteins or peptides.

As used herein, "personal care products" means products used in the cleaning, bleaching and/or disinfecting of hair, skin, scalp, and teeth, including, but not limited to shampoos, body lotions, shower gels, topical moisturizers, toothpaste, and/or other topical cleansers. In some particularly preferred embodiments, these products are utilized on humans, while in other embodiments, these products find use with non-human animals (*e.g.*, in veterinary applications).

As used herein, "cleaning compositions" and "cleaning formulations," unless otherwise indicated, refer to compositions that find use in the removal of undesired compounds from items to be cleaned, such as fabric, dishes, contact lenses, other solid substrates, hair (shampoos), skin (soaps and creams), teeth (mouthwashes, toothpastes) etc. The term encompasses any materials/compounds selected for the particular type of cleaning composition desired and the form of the product (*e.g.*, liquid, gel, granule, or spray composition), as long as the composition is compatible with the neutral metalloprotease and other enzyme(s) used in the composition. The specific selection of cleaning composition materials are readily made by considering the surface, item or fabric to be cleaned, and the desired form of the composition for the cleaning conditions during use.

The terms further refer to any composition that is suited for cleaning, bleaching, disinfecting, and/or sterilizing any object and/or surface. It is intended that the terms include, but are not limited to detergent compositions (*e.g.*, liquid and/or solid laundry detergents and fine fabric detergents; hard surface cleaning formulations, such as for glass, wood, ceramic and metal counter tops and windows; carpet cleaners; oven cleaners; fabric fresheners; fabric softeners; and textile and laundry pre-spotter, as well as dish detergents).

Indeed, the term "cleaning composition" as used herein, includes unless otherwise indicated, granular or powder-form all-purpose or heavy-duty washing agents, especially cleaning detergents; liquid, gel or paste-form all-purpose washing agents, especially the so-called heavy-duty liquid (HDL) types; liquid fine-fabric detergents; hand dishwashing agents or light duty dishwashing agents, especially those of the high-foaming type; machine dishwashing agents, including the various tablet, granular, liquid and rinse-aid types for household and institutional use; liquid cleaning and disinfecting agents, including antibacterial hand-wash types, cleaning bars, mouthwashes, denture cleaners, car or carpet shampoos, bathroom cleaners; hair

shampoos and hair-rinses; shower gels and foam baths and metal cleaners; as well as cleaning auxiliaries such as bleach additives and "stain-stick" or pre-treat types.

As used herein, the terms "detergent composition" and "detergent formulation" are used in reference to mixtures which are intended for use in a wash medium for the cleaning of soiled objects. In some preferred
5 embodiments, the term is used in reference to laundering fabrics and/or garments (*e.g.*, "laundry detergents"). In alternative embodiments, the term refers to other detergents, such as those used to clean dishes, cutlery, etc. (*e.g.*, "dishwashing detergents"). It is not intended that the present invention be limited to any particular detergent formulation or composition. Indeed, it is intended that in addition to neutral metalloprotease, the term encompasses detergents that contain surfactants, transferase(s), hydrolytic enzymes, oxido reductases,
10 builders, bleaching agents, bleach activators, bluing agents and fluorescent dyes, caking inhibitors, masking agents, enzyme activators, antioxidants, and solubilizers.

As used herein, "Applicant Enzyme" refers to the neutral metalloproteases of the present invention.

As used herein, "enhanced performance" in a detergent is defined as increasing cleaning of bleach-sensitive stains (*e.g.*, grass, tea, wine, blood, dingy, etc.), as determined by usual evaluation after a standard
15 wash cycle. In particular embodiments, the neutral metalloprotease of the present invention provides enhanced performance in the removal of colored stains and soils. In further embodiments, the enzyme of the present invention provides enhanced performance in the removal and/or decolorization of stains.

As used herein the term "hard surface cleaning composition," refers to detergent compositions for cleaning hard surfaces such as floors, walls, tile, bath and kitchen fixtures, and the like. Such compositions
20 are provided in any form, including but not limited to solids, liquids, emulsions, etc.

As used herein, "dishwashing composition" refers to all forms for compositions for cleaning dishes, including but not limited to granular and liquid forms.

As used herein, "fabric cleaning composition" refers to all forms of detergent compositions for cleaning fabrics, including but not limited to, granular, liquid and bar forms.

As used herein, "textile" refers to woven fabrics, as well as staple fibers and filaments suitable for conversion to or use as yarns, woven, knit, and non-woven fabrics. The term encompasses yarns made from natural, as well as synthetic (*e.g.*, manufactured) fibers.
25

As used herein, "textile materials" is a general term for fibers, yarn intermediates, yarn, fabrics, and products made from fabrics (*e.g.*, garments and other articles).

As used herein, "fabric" encompasses any textile material. Thus, it is intended that the term encompass garments, as well as fabrics, yarns, fibers, non-woven materials, natural materials, synthetic materials, and any other textile material.
30

As used herein, the term "compatible," means that the cleaning composition materials do not reduce the enzymatic activity of the neutral metalloprotease to such an extent that the neutral metalloprotease is not effective as desired during normal use situations. Specific cleaning composition materials are exemplified in detail hereinafter.
35

As used herein, "effective amount of enzyme" refers to the quantity of enzyme necessary to achieve the enzymatic activity required in the specific application (*e.g.*, personal care product, cleaning composition, etc.). Such effective amounts are readily ascertained by one of ordinary skill in the art and are based on many

factors, such as the particular enzyme variant used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid or dry (*e.g.*, granular, bar) composition is required, and the like.

As used herein, "non-fabric cleaning compositions" encompass hard surface cleaning compositions, dishwashing compositions, personal care cleaning compositions (*e.g.*, oral cleaning compositions, denture
5 cleaning compositions, personal cleansing compositions, etc.), and compositions suitable for use in the pulp and paper industry.

As used herein, "oral cleaning compositions" refers to dentifrices, toothpastes, toothgels, toothpowders, mouthwashes, mouth sprays, mouth gels, chewing gums, lozenges, sachets, tablets, biogels, prophylaxis pastes, dental treatment solutions, and the like.

10 As used herein, the term "transferase" refers to an enzyme that catalyzes the transfer of functional compounds to a range of substrates.

As used herein, "leaving group" refers to the nucleophile which is cleaved from the acyl donor upon substitution by another nucleophile.

As used herein, the term "enzymatic conversion" refers to the modification of a substrate to an
15 intermediate or the modification of an intermediate to an end-product by contacting the substrate or intermediate with an enzyme. In some embodiments, contact is made by directly exposing the substrate or intermediate to the appropriate enzyme. In other embodiments, contacting comprises exposing the substrate or intermediate to an organism that expresses and/or excretes the enzyme, and/or metabolizes the desired substrate and/or intermediate to the desired intermediate and/or end-product, respectively.

20 As used herein, the phrase "detergent stability" refers to the stability of a detergent composition. In some embodiments, the stability is assessed during the use of the detergent, while in other embodiments, the term refers to the stability of a detergent composition during storage.

As used herein, the phrase, "stability to proteolysis" refers to the ability of a protein (*e.g.*, an enzyme) to withstand proteolysis. It is not intended that the term be limited to the use of any particular
25 protease to assess the stability of a protein.

As used herein, "oxidative stability" refers to the ability of a protein to function under oxidative conditions. In particular, the term refers to the ability of a protein to function in the presence of various concentrations of H₂O₂ and/or peracid. Stability under various oxidative conditions can be measured either by standard procedures known to those in the art and/or by the methods described herein. A substantial change in
30 oxidative stability is evidenced by at least about a 5% or greater increase or decrease (in most embodiments, it is preferably an increase) in the half-life of the enzymatic activity, as compared to the enzymatic activity present in the absence of oxidative compounds.

As used herein, "pH stability" refers to the ability of a protein to function at a particular pH. In general, most enzymes have a finite pH range at which they will function. In addition to enzymes that
35 function in mid-range pHs (*i.e.*, around pH 7), there are enzymes that are capable of working under conditions with very high or very low pHs. Stability at various pHs can be measured either by standard procedures known to those in the art and/or by the methods described herein. A substantial change in pH stability is evidenced by at least about 5% or greater increase or decrease (in most embodiments, it is preferably an increase) in the half-life of the enzymatic activity, as compared to the enzymatic activity at the enzyme's

optimum pH. However, it is not intended that the present invention be limited to any pH stability level nor pH range.

As used herein, "thermal stability" refers to the ability of a protein to function at a particular temperature. In general, most enzymes have a finite range of temperatures at which they will function. In addition to enzymes that work in mid-range temperatures (*e.g.*, room temperature), there are enzymes that are capable of working in very high or very low temperatures. Thermal stability can be measured either by known procedures or by the methods described herein. A substantial change in thermal stability is evidenced by at least about 5% or greater increase or decrease (in most embodiments, it is preferably an increase) in the half-life of the catalytic activity of a mutant when exposed to a different temperature (*i.e.*, higher or lower) than optimum temperature for enzymatic activity. However, it is not intended that the present invention be limited to any temperature stability level nor temperature range.

As used herein, the term "chemical stability" refers to the stability of a protein (*e.g.*, an enzyme) towards chemicals that adversely affect its activity. In some embodiments, such chemicals include, but are not limited to hydrogen peroxide, peracids, anionic detergents, cationic detergents, non-ionic detergents, chelants, etc. However, it is not intended that the present invention be limited to any particular chemical stability level nor range of chemical stability.

As used herein, the phrase "neutral metalloprotease activity improvement" refers to the relative improvement of neutral metalloprotease activity, in comparison with a standard enzyme. In some embodiments, the term refers to an improved rate of product formation, while in other embodiments, the term encompasses compositions that produce less hydrolysis product. In additional embodiments, the term refers to neutral metalloprotease compositions with altered substrate specificity.

As used herein, the phrase "alteration in substrate specificity" refers to changes in the substrate specificity of an enzyme. In some embodiments, a change in substrate specificity is defined as a difference between the K_{cat}/K_m ratio observed with an enzyme compared to enzyme variants or other enzyme compositions. Enzyme substrate specificities vary, depending upon the substrate tested. The substrate specificity of an enzyme is determined by comparing the catalytic efficiencies it exhibits with different substrates. These determinations find particular use in assessing the efficiency of mutant enzymes, as it is generally desired to produce variant enzymes that exhibit greater ratios for particular substrates of interest. However, it is not intended that the present invention be limited to any particular substrate composition nor any specific substrate specificity.

As used herein, "surface property" is used in reference to an electrostatic charge, as well as properties such as the hydrophobicity and/or hydrophilicity exhibited by the surface of a protein.

As used herein, the phrase "is independently selected from the group consisting of" means that moieties or elements that are selected from the referenced *Markush* group can be the same, can be different or any mixture of elements as indicated in the following example:

A molecule having 3 R groups wherein each R group is independently selected from the group consisting of A, B and C. Here the three R groups may be: AAA, BBB, CCC, AAB, AAC, BBA, BBC, CCA, CCB, or ABC.

In reference to chemical compositions, the term "substituted" as used herein, means that the organic composition or radical to which the term is applied is:

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(a) made unsaturated by the elimination of at least one element or radical; or

(b) at least one hydrogen in the compound or radical is replaced with a moiety containing one or more (i) carbon, (ii) oxygen, (iii) sulfur, (iv) nitrogen or (v) halogen atoms; or

(c) both (a) and (b).

5 Moieties which may replace hydrogen as described in (b) immediately above, that contain only carbon and hydrogen atoms, are hydrocarbon moieties including, but not limited to, alkyl, alkenyl, alkynyl, alkyldienyl, cycloalkyl, phenyl, alkyl phenyl, naphthyl, anthryl, phenanthryl, fluoryl, steroid groups, and combinations of these groups with each other and with polyvalent hydrocarbon groups such as alkylene, alkylidene and alkylidyne groups. Moieties containing oxygen atoms that may replace hydrogen as described
10 in (b) immediately above include, but are not limited to, hydroxy, acyl or keto, ether, epoxy, carboxy, and ester containing groups. Moieties containing sulfur atoms that may replace hydrogen as described in (b) immediately above include, but are not limited to, the sulfur-containing acids and acid ester groups, thioether groups, mercapto groups and thioketo groups. Moieties containing nitrogen atoms that may replace hydrogen as described in (b) immediately above include, but are not limited to, amino groups, the nitro group, azo
15 groups, ammonium groups, amide groups, azido groups, isocyanate groups, cyano groups and nitrile groups. Moieties containing halogen atoms that may replace hydrogen as described in (b) immediately above include chloro, bromo, fluoro, iodo groups and any of the moieties previously described where a hydrogen or a pendant alkyl group is substituted by a halo group to form a stable substituted moiety.

It is understood that any of the above moieties (b)(i) through (b)(v) can be substituted into each other
20 in either a monovalent substitution or by loss of hydrogen in a polyvalent substitution to form another monovalent moiety that can replace hydrogen in the organic compound or radical.

As used herein, the terms "purified" and "isolated" refer to the removal of contaminants from a sample. For example, neutral metalloprotease are purified by removal of contaminating proteins and other compounds within a solution or preparation that are not neutral metalloprotease. In some embodiments,
25 recombinant neutral metalloprotease are expressed in bacterial or fungal host cells and these recombinant neutral metalloproteases are purified by the removal of other host cell constituents; the percent of recombinant neutral metalloprotease polypeptides is thereby increased in the sample. In particularly preferred embodiments, the metalloprotease of the present invention is substantially purified to a level of at least about 99% of the protein component, as determined by SDS-PAGE or other standard methods known in the art. In
30 alternative preferred embodiments, the metalloprotease of the present invention comprise at least about 99% of the protease component of the compositions. In yet alternative embodiments, the metalloprotease is present in a range of about at least 90-95% of the total protein and/or protease.

As used herein, "protein of interest," refers to a protein (*e.g.*, an enzyme or "enzyme of interest") which is being analyzed, identified and/or modified. Naturally-occurring, as well as recombinant proteins find
35 use in the present invention.

As used herein, "protein" refers to any composition comprised of amino acids and recognized as a protein by those of skill in the art. The terms "protein," "peptide" and polypeptide are used interchangeably herein. Wherein a peptide is a portion of a protein, those skilled in the art understand the use of the term in context.

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As used herein, functionally and/or structurally similar proteins are considered to be "related proteins." In some embodiments, these proteins are derived from a different genus and/or species, including differences between classes of organisms (*e.g.*, a bacterial protein and a fungal protein). In some embodiments, these proteins are derived from a different genus and/or species, including differences between
5 classes of organisms (*e.g.*, a bacterial enzyme and a fungal enzyme). In additional embodiments, related proteins are provided from the same species. Indeed, it is not intended that the present invention be limited to related proteins from any particular source(s). In addition, the term "related proteins" encompasses tertiary structural homologs and primary sequence homologs (*e.g.*, the neutral metalloprotease of the present invention). For example, the present invention encompasses such homologs as those provided in Figures 3-5.
10 Additional homologs are contemplated, including but not limited to metalloprotease enzymes obtained from *B. cereus*, *B. cereus* E33L, *B. caldolyticus*, *B. pumilis*, *B. megaterium*, *B. subtilis* amylosacchariticus, *Brevibacillus brevis*, *Paenibacillus polymyxa* (*Bacillus polymyxa*), *B. stearothermophilus*, *B. thuringiensis*, *B. subtilis* and *S. aureus*, as well as aureolysin, extracellular elastase, and neutral protease B. In further embodiments, the term encompasses proteins that are immunologically cross-reactive.

15 As used herein, the term "derivative" refers to a protein which is derived from a protein by addition of one or more amino acids to either or both the C- and N-terminal end(s), substitution of one or more amino acids at one or a number of different sites in the amino acid sequence, and/or 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. The preparation of a protein
20 derivative is preferably achieved by modifying a DNA sequence which encodes for the native protein, transformation of that DNA sequence into a suitable host, and expression of the modified DNA sequence to form the derivative protein.

Related (and derivative) proteins comprise "variant proteins." In some preferred embodiments, variant proteins differ from a parent protein and one another by a small number of amino acid residues. The
25 number of differing amino acid residues may be one or more, preferably 1, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, or more amino acid residues. In some preferred embodiments, the number of different amino acids between variants is between 1 and 10. In some particularly preferred embodiments, related proteins and particularly variant proteins comprise at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% amino acid sequence identity. Additionally, a related protein or a variant protein as used
30 herein, refers to a protein that differs from another related protein or a parent protein in the number of prominent regions. For example, in some embodiments, variant proteins have 1, 2, 3, 4, 5, or 10 corresponding prominent regions that differ from the parent protein.

Several methods are known in the art that are suitable for generating variants of the enzymes of the present invention, including but not limited to site-saturation mutagenesis, scanning mutagenesis, insertional
35 mutagenesis, random mutagenesis, site-directed mutagenesis, and directed-evolution, as well as various other recombinatorial approaches.

Characterization of wild-type and mutant proteins is accomplished via any means suitable and is preferably based on the assessment of properties of interest. For example, pH and/or temperature, as well as detergent and /or oxidative stability is/are determined in some embodiments of the present invention. Indeed,

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it is contemplated that enzymes having various degrees of stability in one or more of these characteristics (pH, temperature, proteolytic stability, detergent stability, and/or oxidative stability) will find use.

As used herein, "expression vector" refers to a DNA construct containing a DNA sequence that is operably linked to a suitable control sequence capable of effecting the expression of the DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid," "expression plasmid," and "vector" are often used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors that serve equivalent functions and which are, or become, known in the art.

In some preferred embodiments, the neutral metalloprotease gene is ligated into an appropriate expression plasmid. The cloned neutral metalloprotease gene is then used to transform or transfect a host cell in order to express the neutral metalloprotease gene. This plasmid may replicate in hosts in the sense that it contains the well-known elements necessary for plasmid replication or the plasmid may be designed to integrate into the host chromosome. The necessary elements are provided 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), a transcription terminator (a polyadenylation region for eukaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the neutral metalloprotease 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.

The following cassette mutagenesis method may be used to facilitate the construction of the neutral metalloprotease variants of the present invention, although other methods may be used. First, as described herein, a naturally-occurring gene encoding the neutral metalloprotease is obtained and sequenced in whole or in part. Then, the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded neutral metalloprotease. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the protein gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not overly redundant in the neutral metalloprotease gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites are generated by substituting nucleotides in the gene in such a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different restriction enzymes.

Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

Once the naturally-occurring DNA and/or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termin-
5 complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

As used herein, "corresponding 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
10 peptide.

As used herein, "corresponding region," generally refers to an analogous position along related proteins or a parent protein.

The terms "nucleic acid molecule encoding," "nucleic acid sequence encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of
15 deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

As used herein, the term "analogous sequence" refers to a sequence within a protein that provides similar function, tertiary structure, and/or conserved residues as the protein of interest (*i.e.*, typically the original protein of interest). For example, in epitope regions that contain an alpha helix or a beta sheet
20 structure, the replacement amino acids in the analogous sequence preferably maintain the same specific structure. The term also refers to nucleotide sequences, as well as amino acid sequences. In some embodiments, analogous sequences are developed such that the replacement amino acids result in a variant enzyme showing a similar or improved function. In some preferred embodiments, the tertiary structure and/or conserved residues of the amino acids in the protein of interest are located at or near the segment or fragment
25 of interest. Thus, where the segment or fragment of interest contains, for example, an alpha-helix or a beta-sheet structure, the replacement amino acids preferably maintain that specific structure.

As used herein, "homologous protein" refers to a protein (*e.g.*, neutral metalloprotease) that has similar action and/or structure, as a protein of interest (*e.g.*, an neutral metalloprotease from another source). It is not intended that homologs (also referred to herein as "homologues") be necessarily related
30 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. In some preferred embodiments, it is desirable to identify a homolog that has a quaternary, tertiary and/or primary structure similar to the protein of interest, as replacement for the segment or fragment in the protein of interest with an analogous segment from the homolog will reduce the disruptiveness of the change.

As used herein, "homologous genes" refers to at least a pair of genes from different species, which genes correspond to each other and which are identical or very similar to each other. The term encompasses genes that are separated by speciation (*i.e.*, the development of new species) (*e.g.*, orthologous genes), as well as genes that have been separated by genetic duplication (*e.g.*, paralogous genes). These genes encode
35 "homologous proteins."

As used herein, "ortholog" and "orthologous genes" refer to genes in different species that have evolved from a common ancestral gene (*i.e.*, a homologous gene) by speciation. Typically, orthologs retain the same function during the course of evolution. Identification of orthologs finds use in the reliable prediction of gene function in newly sequenced genomes.

5 As used herein, "paralog" and "paralogous genes" refer to genes that are related by duplication within a genome. While orthologs retain the same function through the course of evolution, paralogs evolve new functions, even though some functions are often related to the original one. Examples of paralogous genes include, but are not limited to genes encoding trypsin, chymotrypsin, elastase, and thrombin, which are all serine proteinases and occur together within the same species.

10 As used herein, "wild-type" and "native" proteins are those found in nature. The terms "wild-type sequence," and "wild-type gene" are used interchangeably herein, to refer to a sequence that is native or naturally occurring in a host cell. In some embodiments, the wild-type sequence refers to a sequence of interest that is the starting point of a protein engineering project. The genes encoding the naturally-occurring protein may be obtained in accord with the general methods known to those skilled in the art. The methods
15 generally comprise synthesizing labeled probes having putative sequences encoding regions of the protein of interest, preparing genomic libraries from organisms expressing the protein, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule that is comprised of segments of DNA joined together by means of molecular biological techniques.

20 The term "recombinant oligonucleotide" refers to an oligonucleotide created using molecular biological manipulations, including but not limited to, the ligation of two or more oligonucleotide sequences generated by restriction enzyme digestion of a polynucleotide sequence, the synthesis of oligonucleotides (*e.g.*, the synthesis of primers or oligonucleotides) and the like.

The degree of identity between amino acid sequences is determined using the BLAST algorithm,
25 described by Altschul *et al.*, (Altschul *et al.*, J. Mol. Biol., 215:403-410, ; and Karlin *et al.*, Proc. Natl. Acad. Sci. USA 90:5873-5787). One particularly useful BLAST program is the WU-BLAST-2 program (*See*, Altschul *et al.*, Meth. Enzymol." 266:460-480). 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 and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915) alignments (B) of 50,
30 expectation (E) of 10, M⁵, N⁻⁴, and a comparison of both strands.

As used herein, "percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotide residues in a candidate sequence that is identical with the nucleotide residues of the sequence.

As used herein, the term "hybridization" refers to the process by which a strand of nucleic acid joins with a complementary strand through base pairing, as known in the art.

35 As used herein, the phrase "hybridization conditions" refers to the conditions under which hybridization reactions are conducted. These conditions are typically classified by degree of "stringency" of the conditions under which hybridization is measured. The degree of stringency can be based, for example, on the melting temperature (T_m) of the nucleic acid binding complex or probe. For example, "maximum stringency" typically occurs at about T_m-5°C (5° below the T_m of the probe); "high stringency" at about 5-
40 10° below the T_m; "intermediate stringency" at about 10-20° below the T_m of the probe; and "low

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stringency" at about 20-25° below the T_m . Alternatively, or in addition, hybridization conditions can be based upon the salt or ionic strength conditions of hybridization and/or one or more stringency washes. For example, 6xSSC = very low stringency; 3xSSC = low to medium stringency; 1xSSC = medium stringency; and 0.5xSSC = high stringency. Functionally, maximum stringency conditions may be used to identify
 5 nucleic acid sequences having strict identity or near-strict identity with the hybridization probe; while high stringency conditions are used to identify nucleic acid sequences having about 80% or more sequence identity with the probe.

For applications requiring high selectivity, it is typically desirable to use relatively stringent conditions to form the hybrids (*e.g.*, relatively low salt and/or high temperature conditions are used).

10 The phrases "substantially similar and "substantially identical" in the context of at least two nucleic acids or polypeptides typically means that a polynucleotide or polypeptide comprises a sequence that has at least about 40% identity, more preferable at least about 50% identity, yet more preferably at least about 60% identity, preferably at least about 75% identity, more preferably at least about 80% identity, yet more preferably at least about 90%, still more preferably about 95%, most preferably about 97% identity,
 15 sometimes as much as about 98% and about 99% sequence identity, compared to the reference (*i.e.*, wild-type) sequence. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. Also, databases may be searched using FASTA (Pearson et al., Proc. Natl. Acad. Sci. USA 85:2444-2448). One indication that two polypeptides are substantially identical is that the first polypeptide is immunologically cross-reactive with the second polypeptide. Typically, polypeptides that
 20 differ by conservative amino acid substitutions are immunologically cross-reactive. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions (*e.g.*, within a range of medium to high stringency).

25 As used herein, "equivalent residues" refers to proteins that share particular amino acid residues. For example, equivalent residues may be identified by determining homology at the level of tertiary structure for a protein (*e.g.*, neutral metalloprotease) whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the protein having putative equivalent residues and the protein of
 30 interest (N on N, CA on CA, C on C and O on O) are within 0.13 nm and preferably 0.1 nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the proteins analyzed. The preferred model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available, determined using methods known to those skilled in the art of crystallography and protein
 35 characterization/analysis.

As used herein, the terms "hybrid neutral metalloproteases" and "fusion neutral metalloproteases" refer to proteins that are engineered from at least two different or "parental" proteins. In preferred embodiments, these parental proteins are homologs of one another. For example, in some embodiments, a preferred hybrid neutral metalloprotease or fusion protein contains the N-terminus of a protein and the C-

terminus of a homolog of the protein. In some preferred embodiment, the two terminal ends are combined to correspond to the full-length active protein.

The term "regulatory element" as used herein refers to a genetic element that controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region. Additional regulatory elements include splicing signals, polyadenylation signals and termination signals.

As used herein, "host cells" are generally prokaryotic or eukaryotic hosts which are transformed or transfected with vectors constructed using recombinant DNA techniques known in the art. Transformed host cells are capable of either replicating vectors encoding the protein variants or expressing the desired protein variant. In the case of vectors which encode the pre- or prepro-form of the protein variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means transformation, transduction or transfection. Means of transformation include protoplast transformation, calcium chloride precipitation, electro oration, naked DNA and the like as known in the art. (See, Chang and Cohen, Mol. Gen. Genet., 168:111 - 115 ; Smith et al., Appl. Env. Microbiol., 51:634 ; and the review article by Ferrari et al., in Harwood, Bacillus, Plenum Publishing Corporation, pp. 57-72).

The term "promoter/enhancer" denotes a segment of DNA which contains sequences capable of providing both promoter and enhancer functions (for example, the long terminal repeats of retroviruses contain both promoter and enhancer functions). The enhancer/promoter may be "endogenous" or "exogenous" or "heterologous." An endogenous enhancer/promoter is one which is naturally linked with a given gene in the genome. An exogenous (heterologous) enhancer/promoter is one which is placed in juxtaposition to a gene by means of genetic manipulation (*i.e.*, molecular biological techniques).

The presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York , pp. 16.7-16.8). A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated foreign or exogenous DNA into the genomic DNA of the transfected cell.

The terms "selectable marker" or "selectable gene product" as used herein refer to the use of a gene which encodes an enzymatic activity that confers resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed.

As used herein, the terms "amplification" and "gene amplification" refer to a process by which specific DNA sequences are disproportionately replicated such that the amplified gene becomes present in a higher copy number than was initially present in the genome. In some embodiments, selection of cells by growth in the presence of a drug (*e.g.*, an inhibitor of an inhibitable enzyme) results in the amplification of either the endogenous gene encoding the gene product required for growth in the presence of the drug or by amplification of exogenous (*i.e.*, input) sequences encoding this gene product, or both. Selection of cells by growth in the presence of a drug (*e.g.*, an inhibitor of an inhibitable enzyme) may result in the amplification

of either the endogenous gene encoding the gene product required for growth in the presence of the drug or by amplification of exogenous (*i.e.*, input) sequences encoding this gene product, or both.

"Amplification" is a special case of nucleic acid replication involving template specificity. It is to be contrasted with non-specific template replication (*i.e.*, replication that is template-dependent but not dependent on a specific template). Template specificity is here distinguished from fidelity of replication (*i.e.*, synthesis of the proper polynucleotide sequence) and nucleotide (ribo- or deoxyribo-) specificity. Template specificity is frequently described in terms of "target" specificity. Target sequences are "targets" in the sense that they are sought to be sorted out from other nucleic acid. Amplification techniques have been designed primarily for this sorting out.

As used herein, the term "co-amplification" refers to the introduction into a single cell of an amplifiable marker in conjunction with other gene sequences (*i.e.*, comprising one or more non-selectable genes such as those contained within an expression vector) and the application of appropriate selective pressure such that the cell amplifies both the amplifiable marker and the other, non-selectable gene sequences. The amplifiable marker may be physically linked to the other gene sequences or alternatively two separate pieces of DNA, one containing the amplifiable marker and the other containing the non-selectable marker, may be introduced into the same cell.

As used herein, the terms "amplifiable marker," "amplifiable gene," and "amplification vector" refer to a marker, gene or a vector encoding a gene which permits the amplification of that gene under appropriate growth conditions.

As used herein, the term "amplifiable nucleic acid" refers to nucleic acids which may be amplified by any amplification method. It is contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

As used herein, the term "sample template" refers to nucleic acid originating from a sample which is analyzed for the presence of "target" (defined below). In contrast, "background template" is used in reference to nucleic acid other than sample template which may or may not be present in a sample. Background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

"Template specificity" is achieved in most amplification techniques by the choice of enzyme. Amplification enzymes are enzymes that, under conditions they are used, will process only specific sequences of nucleic acid in a heterogeneous mixture of nucleic acid. For example, in the case of Q β replicase, MDV-1 RNA is the specific template for the replicase (*See e.g.*, Kacian et al., Proc. Natl. Acad. Sci. USA 69:3038). Other nucleic acids are not replicated by this amplification enzyme. Similarly, in the case of T7 RNA polymerase, this amplification enzyme has a stringent specificity for its own promoters (*See*, Chamberlin et al., Nature 228:227). In the case of T4 DNA ligase, the enzyme will not ligate the two oligonucleotides or polynucleotides, where there is a mismatch between the oligonucleotide or polynucleotide substrate and the template at the ligation junction (*See*, Wu and Wallace, Genomics 4:560). Finally, *Taq* and *Pfu* polymerases, by virtue of their ability to function at high temperature, are found to display high specificity for the sequences bounded and thus defined by the primers; the high temperature results in thermodynamic

conditions that favor primer hybridization with the target sequences and not hybridization with non-target sequences.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "probe" refers to an oligonucleotide (*i.e.*, a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labeled with any "reporter molecule," so that is detectable in any detection system, including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

As used herein, the term "target," when used in reference to amplification methods (*e.g.*, the polymerase chain reaction), refers to the region of nucleic acid bounded by the primers used for polymerase chain reaction. Thus, the "target" is sought to be sorted out from other nucleic acid sequences. A "segment" is defined as a region of nucleic acid within the target sequence.

As used herein, the term "polymerase chain reaction" ("PCR") refers to the methods of U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188, which include methods for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target

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sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified".

As used herein, the term "amplification reagents" refers to those reagents (deoxyribonucleotide triphosphates, buffer, etc.), needed for amplification except for primers, nucleic acid template and the
5 amplification enzyme. Typically, amplification reagents along with other reaction components are placed and contained in a reaction vessel (test tube, microwell, etc.).

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of ³²P-labeled
10 deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide or polynucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications.

As used herein, the terms "PCR product," "PCR fragment," and "amplification product" refer to the
15 resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

20 DETAILED DESCRIPTION OF THE PRESENT INVENTION

The present invention provides a neutral metalloprotease enzyme that has improved activity or stability. In some embodiments, the neutral metalloprotease finds use in applications including, but not limited to cleaning, bleaching and disinfecting.

Also as described in more detail in the Examples below, the present invention provides many
25 advantages for cleaning of a wide range of objects, including but not limited to clothing, fabrics, medical devices, etc. In addition, the present invention provides compositions that are effective in cleaning, bleaching, and disinfecting, over a range of wash temperatures and pHs.

In general, proteases hydrolyze amide linkages of proteins via addition of a water molecule to the peptide bond(s). Cleavage occurs at the carbonyl-group of the peptide bond. In bacterial species such as
30 *Bacillus*, there are two main classes of extracellular proteases namely, alkaline or serine proteases and neutral metalloproteases.

Neutral metalloendopeptidases (*i.e.*, neutral metalloproteases) (EC 3.4.24.4) belong to a protease class that has an absolute requirement for zinc ions for catalytic activity. These enzymes are optimally active at neutral pH and are in the 30 to 40 kDa size range. Neutral metalloproteases bind between two and four
35 calcium ions that contribute to the structural stability of the protein. The bound metal ion at the active site of metalloproteases is an essential feature that allows the activation of a water molecule. The water molecule then functions as the nucleophile and cleaves the carbonyl group of the peptide bond.

The neutral zinc-binding metalloprotease family includes the bacterial enzyme thermolysin, and thermolysin-like proteases ("TLPs"), as well as carboxypeptidase A (a digestive enzyme), and the matrix

metalloproteases that catalyze the reactions in tissue remodeling and degradation. The only well characterized of these proteases, with respect to stability and function, is thermolysin and its variants (TLPs). Indeed, much research has been focused on the engineering *Bacillus subtilis* neutral proteases to increase the thermal stability of the enzyme (*See e.g.*, Vriend et al., In, Tweel et al. (eds), Stability and Stabilization of enzymes, Elsevier, pp. 93-99).

Most effort has been focused on increasing the stability of the protease by altering structural determinants identified through the use of molecular modeling suggested to prevent local unfolding processes that would result in autolysis of the protein and cause the neutral protease to denature at high temperatures (*See e.g.*, van den Burg et al., in Hopsu-Havu et al., (eds), Proteolysis in Cell Functions Manipulating the Autolytic Pathway of a Bacillus Protease. Biomedical and Health Research Vol. 13, IOS Press p. 576).

Compositions and methods to engineer neutral metalloproteases with improved characteristics are provided herein. As indicated herein, calcium ions have been reported for other proteases such as thermolysin, to prevent autolysis. The *B. stearothermophilus* neutral protease has been stabilized against autolysis and proteolytic degradation by addition of calcium (*See*, Dürrschmidt et al., FEBS J., 272:1523-1534).

Indeed, the present invention provides compositions and methods suitable for the engineering of neutral metalloproteases that are independent of calcium in order to maintain their structural stability. In some embodiments, engineering prevents the local unfolding in a particular secondary structural element that may prevent proteolysis.

Natural and engineered proteases, such as subtilisin are often expressed in *Bacillus subtilis* and several have been applied in detergent formulations to remove proteinaceous stains. Others have been applied for example in the baking industry (*e.g.*, thermolysin from *Bacillus thermoproteolyticus*; *See e.g.*, Galante and Formantici, Curr. Organic Chem., 7, 1399-1422). In general, the serine proteases have been more widely utilized in detergents, at least partially due to the relative ease with which these proteases can be stabilized.

Indeed, metalloproteases are less frequently used in industry, and particularly in the detergent industry for a number of reasons. These enzymes involve more complex protein systems, as the enzymes have the absolute requirement for calcium and zinc ions for stability and function, respectively. Further, the detergent solution as well as the water used in the laundry process often contains components that often interfere with the binding of the ions by the enzyme or chelate these ions, resulting in a decrease or loss of proteolytic function and destabilization of the protease.

In contrast to the currently used metalloprotease enzyme systems, the present invention provides neutral metalloproteases that are sufficiently stabilized to facilitate long-term shelf storage in liquid laundry detergent compositions. In particularly preferred embodiments, the metalloprotease stability and activity are preserved through complexing the enzyme with its obligatory active-site zinc molecule. Importantly, the combination of calcium and zinc ions does not have a deleterious effect on the enzyme's function. In some embodiments, the neutral metalloprotease stabilized is the wild-type metalloprotease from *Bacillus amyloliquefaciens* (*e.g.*, purified MULTIFECT® Neutral; "PMN"). In alternative preferred embodiments, recombinant neutral metalloprotease (*e.g.*, *Bacillus amyloliquefaciens* neutral metalloprotease cloned into *Bacillus subtilis* ("nprE")). In additional embodiments, metalloproteases with improved stability encompass enzymes with increased affinity for one or more of the calcium binding sites of the enzyme. In preferred

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embodiments, the neutral metalloproteases of the present invention find use in general detergent applications, including but not limited to cold water temperatures, grass stains, and/or low pH conditions.

The present disclosure provides conditions that stabilize zinc-binding neutral metalloprotease for increased storage stability in detergent bases and/or compositions. The detergent compositions may comprise at least one metalloprotease (*e.g.*, any *Bacillus* neutral metalloprotease) that is stabilized against autolysis and unfolding, by the inclusion within the detergent formulation of the essential zinc and/or calcium ions.

Although some experimental results indicated that nprE loses some proteolytic activity (~ 20 %) after one hour of adding the detergent base, nprE incubated at 32 °C in the presence of zinc ions showed significant stabilization over the test conditions with no additional salts or calcium ions. The presence of both calcium and zinc ions did not show an additive effect. At zinc ion concentrations lower than 15 mM neutral metalloprotease is sufficiently stable over approximately 4 weeks. Thus, the addition of zinc may increase the storage life of neutral metalloprotease in the presence of detergent components.

Furthermore, the zinc cation may be replaced with Co^{2+} , Mn^{2+} or Fe^{2+} , since all of these ions have been shown to bind and restore the protease activity of neutral metalloproteases. However, it was determined that Mn^{2+} and Fe^{2+} do not restore all of the native activity. While Co^{2+} restores the highest percentage of the activity, it is apparently less firmly bound than Zn^{2+} . The zinc cation is an essential feature in the active site of all neutral metalloproteases, as it is known to play a role in substrate binding and enzyme catalysis (*See e.g.*, Holmquist and Vallee, *J. Biol. Chem.*, 249:4601-4607). The relatively tight affinity of the neutral metalloprotease for the zinc cation (~ μM range) and the approximately 10-fold greater affinity for this ion relative to calcium, suggest that zinc functions as a stabilizer, thereby preventing autolysis, proteolysis and unfolding. However, it is not intended that the present invention be limited to any particular mechanisms.

The present invention provides extremely beneficial opportunities for application in the production and development of industrial detergents. Many detergents are available with high specificity towards the removal of protein, starch and grease stains. In particular, the better wash performance of PMN or neutral metalloprotease from *B. amyloliquefaciens* on Equest Grass (Warwick) indicates that the neutral metalloproteases of the present invention in a detergent base that also contains zinc finds use in improved detergent compositions.

DETAILED DESCRIPTION OF CLEANING AND DETERGENT FORMULATIONS OF THE PRESENT INVENTION

Unless otherwise noted, all component or composition levels provided herein are made in reference to the active level of that component or composition, and are exclusive of impurities, for example, residual solvents or by-products, which may be present in commercially available sources.

Enzyme components weights are based on total active protein.

All percentages and ratios are calculated by weight unless otherwise indicated. All percentages and ratios are calculated based on the total composition unless otherwise indicated.

In the exemplified detergent compositions, the enzymes levels are expressed by pure enzyme by weight of the total composition and unless otherwise specified, the detergent ingredients are expressed by weight of the total compositions.

CLEANING COMPOSITIONS COMPRISING NEUTRAL METALLOPROTEASE

The stabilized neutral metalloproteases of the present invention are useful in formulating various detergent compositions. The cleaning composition of the present invention may be advantageously employed for example, in laundry applications, hard surface cleaning, automatic dishwashing applications, as well as
5 cosmetic applications such as dentures, teeth, hair and skin. However, due to the unique advantages of increased effectiveness in lower temperature solutions and the superior color-safety profile, the enzymes of the present invention are ideally suited for laundry applications such as the bleaching of fabrics. Furthermore, the enzymes of the present invention find use in both granular and liquid compositions.

The enzymes of the present invention also find use in cleaning additive products. A cleaning
10 additive product including at least one enzyme of the present invention is ideally suited for inclusion in a wash process when additional bleaching effectiveness is desired. Such instances include, but are not limited to low temperature solution cleaning applications. The additive product may be, in its simplest form, one or more neutral metalloprotease enzyme as provided by the present invention. In some embodiments, the additive is packaged in dosage form for addition to a cleaning process where a source of peroxygen is
15 employed and increased bleaching effectiveness is desired. In some embodiments, the single dosage form comprises a pill, tablet, gelcap or other single dosage unit including premeasured powders and/or liquids. In some embodiments, filler and/or carrier material(s) are included, in order to increase the volume of such composition. Suitable filler or carrier materials include, but are not limited to, various salts of sulfate, carbonate and silicate as well as talc, clay and the like. In some embodiments filler and/or carrier materials
20 for liquid compositions include water and/or low molecular weight primary and secondary alcohols including polyols and diols. Examples of such alcohols include, but are not limited to, methanol, ethanol, propanol and isopropanol. In some embodiments, the compositions comprise from about 5% to about 90% of such materials. In additional embodiments, acidic fillers are used to reduce the pH of the composition. In some alternative embodiments, the cleaning additive includes at least one activated peroxygen source as described
25 below and/or adjunct ingredients as more fully described below.

The cleaning compositions and cleaning additives of the present invention require an effective amount of neutral metalloprotease enzyme as provided in the present invention. In some embodiments, the required level of enzyme is achieved by the addition of one or more species of neutral metalloprotease provided by the present invention. Typically, the cleaning compositions of the present invention comprise at
30 least 0.0001 weight percent, from about 0.0001 to about 1, from about 0.001 to about 0.5, or even from about 0.01 to about 0.1 weight percent of at least one neutral metalloprotease provided by the present invention.

In some preferred embodiments, the cleaning compositions provided herein are typically formulated such that, during use in aqueous cleaning operations, the wash water has a pH of from about 5.0 to about 11.5, or in alternative embodiments, even from about 6.0 to about 10.5. In some preferred embodiments,
35 liquid product formulations are typically formulated to have a neat pH from about 3.0 to about 9.0, while in some alternative embodiments the formulation has a neat pH from about 3 to about 5. In some preferred embodiments, granular laundry products are typically formulated to have a pH from about 8 to about 11. Techniques for controlling pH at recommended usage levels include the use of buffers, alkalis, acids, etc., and are well known to those skilled in the art.

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In some particularly preferred embodiments, when at least one neutral metalloprotease is employed in a granular composition or liquid, the neutral metalloprotease is in the form of an encapsulated particle to protect the enzyme from other components of the granular composition during storage. In addition, encapsulation also provides a means of controlling the availability of the neutral metalloprotease(s) during the cleaning process and may enhance performance of the neutral metalloprotease(s). It is contemplated that the encapsulated neutral metalloproteases of the present invention will find use in various settings. It is also intended that the neutral metalloprotease be encapsulated using any suitable encapsulating material(s) and method(s) known in the art.

In some preferred embodiments, the encapsulating material typically encapsulates at least part of the neutral metalloprotease catalyst. In some embodiments, the encapsulating material is water-soluble and/or water-dispersible. In some additional embodiments, the encapsulating material has a glass transition temperature (T_g) of 0°C or higher (*See e.g.*, WO 97/11151, particularly from page 6, line 25 to page 7, line 2, for more information regarding glass transition temperatures).

In some embodiments, the encapsulating material is selected from the group consisting of carbohydrates, natural or synthetic gums, chitin and chitosan, cellulose and cellulose derivatives, silicates, phosphates, borates, polyvinyl alcohol, polyethylene glycol, paraffin waxes and combinations thereof. In some embodiments in which the encapsulating material is a carbohydrate, it is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and combinations thereof. In some preferred embodiments, the encapsulating material is a starch (*See e.g.*, EP 0 922 499; US 4,977,252. US 5,354,559, and US 5,935,826, for descriptions of some exemplary suitable starches).

In additional embodiments, the encapsulating material comprises a microsphere made from plastic (*e.g.*, thermoplastics, acrylonitrile, methacrylonitrile, polyacrylonitrile, polymethacrylonitrile and mixtures thereof; commercially available microspheres that find use include, but are not limited to EXPANCEL® [Casco Products, Stockholm, Sweden], PM 6545, PM 6550, PM 7220, PM 7228, EXTENDOSPHERES®, and Q-CEL® [PQ Corp., Valley Forge, PA], LUXSIL® and SPHERICEL1® [Potters Industries, Inc., Carlstadt, NJ and Valley Forge, PA]).

PROCESSES OF MAKING AND USING OF APPLICANTS' CLEANING COMPOSITION

In some preferred embodiments, the cleaning compositions of the present invention are formulated into any suitable form and prepared by any process chosen by the formulator, (*See e.g.*, U.S. 5,879,584, U.S. 5,691,297, U.S. 5,574,005, U.S. 5,569,645, U.S. 5,565,422, U.S. 5,516,448, U.S. 5,489,392, and U.S. 5,486,303, for some non-limiting examples). In some embodiments in which a low pH cleaning composition is desired, the pH of such composition is adjusted via the addition of an acidic material such as HCl.

ADJUNCT MATERIALS

While not essential for the purposes of the present invention, in some embodiments, the non-limiting list of adjuncts described herein are suitable for use in the cleaning compositions of the present invention. Indeed, in some embodiments, adjuncts are incorporated into the cleaning compositions of the present invention. In some embodiments, adjunct materials assist and/or enhance cleaning performance, treat the substrate to be cleaned, and/or modify the aesthetics of the cleaning composition (*e.g.*, perfumes, colorants, dyes, etc.). It is understood that such adjuncts are in addition to the neutral metalloproteases of the present invention. The precise nature of these additional components, and levels of incorporation thereof, depends on

the physical form of the composition and the nature of the cleaning operation for which it is to be used. Suitable adjunct materials include, but are not limited to, surfactants, builders, chelating agents, dye transfer inhibiting agents, deposition aids, dispersants, additional enzymes, and enzyme stabilizers, catalytic materials, bleach activators, bleach boosters, hydrogen peroxide, sources of hydrogen peroxide, preformed peracids, polymeric dispersing agents, clay soil removal/anti-redeposition agents, brighteners, suds suppressors, dyes, perfumes, structure elasticizing agents, fabric softeners, carriers, hydrotropes, processing aids and/or pigments. In addition to those provided explicitly herein, additional examples are known in the art (*See e.g.*, U.S. Patent Nos. 5,576,282, 6,306,812 B1 and 6,326,348 B1). In some embodiments, the aforementioned adjunct ingredients constitute the balance of the cleaning compositions of the present invention.

10 SURFACTANTS - In some embodiments, the cleaning compositions of the present invention comprise at least one surfactant or surfactant system, wherein the surfactant is selected from nonionic surfactants, anionic surfactants, cationic surfactants, ampholytic surfactants, zwitterionic surfactants, semi-polar nonionic surfactants, and mixtures thereof. In some low pH cleaning composition embodiments (*e.g.*, compositions having a neat pH of from about 3 to about 5), the composition typically does not contain alkyl
15 ethoxylated sulfate, as it is believed that such surfactant may be hydrolyzed by such compositions the acidic contents.

In some embodiments, the surfactant is present at a level of from about 0.1% to about 60%, while in alternative embodiments, the level is from about 1% to about 50% , while in still further embodiments, the level is from about 5% to about 40%, by weight of the cleaning composition.

20 BUILDERS - In some embodiments, the cleaning compositions of the present invention comprise one or more detergent builders or builder systems. In some embodiments incorporating at least one builder, the cleaning compositions comprise at least about 1%, from about 3% to about 60% or even from about 5% to about 40% builder by weight of the cleaning composition.

Builders include, but are not limited to, the alkali metal, ammonium and alkanolammonium salts of
25 polyphosphates, alkali metal silicates, alkaline earth and alkali metal carbonates, aluminosilicate builders polycarboxylate compounds, ether hydroxypolycarboxylates, copolymers of maleic anhydride with ethylene or vinyl methyl ether, 1, 3, 5-trihydroxy benzene-2, 4, 6-trisulphonic acid, and carboxymethyloxysuccinic acid, the various alkali metal, ammonium and substituted ammonium salts of polyacetic acids such as ethylenediamine tetraacetic acid and nitrilotriacetic acid, as well as polycarboxylates such as mellitic acid,
30 succinic acid, citric acid, oxydisuccinic acid, polymaleic acid, benzene 1,3,5-tricarboxylic acid, carboxymethyloxysuccinic acid, and soluble salts thereof. Indeed, it is contemplated that any suitable builder will find use in various embodiments of the present invention.

CHELATING AGENTS - In some embodiments, the cleaning compositions of the present invention contain at least one chelating agent. Suitable chelating agents include, but are not limited to copper, iron
35 and/or manganese chelating agents and mixtures thereof. In embodiments in which at least one chelating agent is used, the cleaning compositions of the present invention comprise from about 0.1% to about 15% or even from about 3.0% to about 10% chelating agent by weight of the subject cleaning composition.

DEPOSITION AID - In some embodiments, the cleaning compositions of the present invention include at least one deposition aid. Suitable deposition aids include, but are not limited to polyethylene

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glycol, polypropylene glycol, polycarboxylate, soil release polymers such as polytelephthalic acid, clays such as kaolinite, montmorillonite, atapulgite, illite, bentonite, halloysite, and mixtures thereof.

DYE TRANSFER INHIBITING AGENTS - In some embodiments, the cleaning compositions of the present invention include one or more dye transfer inhibiting agents. Suitable polymeric dye transfer inhibiting agents include, but are not limited to, polyvinylpyrrolidone polymers, polyamine N-oxide polymers, copolymers of N-vinylpyrrolidone and N-vinylimidazole, polyvinylloxazolidones and polyvinylimidazoles or mixtures thereof.

In embodiments in which at least one dye transfer inhibiting agent is used, the cleaning compositions of the present invention comprise from about 0.0001% to about 10%, from about 0.01% to about 5%, or even from about 0.1% to about 3% by weight of the cleaning composition. Dispersants - In some embodiments, the cleaning compositions of the present invention contains at least one dispersants. Suitable water-soluble organic materials include, but are not limited to the homo- or co-polymeric acids or their salts, in which the polycarboxylic acid comprises at least two carboxyl radicals separated from each other by not more than two carbon atoms.

ENZYMES - In some embodiments, the cleaning compositions of the present invention comprise one or more detergent enzymes which provide cleaning performance and/or fabric care benefits. Examples of suitable enzymes include, but are not limited to, hemicellulases, peroxidases, proteases, cellulases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, keratinases, reductases, oxidases, phenoloxidases, lipoxigenases, ligninases, pullulanases, tannases, pentosanases, malanases, β -glucanases, arabinosidases, hyaluronidase, chondroitinase, laccase, and amylases, or mixtures thereof. In some embodiments, a combination of enzymes is used (*i.e.*, a "cocktail") comprising conventional applicable enzymes like protease, lipase, cutinase and/or cellulase in conjunction with amylase is used.

ENZYME STABILIZERS - In some embodiments of the present invention, the enzymes used in the detergent formulations of the present invention are stabilized. It is contemplated that various techniques for enzyme stabilization will find use in the present invention. For example, in some embodiments, the enzymes employed herein are stabilized by the presence of water-soluble sources of zinc (II), calcium (II) and/or magnesium (II) ions in the finished compositions that provide such ions to the enzymes, as well as. other metal ions (e.g., barium (II), scandium (II), iron (II), manganese (II), aluminum (III), Tin (II), cobalt (II), copper (II), Nickel (II), and oxovanadium (IV)).

CATALYTIC METAL COMPLEXES -- In some embodiments, the cleaning compositions of the present invention contain one or more catalytic metal complexes. In some embodiments, a metal-containing bleach catalyst finds use. In some preferred embodiments, the metal bleach catalyst comprises a catalyst system comprising a transition metal cation of defined bleach catalytic activity, (*e.g.*, copper, iron, titanium, ruthenium, tungsten, molybdenum, or manganese cations), an auxiliary metal cation having little or no bleach catalytic activity (*e.g.*, zinc or aluminum cations), and a sequester having defined stability constants for the catalytic and auxiliary metal cations, particularly ethylenediaminetetraacetic acid, ethylenediaminetetra (methylenephosphonic acid) and water-soluble salts thereof are used (*See e.g.*, U.S. 4,430,243).

In some embodiments, the cleaning compositions of the present invention are catalyzed by means of a manganese compound. Such compounds and levels of use are well known in the art (*See e.g.*, U.S. 5,576,282).

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In additional embodiments, cobalt bleach catalysts find use in the cleaning compositions of the present invention. Various cobalt bleach catalysts are known in the art (*See e.g.*, U.S. 5,597,936, and U.S. 5,595,967). Such cobalt catalysts are readily prepared by known procedures (*See e.g.*, U.S. 5,597,936, and U.S. 5,595,967).

5 In additional embodiments, the cleaning compositions of the present invention include a transition metal complex of a macropolycyclic rigid ligand ("MRL"). As a practical matter, and not by way of limitation, in some embodiments, the compositions and cleaning processes provided by the present invention are adjusted to provide on the order of at least one part per hundred million of the active MRL species in the aqueous washing medium, and in some preferred embodiments, provide from about 0.005 ppm to about 25
10 ppm, more preferably from about 0.05 ppm to about 10 ppm, and most preferably from about 0.1 ppm to about 5 ppm, of the MRL in the wash liquor.

Preferred transition-metals in the instant transition-metal bleach catalyst include, but are not limited to manganese, iron and chromium. Preferred MRLs also include, but are not limited to special ultra-rigid ligands that are cross-bridged (*e.g.*, 5,12-diethyl-1,5,8,12-tetraazabicyclo[6.6.2]hexadecane). Suitable
15 transition metal MRLs are readily prepared by known procedures (*See e.g.*, WO 00/32601, and U.S. 6,225,464).

PROCESSES OF MAKING AND USING CLEANING COMPOSITIONS

The cleaning compositions of the present invention are formulated into any suitable form and prepared by any suitable process chosen by the formulator, (*See e.g.*, U.S. 5,879,584, U.S. 5,691,297, U.S.
20 5,574,005, U.S. 5,569,645, U.S. 5,565,422, U.S. 5,516,448, U.S. 5,489,392, U.S. 5,486,303, U.S. 4,515,705, U.S. 4,537,706, U.S. 4,515,707, U.S. 4,550,862, U.S. 4,561,998, U.S. 4,597,898, U.S. 4,968,451, U.S. 5,565,145, U.S. 5,929,022, U.S. 6,294,514, and U.S. 6,376,445 for some non-limiting examples).

METHOD OF USE

In preferred embodiments, the cleaning compositions of the present invention find use in cleaning
25 surfaces and/or fabrics. In some embodiments, at least a portion of the surface and/or fabric is contacted with at least one embodiment of the cleaning compositions of the present invention, in neat form or diluted in a wash liquor, and then the surface and/or fabric is optionally washed and/or rinsed. For purposes of the present invention, "washing" includes, but is not limited to, scrubbing, and mechanical agitation. In some embodiments, the fabric comprises any fabric capable of being laundered in normal consumer use conditions.
30 In preferred embodiments, the cleaning compositions of the present invention are used at concentrations of from about 500 ppm to about 15,000 ppm in solution. In some embodiments in which the wash solvent is water, the water temperature typically ranges from about 5 °C to about 90 °C. In some preferred embodiments for fabric cleaning, the water to fabric mass ratio is typically from about 1:1 to about 30:1.

EXPERIMENTAL

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

In the experimental disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); rpm (revolutions per minute); H₂O (water); HCl (hydrochloric acid); aa and AA (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); gm (grams); µg and ug (micrograms); mg (milligrams); ng

(nanograms); μ l and μ l (microliters); ml (milliliters); mm (millimeters); nm (nanometers); μ m and μ m (micrometer); M (molar); mM (millimolar); μ M and μ M (micromolar); U (units); V (volts); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); $MgCl_2$ (magnesium chloride); NaCl (sodium chloride); OD₂₈₀ (optical density at 280 nm); OD₄₀₅ (optical density at 405 nm); OD₆₀₀ (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); EtOH (ethanol); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); LAS (lauryl sodium sulfonate); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); TAED (N,N,N',N'-tetraacetythylenediamine); BES (polyestersulfone); MES (2-morpholinoethanesulfonic acid, monohydrate; f.w. 195.24; Sigma # M-3671); $CaCl_2$ (calcium chloride, anhydrous; f.w. 110.99; Sigma # C-4901); DMF (N,N-dimethylformamide, f.w. 73.09, d = 0.95); Abz-AGLA-Nba (2-Aminobenzoyl-L-alanylglycyl-L-leucyl-L-alanine-4-nitrobenzylamide, f.w. 583.65; Bachem # H-6675, VWR catalog # 100040-598); SBG1% ("Super Broth with Glucose"; 6 g Soytone [Difco], 3 g yeast extract, 6 g NaCl, 6 g glucose); the pH was adjusted to 7.1 with NaOH prior to sterilization using methods known in the art; w/v (weight to volume); v/v (volume to volume); Npr and npr (neutral metalloprotease); SEQUEST® (SEQUEST database search program, University of Washington); *Npr* and *npr* (neutral metalloprotease gene); nprE and NprE (*B. amyloliquefaciens* neutral metalloprotease); PMN (purified MULTIFECT® metalloprotease); MS (mass spectroscopy); SRI (Stain Removal Index); TIGR (The Institute for Genomic Research, Rockville, MD); AATCC (American Association of Textile and Coloring Chemists); Amersham (Amersham Life Science, Inc., Arlington Heights, IL); Coming (Coming International, Coming, NY); ICN (ICN Pharmaceuticals, Inc., Costa Mesa, CA); Pierce (Pierce Biotechnology, Rockford, IL); Equest (Equest, Warwick International Group, Inc., Flintshire, UK); EMPA (Eidgenossische Material Prüfungs und Versuch Anstalt, St. Gallen, Switzerland); CFT (Center for Test Materials, Vlaardingen, The Netherlands); Amicon (Amicon, Inc., Beverly, MA); ATCC (American Type Culture Collection, Manassas, VA); Becton Dickinson (Becton Dickinson Labware, Lincoln Park, NJ); Perkin-Elmer (Perkin-Elmer, Wellesley, MA); Rainin (Rainin Instrument, LLC, Woburn, MA); Eppendorf (Eppendorf AG, Hamburg, Germany); Waters (Waters, Inc., Milford, MA); Geneart (Geneart GmbH, Regensburg, Germany); Perseptive Biosystems (Perseptive Biosystems, Ramsey, MN); Molecular Probes (Molecular Probes, Eugene, OR); BioRad (BioRad, Richmond, CA); Clontech (CLONTECH Laboratories, Palo Alto, CA); Cargill (Cargill, Inc., Minneapolis, MN); Difco (Difco Laboratories, Detroit, MI); GIBCO BRL or Gibco BRL (Life Technologies, Inc., Gaithersburg, MD); New Brunswick (New Brunswick Scientific Company, Inc., Edison, NJ); Thermoelectron (Thermoelectron Corp., Waltham, MA); BMG (BMG Labtech, GmbH, Offenburg, Germany); Greiner (Greiner Bio-One, Kremsmuenster, Austria); Novagen (Novagen, Inc., Madison, WI); Novex (Novex, San Diego, CA); Finnzymes (Finnzymes OY, Finland) Qiagen (Qiagen, Inc., Valencia, CA); Invitrogen (Invitrogen Corp., Carlsbad, CA); Sigma (Sigma Chemical Co., St. Louis, MO); DuPont Instruments (Asheville, NY); Global Medical Instrumentation or GMI (Global Medical Instrumentation; Ramsey, MN); MJ Research (MJ Research, Waltham, MA); Infors (Infors AG, Bottmingen, Switzerland); Stratagene (Stratagene Cloning Systems, La Jolla, CA); Roche (Hoffmann La Roche, Inc., Nutley, NJ); Agilent (Agilent Technologies, Palo Alto, CA); S-Matrix (S-Matrix Corp., Eureka, CA); US Testing (United States Testing Co., Hoboken, NY); West Coast Analytical Services (West Coast Analytical Services, Inc., Santa Fe Springs, CA); Ion Beam Analysis Laboratory (Ion Beam Analysis Laboratory, The University of Surrey Ion Beam Centre (Guildford,

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UK); TOM (Terg-o-Meter); BMI (blood, milk, ink); BaChem (BaChem AG, Bubendorf, Switzerland); Molecular Devices (Molecular Devices, Inc., Sunnyvale, CA); Coming (Coming International, Coming, NY); MicroCal (Microcal, Inc., Northhampton, MA); Chemical Computing (Chemical Computing Corp., Montreal, Canada); NCBI (National Center for Biotechnology Information); Argo Bioanalytica (Argo Bioanalytica, Inc., New Jersey); Vydac (Grace Vydac, Hesperia, CA); Minolta (Konica Minolta, Ramsey, NJ); and Zeiss (Carl Zeiss, Inc., Thornwood, NY).

In these experiments, a spectrophotometer was used to measure the absorbance of the products formed after the completion of the reactions. A reflectometer was used to measure the reflectance of the swatches. Unless otherwise indicated, protein concentrations were estimated by Coomassie Plus (Pierce), using BSA as the standard.

The following assays were used in the Examples described below.

A. BRADFORD ASSAY FOR PROTEIN CONTENT DETERMINATION IN 96-WELL MICROTITER PLATES (MTPS)

In these assays, the Bradford dye reagent (Quick Start) assay was used to determine the protein concentration in NprE protease samples on MTP scale.

In this assay system, the chemical and reagent solutions used were:

Quick Start Bradford Dye Reagent

BIO-RAD, #500-0205

Dilution buffer

10mM NaCl, 0.1mM CaCl₂, 0.005% TWEEN®-80

The equipment used was a Biomek FX Robot (Beckman) and a SpectraMAX (type 340) MTP reader; the MTPs were from Costar (type 9017).

In the test, 200 µl Bradford Dye Reagent was pipetted into each well, followed by 15 µl dilution buffer. Finally 10 µl of filtered culture broth were added to the wells. After thorough mixing, the MTPs were incubated for at least 10 minutes at room temperature. Possible air bubbles were blown away and the ODs of the wells were read at 595 nm.

To determine the protein concentration, the background reading (*i.e.*, from uninoculated wells) was subtracted from the sample readings. The obtained OD₅₉₅ values provide a relative measure of the protein content in the samples. The linearity of the NprE calibration lines between 0 to 5 µg enabled the use of OD₅₉₅ nm values as a relative measure for the protein content. As the expected content of NprE in supernatant was 200-300 µg/ml, the 10µl sample volume used in the test contains less than 5 µg protein, providing values in the linear range.

B. MICROSWATCH ASSAY FOR TESTING PROTEASE PERFORMANCE

The detergents used in this assay did not contain enzymes. The equipment used was a Biomek FX Robot (Beckman) and a SpectraMAX (type 340) MTP reader; the MTPs were from Costar (type 9017).

DETERGENT PREPARATION (COLD WATER LIQUID DETERGENT; US CONDITIONS):

Milli-Q water was adjusted to 6 gpg water hardness (Ca/Mg=3/1), and 0.78 g/l TIDE® 2007-2x detergent was added. The detergent solution was vigorously stirred for at least 15 minutes. Then, 5 mM HEPES (free acid) was added and the pH adjusted to 8.2.

MICROSWATCHES

Microswatches of ¼" circular diameter were obtained from CFT. Before cutting of the swatches, the fabric (EMPA 116) was washed with water. Two microswatches were placed in each well of a 96-well microtiter plate vertically to expose the whole surface area (*i.e.*, not flat on the bottom of the well).

5 TEST METHOD

The incubator was set to 20°C. The filtered culture broth samples were tested at an appropriate concentration by dilution with a mixture of 10 mM NaCl, 0.1 mM CaCl₂ and 0.005% TWEEN®-80 solution. The desired detergent solution was prepared as described above. Then, 190 µl of detergent solution was added to each well of the MTP, containing microswatches. To this mixture, 10 µl of the diluted enzyme solution were added (to provide a total volume of 200 µl/well). The MTP was sealed with tape and placed in the incubator for 30 minutes, with agitation at 1400 rpm. Following incubation under the appropriate conditions, 100 µl of solution from each well were removed and placed into a fresh MTP. The new MTP containing 100 µl of solution/well was read at 405 nm in a MTP reader. Blank controls, as well as a control containing two microswatches and detergent but no enzyme were also included.

15 CALCULATION OF THE BMI PERFORMANCE:

The obtained absorbance value was corrected for the blank value (*i.e.*, obtained after incubation of microswatches in the absence of enzyme). The resulting absorbance was a measure for the hydrolytic activity. For each sample (*e.g.*, nprE or variant) the performance index was calculated. The performance index compared the performance of the variant (actual value) and the standard enzyme (theoretical value) at the same protein concentration. In addition, the theoretical values were calculated, using the parameters of the Langmuir equation of the standard enzyme. A performance index (PI) that is greater than 1 (PI>1) identified a better variant (as compared to the standard [*e.g.*, wild-type]), while a PI of 1 (PI=1) identified a variant that performed the same as the standard, and a PI that is less than 1 (PI<1) identified a variant that performed worse than the standard. Thus, the PI identified winners, as well as variants that are less desirable for use under certain circumstances.

C. CITRATE STABILITY ASSAY FOR NPPE PROTEASE.

Citrate stability was measured after incubation of wild-type NprE and variants in the presence of 50 mM citrate. The initial and residual activity was determined using the DMC hydrolysis assay. In this assay system, the chemical and reagent solutions used were:

Citric acid monohydrate	Merck 1.00244
Pipes (free acid)	Sigma P-1851
Tris (free acid)	Sigma T-1378
HEPES (Ultra>99.5%)	Sigma-H7523
TWEEN®-80	Sigma P-8074
Dimethylcasein(DMC)	Sigma C-9801
Tris buffer (free acid)	6.04 g dissolved in 1000 ml water (= 50 mM)
HEPES buffer	11.9 g. dissolved in 1000 ml water (= 50 mM)

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Citrate buffer (free acid)	21.0 g. dissolved in 1000 ml water (= 100 mM),
PIPES buffer (free acid):	3.32 g dissolved in about 960 ml water,
DMC solution	1% w/v in 55 mM PIPES buffer, final pH = 6.0
Dilution buffer 1	0.1 mM CaCl ₂ /25 mM Tris; pH 8.2
Dilution buffer 2	0.1 mM CaCl ₂ /50 mM Citrate/25 mM Tris; pH 8.2

The concentrations of these dilution buffers are indicated as final concentrations. The initial concentration was proportionally higher and dependent on the dilution rate. The initial concentration was proportionally higher and dependent on the dilution rate. In alternative experiments, HEPES finds use in exchange for Tris. The equipment used was a Biomek FX Robot (Beckman), and an incubator/shaker (Innova, type 4230; New Brunswick). The PIPES buffer was adjusted to pH 5.8 with 4 N HCl (final concentration of 55 mM). The Tris buffer was adjusted to pH 8.2 with 4 N HCl (final concentration of 25 mM). The 50 mM citrate/25 mM Tris buffer was adjusted to pH 8.2 with 4 N NaOH. The HEPES buffer was adjusted to pH 8.2 with 4 N NaOH (final concentration of 25 mM). The 50 mM citrate/25 mM HEPES buffer was adjusted to pH 8.2 with 4 N NaOH.

10 PROTEIN DETERMINATION

In order to establish the desired dilution rate in the citrate stability assay the protease concentration of the wild-type NprE controls for each plate were determined with the TCA assay. In this method, 25 µl filtered culture broth were added to 200 µl 16.875% (w/v) TCA. After incubation for 10 to 15 minutes at ambient temperature, the light scattering/absorbance at 405 nm was determined. The protein concentration was determined by using a calibration line, constructed with purified NprE.

TEST METHOD

The dilution rate of the filtered culture broth was determined using the TCA assay, as described above.

STRESSED CONDITIONS:

The filtered culture broth was diluted with dilution buffer 2. The MTP was covered with tape, shaken for a few seconds and placed in the incubator at 25°C for 60 minutes at 200 rpm. After incubation, 20 µl of the mixture were taken from each well and transferred into a new MTP, containing 180 µl 1% DMC preheated substrate solution (the substrate was preheated at 25°C). The MTP was placed directly in the incubator/shaker and incubated at 25°C for 30 minutes at 200 rpm agitation. The residual protease activity was determined using the dimethylcasein hydrolysis assay, described below.

UNSTRESSED CONDITIONS

The filtered culture broth was diluted with dilution buffer 1. Immediately, 20 µl of the mixture were taken from each well and transferred into a new MTP, containing 180 µl of preheated 1% DMC substrate solution (the substrate was preheated at 25°C). The MTP was placed directly in the incubator/shaker and incubated for 25°C for 30 minutes at 200 rpm agitation. The initial protease activity as determined with TNBS, using the dimethylcasein hydrolysis assay, described below.

All residual activity values (determined with the dimethylcasein hydrolysis assay) were calculated using the following equation.

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$$\% \text{ Residual Activity} = \text{OD}_{60 \text{ min value}} * 100 / \text{OD}_{00 \text{ min value}}$$

D. DIMETHYLCASEIN HYDROLYSIS ASSAY

In this assay system, the chemicals and reagent solutions used were:

Dimethylcasein (DMC)	Sigma C-9801
TWEEN®-80	Sigma P-8074
PIPES buffer (free acid)	Sigma P-1851; 15.1 g dissolved in about 960 ml
	water; pH adjusted to 6.0 with 4N NaOH, 1 ml of
	5% TWEEN®-80 added and the volume
brought	up to 1000 ml. Final concentration
of PIPES and	TWEEN®-80: 50 mM and 0.005%
respectively.	
Picrylsulfonic acid (TNBS)	Sigma P-2297 (5% solution in water)
Reagent A	45.4 g Na ₂ B ₄ O ₇ ·10 H ₂ O (Merck 6308) and
	15 ml of 4N NaOH dissolved together to a
final	volume of 1000 ml (by heating if
needed)	
Reagent B	35.2 g NaH ₂ PO ₄ ·1H ₂ O (Merck 6346) and 0.6 g
	Na ₂ SO ₃ (Merck 6657) dissolved together to a
	final volume of 1000 ml.

METHOD

- 5 To prepare the substrate, 4 g dimethylcasein was dissolved in 400 ml PIPES buffer. The filtered culture supernatants were diluted with PIPES buffer. Then, 10 µl of each diluted supernatant were added to 200 µl substrate in the wells of a MTP. The MTP was covered with tape, shaken for a few seconds and placed in an oven at 25°C for 30 minutes without agitation. About 15 minutes before removal of the 1st plate from the oven, the TNBS reagent was prepared by mixing 1 ml TNBS solution per 50 ml of Reagent A. MTPs
- 10 were filled with 60 µl TNBS Reagent A per well. The incubated plates were shaken for a few seconds, after which 10 µl was transferred to the MTPs with TNBS Reagent A. The plates were covered with tape and shaken for 20 minutes in a bench shaker (BMG ThermoStar) at room temperature and 500 rpm. Finally, 200 µl Reagent B was added to the wells, mixed for 1 minute on a shaker, and the absorbance at 405 nm was determined using a MTP reader.
- 15 The obtained absorbance value was corrected for the blank value (*i.e.*, substrate without enzyme). The resulting absorbance was a measure of the hydrolytic activity. The (arbitrary) specific activity of a sample was calculated by dividing the absorbance and the determined protein concentration.

E. TIDE® STABILITY ASSAY

- The stability of NprE and variants was measured after an incubation step in the presence of 25% TIDE® compact detergent. The initial and residual activity was determined using the AGLA-assay described below. The equipment used was a Biomek FX Robot (Beckman), a fluorescence meter (FLUOstar Optima; BMG), an incubator/shaker (iEMS; Thermoelectron) and an incubator/shaker (Innova; New Brunswick (type 4230)); the MTPs were from Costar (type 9017) and from Greiner (black plates, type 655076).

CHEMICALS AND REAGENTS:

In this assay system, the chemical and reagent solutions used were:

TIDE®-compact detergent	With and without DTPA
TIDE®-compact detergent solution	125 g TIDE®-compact dissolved in a mixture of 50 g of 50 mM HEPES pH 8.2 and 275 ml water;
	concentration of TIDE® was 27.7%,
after dilution	with supernatant 25 %
MES dilution buffer	52.6 mM MES/NaOH, 2.6 mM CaCl ₂ , 0.005% TWEEN®-80, pH 6.5
AGLA substrate	BaChem, cat no. H-6675 or American Peptide Company, cat no. 81-0-31
AGLA substrate solution	451 mg of AGLA dissolved in 16 ml N,N-dimethylformamide; this solution was
poured into	304 ml of MES-buffer
(52.6 mM MES/NaOH, 2.6 mM CaCl ₂ , 0.005% TWEEN®-80, pH 6.5) with	
stirring	

TEST METHOD:

10 UNSTRESSED CONDITIONS:

First, 20 µl filtered culture broth was diluted with 180 µl MES dilution buffer. Then, 20 µl of this diluted broth was diluted with 180 µl MES dilution buffer. Then, 10 µl of this dilution was diluted with 190 µl AGLA-substrate solution in a pre-warmed plate at 25°C. Any air bubbles present were blown away and the plate was measured according to the AGLA protease assay protocol.

15 STRESSED CONDITIONS:

First, 20 µl filtered culture broth was diluted with 180 µl TIDE®-compact detergent solution without DTPA and after premixing in the iEMS shaker for 5 minutes, were incubated further in the Innova shaker. The plate was incubated for a total of 60 minutes at 32°C, at 200 rpm. In addition, 20 µl filtered culture broth were diluted with 180 µl TIDE®-compact detergent solution with DTPA and after premixing in the iEMS

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shaker for 5 minutes, were incubated further in the Innova shaker. The plate was incubated for a total of 40 minutes at 20°C, at 200 rpm. Then, 20 µl of either of these solutions were diluted with 180 µl MES dilution buffer and 10 µl of this dilution were diluted with 190 µl AGLA-substrate solution in a pre-warmed plate at 25°C. Any air bubbles present were blown away and the plate was measured according to the AGLA protease assay protocol.

CALCULATIONS:

Fluorescence measurements were taken at excitation of 350 nm and emission of 415 nm. The spectrofluorometer software calculated the reaction rates of the increase in fluorescence for each well to a linearly regressed line of milli-RFU / min:

$$\text{Percentage of residual activity: } \frac{(\text{Slope of stressed condition}) * 100}{(\text{Slope of unstressed condition})}$$

F. 2-AMINOBENZOYL-L-ALANYLGLYCYL-L-LEUCYL-L-ALANINO-4-NITROBENZYLAMIDE PROTEASE ASSAY (ABZ-AGLA-NBA)

The method provided below provides a degree of technical detail that yields reproducible protease assay data independent of time and place. While the assay can be adapted to a given laboratory condition, any data obtained through a modified procedure must be reconciled with results produced by the original method. Neutral metalloproteases cleave the peptide bond between glycine and leucine of 2-aminobenzoyl-L-alanylglycyl-L-leucyl-L-alanino-4-nitrobenzylamide (Abz-AGLA-Nba). Free 2-aminobenzoyl-L-alanylglycine (Abz-AG) in solution has a fluorescence emission maximum at 415 nm with an excitation maximum of 340 nm. Fluorescence of Abz-AG is quenched by nitrobenzylamide in the intact Abz-AGLA-Nba molecule.

In these experiments, the liberation of Abz-AG by protease cleavage of Abz-AGLA-Nba was monitored by fluorescence spectroscopy (Ex. 340 / Em. 415). The rate of appearance of Abz-AG was a measure of proteolytic activity. Assays were performed under non-substrate limited initial rate conditions.

A microplate mixer with temperature control (*e.g.*, Eppendorf Thermomixer) was required for reproducible assay results. The assay solutions were incubated to desired temperature (*e.g.*, 25°C) in the microplate mixer prior to enzyme addition. Enzyme solutions were added to the plate in the mixer, mixed vigorously and rapidly transferred to the plate reader.

A spectrofluorometer with capability of continuous data recording, linear regression analysis, and with temperature control was required (*e.g.*, SpectraMax M5, Gemini EM, Molecular Devices). The reader was always maintained at the desired temperature (*e.g.*, 25°C). The reader was set for top-read fluorescence detection and the excitation was set to 350 nm and emission to 415 nm without the use of a cut-off filter. The PMT was set to medium sensitivity and 5 readings per well. Autocalibration was turned on, but only to calibrate before the first reading. The assay was measured for 3 minutes with the reading interval minimized according to the number of wells selected to be monitored. The reader was set to calculate the rate of milli-RFU/min (thousandths of relative fluorescence units per minute). The number of readings used to calculate the rate (Vmax points) was set to the number equivalent to 2 minutes, as determined by the reading interval (*e.g.*, a reading every 10 seconds would use 12 points to calculate the rate). The max RFU was set to 50,000.

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All pipeting of enzyme and substrate stock solutions were done with positive displacement pipets (Rainin Microman). Buffer, assay, and enzyme working solutions were pipetted by single or multi-channel air-displacement pipets (Rainin LTS) from tubes, reagent reservoirs or stock microplates. A repeater pipet (Eppendorf) finds use in transferring the assay solution to microplate wells when few wells are used, to minimize reagent loss. Automated pipetting instruments such as the Beckman FX or Cybio Cybi-well also find use in transferring enzyme solutions from a working stock microplate to the assay microplate in order to initiate an entire microplate at once.

REAGENTS AND SOLUTIONS:

52.6 mM MES/NaOH, 2.6 mM CaCl₂, pH 6.5 - MES Buffer

MES acid (10.28 g) and 292 mg anhydrous CaCl₂ were dissolved in approximately 900mL purified water. The solution was titrated with NaOH to pH 6.5 (at 25°C or with temperature adjustment pH probe). The pH-adjusted buffer was made up to 1L total volume. The final solution was filtered through a 0.22 µm sterile filter and kept at room temperature.

48 mM Abz-AGLA-Nba in DMF - Abz-AGLA-Nba Stock

Approximately 28 mg of Abz-AGLA-Nba was placed in a small tube. It was dissolved in mL of DMF (volume will vary depending upon Abz-AGLA-Nba massed) and vortexed for several minutes. The solution was stored at room temperature shielded from light.

50 mM MES, 2.5 mM CaCl₂, 5% DMF, 2.4 mM Abz-AGLA-Nba pH 6.5 - Assay Solution

One mL Abz-AGLA-Nba stock was added to 19 mL MES Buffer and vortexed. The solution was stored at room temperature shielded from light.

50 mM MES, 2.5 mM CaCl₂, pH 6.5 - Enzyme Dilution Buffer

This buffer was produced by adding 5 mL purified water to 95 mL MES Buffer.

50 mM MES, 2.5 mM CaCl₂, 5% DMF, pH 6.5 - Substrate Dilution Buffer

Five mL pure DMF were added to 95 mL MES Buffer. This buffer was used to determine kinetic parameters.

ENZYME SOLUTIONS

The enzyme stock solutions were diluted with enzyme dilution buffer to a concentration of approximately 1 ppm (1 µg/mL). MULTIFECT® neutral protease (wild-type NprE) was diluted to concentrations below 6 ppm (6 µg/mL). Serial dilutions were preferred. Solutions were stable at room temperature for 1 hour, but for longer term storage, the solutions were maintained on ice.

PROCEDURE

First all buffers, stock, and working solutions were prepared. Each enzyme dilution was assayed in triplicate, unless otherwise indicated. When not completely full, the enzyme working solution stock microplate was arranged in full vertical columns starting from the left of the plate (to accommodate the plate reader). The corresponding assay plate was similarly set up. The microplate spectrofluorometer was set up as previously described.

First, a 200 µL aliquot of assay solution were placed in the wells of a 96-well microplate. The plate was incubated for 10 min at 25°C in a temperature controlled microplate mixer, shielded from light. The assay was initiated by transferring 10 µL of the working enzyme solutions from the stock microplate to the

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assay microplate in the mixer. Optimally, 96-well pipetting head finds use, or an 8-well multi-channel pipet was used to transfer from the left-most column first. The solutions were vigorously mixed for 15 seconds (900rpm in Eppendorf Thermomixer). Immediately, the assay microplate was transferred to the microplate spectrofluorometer and recording of fluorescence measurements at excitation of 350 nm and emission of 415 nm were begun. The spectrofluorometer software calculated the reaction rates of the increase in fluorescence for each well to a linearly regressed line of milli-RFU / min. In some experiments, a second plate was placed in the microplate mixer for temperature equilibration while the first plate was being read.

The rate initial velocities were linear with respect to product concentration (i.e., liberated 2-aminobenzoyl fluorescence) up to 0.3 mM product, which corresponded to approximately 50,000 RFU in a solution starting at 2.3mM Abz-AGLA-Nba with background fluorescence of approximately 22,000 RFU. Abz-AGLA-Nba was dissolved in DMF and was been used the day it was prepared.

DETERGENT COMPOSITIONS:

In the exemplified detergent compositions, the enzymes levels are expressed by pure enzyme by weight of the total composition and unless otherwise specified, the detergent ingredients are expressed by weight of the total compositions. The abbreviated component identifications therein have the following meanings:

Abbreviation	Ingredient
LAS	: Sodium linear C ₁₁₋₁₃ alkyl benzene sulfonate.
NaC16-17HSAS	: Sodium C ₁₆₋₁₇ highly soluble alkyl sulfate
TAS	: Sodium tallow alkyl sulphate.
CxyAS	: Sodium C _{1x} - C _{1y} alkyl sulfate.
CxyEz	: C _{1x} - C _{1y} predominantly linear primary alcohol condensed with an average of z moles of ethylene oxide.
CxyAEzS	: C _{1x} - C _{1y} sodium alkyl sulfate condensed with an average of z moles of ethylene oxide. Added molecule name in the examples.
Nonionic	: Mixed ethoxylated/propoxylated fatty alcohol e.g. Plurafac LF404 being an alcohol with an average degree of ethoxylation of 3.8 and an average degree of propoxylation of 4.5.
QAS	: R ₂ .N+(CH ₃) ₂ (C ₂ H ₄ OH) with R ₂ = C ₁₂ -C ₁₄ .
Silicate	: Amorphous Sodium Silicate (SiO ₂ :Na ₂ O ratio = 1.6-3.2:1).
Metasilicate	: Sodium metasilicate (SiO ₂ :Na ₂ O ratio = 1.0).
Zeolite A	: Hydrated Aluminosilicate of formula Na ₁₂ (AlO ₂ SiO ₂) ₁₂ . 27H ₂ O
SKS-6	: Crystalline layered silicate of formula δ-Na ₂ Si ₂ O ₅ .
Sulfate	: Anhydrous sodium sulphate.
STPP	: Sodium Tripolyphosphate.

MA/AA	: Random copolymer of 4:1 acrylate/maleate, average molecular weight about 70,000-80,000.
AA	: Sodium polyacrylate polymer of average molecular weight 4,500.
Polycarboxylate	: Copolymer comprising mixture of carboxylated monomers such as acrylate, maleate and methacrylate with a MW ranging between 2,000-80,000 such as Sokolan commercially available from BASF, being a copolymer of acrylic acid, MW4,500.
BB1	: 3-(3,4-Dihydroisoquinolinium)propane sulfonate
BB2	: 1-(3,4-dihydroisoquinolinium)-decane-2-sulfate
PB 1	: Sodium perborate monohydrate.
PB4	: Sodium perborate tetrahydrate of nominal formula $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$.
Percarbonate	: Sodium percarbonate of nominal formula $2\text{Na}_2\text{CO}_3 \cdot 3\text{H}_2\text{O}_2$.
TAED	: Tetraacetyl ethylene diamine.
NOBS	: Nonanoyloxybenzene sulfonate in the form of the sodium salt.
DTPA	: Diethylene triamine pentaacetic acid.
HEDP	: 1,1-hydroxyethane diphosphonic acid.
DETPMP	: Diethyltriamine penta (methylene) phosphonate, marketed by Monsanto under the Trade name Dequest 2060.
EDDS	: Ethylenediamine-N,N'-disuccinic acid, (S,S) isomer in the form of its sodium salt
Diamine	: Dimethyl aminopropyl amine; 1,6-hexane diamine; 1,3-propane diamine; 2-methyl-1,5-pentane diamine; 1,3-pentanediamine; 1-methyl-diaminopropane.
DETBCHD	: 5, 12- diethyl-1,5,8,12-tetraazabicyclo [6,6,2] hexadecane, dichloride, Mn(II) SALT
PAAC	: Pentaamine acetate cobalt(III) salt.
Paraffin	: Paraffin oil sold under the tradename Winog 70 by Wintershall.
Paraffin Sulfonate	: A Paraffin oil or wax in which some of the hydrogen atoms have been replaced by sulfonate groups.
Aldose oxidase	: Oxidase enzyme sold under the tradename Aldose Oxidase by Novozymes A/S
Galactose oxidase	: Galactose oxidase from Sigma
nprE	: The recombinant form of neutral metalloprotease expressed in <i>Bacillus subtilis</i> .
PMN	: Purified neutral metalloprotease from <i>Bacillus amyloliquefaciens</i> .
Amylase	: Amylolytic enzyme sold under the tradename PURAFECT® Ox described in WO 94/18314, WO96/05295 sold by Genencor; NATALASE®, TERMAMYL®, FUNGAMYL® and DURAMYL™, all available from Novozymes A/S.

Lipase	: Lipolytic enzyme sold under the tradename LIPOLASE®, LIPOLASE® Ultra by Novozymes A/S and Lipomax™ by Gist-Brocades.
Cellulase	: Cellulytic enzyme sold under the tradename Carezyme, Celluzyme and/or Endolase by Novozymes A/S.
Pectin Lyase	: PECTAWAY® and PECTAWASH® available from Novozymes A/S.
PVP	: Polyvinylpyrrolidone with an average molecular weight of 60,000
PVNO	: Polyvinylpyridine-N-Oxide, with an average molecular weight of 50,000.
PVPVI	: Copolymer of vinylimidazole and vinylpyrrolidone, with an average molecular weight of 20,000.
Brightener 1	: Disodium 4,4'-bis(2-sulphostyryl)biphenyl.
Silicone antifoam	: Polydimethylsiloxane foam controller with siloxane-oxyalkylene copolymer as dispersing agent with a ratio of said foam controller to said dispersing agent of 10:1 to 100:1.
Suds Suppressor	: 12% Silicone/silica, 18% stearyl alcohol, 70% starch in granular form.
SRP 1	: Anionically end capped poly esters.
PEG X	: Polyethylene glycol, of a molecular weight of x.
PVP K60 ®	: Vinylpyrrolidone homopolymer (average MW 160,000)
Jeffamine ® ED-2001	: Capped polyethylene glycol from Huntsman
Isachem ® AS	: A branched alcohol alkyl sulphate from Enichem
MME PEG (2000)	: Monomethyl ether polyethylene glycol (MW 2000) from Fluka Chemie AG.
DC3225C	: Silicone suds suppresser, mixture of Silicone oil and Silica from Dow Coming.
TEPAE	: Tetraethylenepentaamine ethoxylate.
BTA	: Benzotriazole.
Betaine	: $(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{COO}^-$
Sugar	: Industry grade D-glucose or food grade sugar
CFAA	: $\text{C}_{12}\text{-C}_{14}$ alkyl N-methyl glucamide
TPKFA	: $\text{C}_{12}\text{-C}_{14}$ topped whole cut fatty acids.
Clay	: A hydrated aluminum silicate in a general formula $\text{Al}_2\text{O}_3\text{SiO}_2 \cdot x\text{H}_2\text{O}$. Types: Kaolinite, montmorillonite, atapulgite, illite, bentonite, halloysite.
pH	: Measured as a 1% solution in distilled water at 20°C.

EXAMPLE 1

CLONING OF THE NEUTRAL METALLOPROTEASE GENE FROM *B. AMYLOLIQUEFACIENS*.

In this Example, methods used to clone the *B. amyloliquefaciens* neutral metalloprotease gene are described. The gene-encoding neutral metalloprotease was cloned from *B. amyloliquefaciens* using well-established methods in this art. The non-exempt (i.e., the strain carries extragenomic DNA (besides the chloramphenicol selectable marker which is allowed in an exempt strain), specifically the plasmid pJM102 sequences) strain BC91504 (*aprE/nprE*-pJM102 in BG3594::comK) carries the *B. subtilis* *aprE* promoter and signal sequence fused to *B. amyloliquefaciens* *nprE* propeptide/mature gene in integrating plasmid pJM102.

The following two sequences (SEQ ID NO:1 and SEQ ID NO:2) of *B. subtilis* and *B. amyloliquefaciens* were generated via PCR with the oligonucleotide primers corresponding to the underlined sequences.

B. subtilis chromosomal *EcoRI* restriction site (GAATTC) and *aprE* start codon (GTG) and *B. amyloliquefaciens* *nprE* stop codon are shown in the following sequences in boldface type as well as a synthetically introduced *HindIII* restriction site (AAGCTT) designed into primer # 4.

The *B. amyloliquefaciens* *aprE* 5' upstream sequence, promoter and signal sequence coding region are shown in the following sequence (SEQ ID NO:1). Primer 1 (*apr*-f; GAGCTGGGTAAAGCCTATGAAT; SEQ ID NO:5) is shown underlined, at the beginning of the sequence, while the *aprE* portion of primers 2 and 3 (*npr*-f and *npr*-r; GTTCAGCAACATGTCTGCGCAGGCT; SEQ ID NO:6) are shown double underlined at the end of the sequence.

GAGCTGGGTAAAGCCTATGAATTCTCCATTTTCTTCTGCTATCAAAATAACAGAC
TCGTGATTTTCCAAACGAGCTTTCAAAAAAGCCTCTGCCCCTTGCAAATCGGATG
CCTGTCTATAAAATTCCCAGATATTGGTTAAACAGCGGCGCAATGGCGGCCGCATC
TGATGTCTTTGCTTGGCGAATGTTTCATCTTATTTCTTCTCCCTCTCAATAATTTTT
TCATTCTATCCCTTTTCTGTAAAGTTTATTTTTTCAGAATACTTTTATCATCATGCTT
TGAAAAAATATCACGATAATATCCATTGTTCTCACGGAAGCACACGCAGGTCATT
TGAACGAATTTTTTCGACAGGAATTTGCCGGGACTCAGGAGCATTAACTAAAA
AAGCATGACATTTTCAGCATAATGAACATTTACTCATGTCTATTTTCGTTCTTTTCT
GTATGAAAATAGTTATTTTCGAGTCTCTACGGAATAGCGAGAGATGATATACCTA
AATAGAGATAAAATCATCTCAAAAAATGGGTCTACTAAATATTATTCATCTA
TTACAATAAAATTCACAGAATAGTCTTTTAAAGTAAGTCTACTCTGAATTTTTTTAAA
AGGAGAGGGTAAAGAGTGAGAAGCAAAAAATTGTGGATCAGCTTGTTGTTTGCG
TTAACGTTAATCTTTACGATGGCGTTCAGCAACATGTCTGCGCAGGCT (SEQ ID
NO:1)

The sequence of the *B. amyloliquefaciens* propeptide and mature *nprE* coding sequence and transcription terminator are provided in the sequence below. In this sequence, the *nprE* portion of primers 2 and 3 is underlined (GCTGAGAATCCTCAGCTTAAAGAAAACCTG; SEQ ID NO:7), while the *npr*-r portion of primer 4 (GGCTTACCATGATCATATATGTCAAGCTTGGGGGG; SEQ ID NO:8) is shown double underlined.

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GCTGAGAATCCTCAGCTTAAAGAAAACCTGACGAATTTTGTACCGAAGCATTCTT
 TGGTGCAATCAGAATTGCCTTCTGTCAAGTACAAAGCTATCAAGCAATACTTGAA
 AAAAAACGGCAAAGTCTTTAAAGGCAATCCTTCTGAAAGATTGAAGCTGATTGAC
 CAAACGACCGATGATCTCGGCTACAAGCACTTCCGTTATGTGCCTGTCGTAAACG
 GTGTGCCTGTGAAAGACTCTCAAGTCATTATTACGTCGATAAATCCAACAACGT
 CTATGCGATTAACGGTGAATTAACAACGATGTTTCCGCCAAAACGGCAAACAGC
 AAAAAATTATCTGCAAATCAGGCGCTGGATCATGCTTATAAAGCGATCGGCAAAT
 CACCTGAAGCCGTTTCTAACGGAACCGTTGCAAACAAAAACAAAGCCGAGCTGA
 AAGCAGCAGCCACAAAAGACGGCAAATACCGCCTCGCCTATGATGTAACCATCC
 GCTACATCGAACCGGAACCTGCAAACCTGGGAAGTAACCGTTGATGCGGAAACAG
 GAAAAATCCTGAAAAAGCAAACAAAGTGGAGCATGCCGCCACAACCGGAACAG
 GTACGACTCTTAAAGGAAAAACGGTCTCATTAAATATTTCTTCTGAAAGCGGCAA
 ATATGTGCTGCGCGATCTTTCTAAACCTACCGGAACACAAATTATTACGTACGAT
 CTGCAAACCGCGAGTATAACCTGCCGGGCACACTCGTATCCAGCACCACAAACC
 AGTTTACAACCTTCTTCTCAGCGCGCTGCCGTTGATGCGCATTACAACCTCGGCAAA
 GTGTATGATTATTTCTATCAGAAGTTTAATCGCAACAGCTACGACAATAAAGGCG
 GCAAGATCGTATCCTCCGTTTATTACGGCAGCAGATACAATAACGCAGCCTGGAT
 CGGCGACCAAATGATTTACGGTGACGGCGACGGTTTATTCTTCTCACCTCTTTCCG
 GTTCAATGGACGTAACCGCTCATGAAATGACACATGGCGTTACACAGGAAACAG
 CCAACCTGAACTACGAAAATCAGCCGGGCGCTTTAAACGAATCCTTCTCTGATGT
 ATTCGGGTACTTCAACGATACTGAGGACTGGGATATCGGTGAAGATATTACGGTC
 AGCCAGCCGGCTCTCCGAGCTTATCCAATCCGACAAAATACGGACAGCCTGATA
 ATTTCAAAAATTACAAAACCTTCCGAACACTGATGCCGGCGACTACGGCGGCGT
 GCATACAAACAGCGGAATCCCGAACAAGCCGCTTACAATACGATTACAAAAT
 CGGCGTGAACAAAGCGGAGCAGATTTACTATCGTGCTCTGACGGTATACCTCACT
 CCGTCATCACTTTTAAAGATGCAAAAGCCGCTTTGATTCAATCTGCGCGGGACC
 TTTACGGTCTCAAGATGCTGCAAGCGTAGAAGCTGCCTGGAATGCAGTCGGATT
 GTAAACAAGAAAAGAGACCGGAAATCCGGTCTCTTTTTTATATCTAAAAACATTT
 CACAGTGGCTTCACCATGATCATATATGTCAAGCTTGGGGGG (SEQ ID NO:2)

The amino acid sequence of the full-length NprE (pre-, pro- and mature sequence) is provided

below:

MGLGKKLSVAVAAAFMSLTISLPGVQAAENPQLKENLTNFVPKHSLVQSELPVSVDK
 AIKQYLKQNGKVKGNPSERLKLIDQTTDDLGYKHFRYPVNVNGVPVKDSQVVIHVDK
 SNNVYAINGELNNDVSAKTANSKKLSANQALDHAYKAIGKSPEAVSNGTVANKNKA
 ELKAAATKDGYRLAYDVTIRYIEPEPANWEVTVD AETGKILKKQNKVEHAATTGT
 GTTLKGKTVSLNISSESGKYVLRDL SKPTGTQIITYDLQNREYNLPGLTVSSTTNQFTT
 SSQRAAVDAHYNL GKVDYDFYQKFNRSYDNKGGKIVSSVHYGSRYNNAAWIGDQ
 MIYGDGDSFFSPLSGSMDVTAHEMTHGVTQETANLNYENQPGALNESFSDFGYF
 NDTEDWDIGEDITVSQPALRSLSNPTKYGPDPNFKNYKNLPNTDAGDYGGVHTNSGI
 PNKAA YNTITKIGVNKAEQIYYRALTVYLT PPSSTFKDAKAALIQSARDLYGSQDAASV
 EAAWNAVGL (SEQ ID NO:3)

5

In some alternative embodiments, the following NprE sequence finds use in the present invention.

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VRSKKLWISLLFALTLIFTMAFSNMSAQAAENPQLKENLTNFVPKHSLVQSELPVSVD
 KAIKQYLKQNGKVFKGNPSERLKLIDQTTDDLGYKHFRYVPVNVGVPVKDSQVIIHV
 DKSNNVYAINGELNNDVSAKTANSKKLSANQALDHAYKAIGKSPEAVSNGTVANKN
 KAEKAAAATKDGKYRLAYDVTIRYIEPEPANWEVTVDAETGKILKKQNKVEHAATT
 GTGTTLKGKTVSLNISSESGKYVLRDL SKPTGTQIITYDLQNREYNLPGLVSSTTNQF
 TTSSQRAAVDAHYNLGKVYDYFYQKFNRNSYDNKGGKIVSSVHYGSRYNNAAWIG
 DQMIYGDGDGSFFSPLSGSMDVTAHEMTHGVTQETANLNYENQPGALNESFSDVFG
 YFNDTEDWDIGEDITVSQPALRSLSNPTKYGQPDNFKNYKNLPNTDAGDYGGVHTNS
 GIPNKAAAYNTITKIGVNAEQIYYRALTVYLTPSSTFKDAKAALIQSARDLYGSQDAA
 SVEAAWNAVGL (SEQ ID NO:4)

The primer sequences used in these PCR experiments are provided below:

Primers Used in PCR Experiments		
Primer Number	Sequence	SEQ ID NO:
1	5'-GAGCTGGGTAAAGCCTATGAAT-3'	SEQ ID NO:5
2	5'-CAGGTTTCTTTAAGCTGAGGATTCTCAGC- AGCCTGCGCAGACATGTTGCTGAAC-3'	SEQ ID NO:9
3	5'-GTTCAGCAACATGTCTGCGCAGGCT- GCTGAGAATCCTCAGCTTAAAGAAAACCTG-3'	SEQ ID NO:10
4	5'-CCCCCAAGCTTGACATATATGATCATGGTGAAGCC-3'	SEQ ID NO:11

Primers 2 and 3 are reverse complements of each other and correspond to either noncoding (#2) or
 5 coding (#3) strands of the chromosomal DNAs. For the coding strand, they correspond to the last 25 base
 pairs of the *aprE* signal sequence and the first 30 base pairs of the *nprE* propeptide. Primer #4 is the reverse
 complement to the underlined sequence, comprising 24 base pairs 3' of the *nprE* stop codon and terminator
 with an introduced *Hind*III site preceded by six dCTP residues, to provide a so-called "clamp," allowing more
 efficient cleavage with *Hind*III restriction endonuclease, as some restriction enzymes cleave inefficiently if
 10 their recognition sequence is located at the very ends of DNA fragments.

The two PCR fragments were generated with the following protocol and reagents (except DNA template and oligonucleotide primers) from Applied Biosystems' rTTH DNA Polymerase, XL Kit:

40.6 µl H₂O
 30 µl 3.3x *rTth* PCR buffer
 15 10 µl 2 mM dNTP mix
 4.4 µl 25 mM Mg-acetate
 5 µl 50 µM primer # 1 or # 3 (forward primers)
 5 µl 50 µM primer # 2 or # 4 (reverse primers)
 2 µl *B. subtilis* or *B. amyloliquefaciens* chromosomal DNA
 20 2 µl *rTth* polymerase
 1 µl *Pfu* Turbo polymerase
 100 µl total reaction volume

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The PCR conditions used in these experiments were (95°C, 30 sec./58°C, 30sec/68°C, 1 min.) x 30 cycles followed by rapid cooling to 4°C. Reactions were run on 1.2% agarose/TBE preparative gels, the appropriately-sized fragments excised and purified using the QIAGEN® Gel Extraction Kit. In a second fusion, PCR reactions were conducted in which chromosomal DNAs were replaced by 1 ul each of the two
5 separate fragments and only outside primers #s 1 and #2 were used. The same PCR conditions as described above were used. Due to the complementary ends formed on the two fragments from the use of complementary primers 2 and 3 in the first PCRs, the two fragments were precisely fused.

The fusion fragment was digested with *EcoRI* and *HindIII* and gel purified as described above. The integration plasmid pJM102 was also digested with *EcoRI* and *HindIII*, and the linear plasmid was then gel
10 purified and ligated by standard techniques to the digested apr/npr fusion fragment. This ligation reaction was subsequently used to directly transform a xylose-induced *B. subtilis* strain.

After purification, the two fragments were generated by PCR with primers 1 and 2 from wild-type *B. subtilis* chromosomal DNA, and with primers 3 and 4 from chromosomal DNA from a *B. amyloliquefaciens* strain. This fragment was again purified as described above, followed by cutting with *EcoRI* and *HindIII* as in
15 the same digestion of the integrating plasmid pJM102 and subsequent ligation of the fusion fragment to the plasmid. Several transformants had the fusion sequenced from the chromosome to verify the absence of any PCR-derived mutations. One of these was then amplified stepwise from 5 - 25 mg/mL chloramphenicol, the selectable marker on pJM102, to co-amplify the linked expression cassette.

The selected sequence verified transformant was obtained by selection for pJM102's
20 chloramphenicol (CMP) resistance marker on LB/agar plates containing 5 mg/ml CMP. This was then inoculated into LB broth at 10 mg/ml CMP overnight at 37°C, with shaking at 250 RPM. This culture was then streaked onto LB/agar plates with 10 mg/ml CMP to isolate single colonies. One colony was then inoculated into LB broth at 25 mg/ml CMP overnight at 37°C, with shaking at 250 RPM. This culture was then streaked to LB/agar plates with 25 mg/ml CMP to isolate single colonies. These colonies were harvested
25 and stored in glycerol at -70°C until use, as known in the art.

The deletion of the two non-essential proteases present in *B. subtilis* (aprE and nprE), as well as amylase, reduced the total extracellular protease level during the production of metalloprotease. The DNA encoding the neutral metalloprotease was cloned into an amylase-deleted host. The inducible comK for competence development was inserted in the middle of the amylase locus, making the strain "amy." The
30 secretion of the expressed protein was ensured by insertion of the nucleotides encoding the signal sequence prior to the coding sequence of the gene.

EXAMPLE 2

EXPRESSION AND FERMENTATION OF THE PURIFIED MULTIFECT® NEUTRAL AND RECOMBINANT NEUTRAL METALLOPROTEASE (NPPE).

The recombinant *Bacillus subtilis* produced as described in Example 1 was cultivated by
35 conventional batch fermentation in a nutrient medium as described below. One glycerol vial (prepared as described in Example 1) of *B. subtilis* culture containing the *B. amyloliquefaciens* neutral metalloprotease was used to inoculate 600 ml of SBG1% medium containing 200 mg/L chloramphenicol. The cultures were grown for 48 hours at 37°C, after which time, the culture fluid was recovered by centrifugation at 12,000

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rpm, as known in the art. This procedure was done in duplicate. The final enzyme concentrations obtained were in the range of about 1.4 and 2 g/L.

EXAMPLE 3

PURIFICATION AND CHARACTERIZATION OF NEUTRAL METALLOPROTEASE

5 This Example describes the methods used to purify the neutral metalloprotease expressed by the organisms described in Example 2. After 36 hours of incubation at 37 °C, the fermentation broth was recovered and centrifuged at 12 000 rpm (SORVALL® centrifuge model RC5B). The secreted neutral metalloproteases were isolated from the culture fluid and concentrated approximately 10-fold using an Amicon filter system 8400 with a BES (polyethersulfone) 10kDa cutoff.

10 The concentrated supernatant was dialyzed overnight at 4 °C against 25 mM MES buffer, pH 5.4, containing 10 mM NaCl. The dialysate was then loaded onto a cation-exchange column Porous HS20 (total volume ~ 83 mL; binding capacity ~ 4.5g protein/mL column; Waters) as described below. The column was pre-equilibrated with 25 mM MES buffer, pH 5.4, containing 10 mM NaCl. Then, approximately 200-300 mL of sample was loaded onto the column. The bound protein was eluted using a pH gradient from 5.4 to 6.2
15 over 10-column volumes of MES buffer. Elution of the protein was between pH 5.82 and 6.0, and was assessed using proteolytic activity as described herein and 10 % (w/v) NUPAGE® SDS-PAGE (Novex). The neutral protease containing fractions were then pooled. Calcium and zinc chloride salts in the ratio of 3:1 were added prior to the adjustment of the pH to 5.8. The Perceptive Biosystems BIOCAD® Vision (GMI) was used for protein purification.

20 The purified protein, assessed using a 10 % (w/v) NUPAGE® SDS-PAGE, was determined to homogenous, with greater than 95 % purity. Typically, less than 1% of the purified preparations showed serine protease activity when assessed using the standard protease assay with the small substrate, suc-p-AAPF-pNA (N-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide) (Sigma). This assay was performed in microtiter plate format (96 well) using a 100 mM Tris-HCl buffer, pH 8.5, containing 10 mM CaCl₂ and
25 0.005 % TWEEN®-80. The substrate (p-AAPF NA) was prepared by making a 160 mM stock in DMSO (dimethylsulfoxide) (100 mg/ml) and diluting this stock 100-fold with the Tris-HCl buffer containing CaCl₂ and 0.005 % TWEEN®-80. Then, 10 uL of diluted protease solution (dilutions were prepared using 100 mM Tris-HCl buffer, pH 8.5, containing 10 mM CaCl₂ and 0.005 % TWEEN-80) was added to 190 uL 1mg/ml p-AAPF solution. The assay was mixed for 5 minutes and the kinetic change at 410 nm was read over 2 to 5
30 minutes. The slope of the response was measured and used as an indication of the amount of serine protease, activity. The protein was formulated for storage using 25 mM MES buffer, pH 5.8, containing 1 mM zinc chloride, 4 mM calcium chloride, and 40 % propylene glycol.

EXAMPLE 4

AFFINITY OF PURIFIED MULTIFECT® NEUTRAL METALLOPROTEASE (PMN) FOR CALCIUM 35 AND ZINC CATIONS

In this Example, methods to determine the affinity of the neutral metalloprotease (PMN) prepared as described in the above Examples are described. The affinities of PMN for calcium and zinc ions were performed using the fluorescent indicators Fluo-3 and FluoZin-3, respectively obtained from Molecular Probes. All fluorescence measurements were recorded on a LS50B Luminescence spectrophotometer (Perkin-
40 Elmer). The binding of Fluo-3 was monitored by excitation at 500 nm and the emission spectra were recorded

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from 505 to 550 nm. Similarly, the binding of FluoZin-3 was monitored by excitation at 495 nm and the emission spectra were collected from 500 to 550 nm. The excitation and emission slit width were both set at 2.5 nm.

In these determinations, 100 μ M neutral metalloprotease in 50 mM Tris-HCl buffer, pH 8.4, was
 5 titrated with increasing amounts of the relevant indicator. The titration curves are shown in Figure 1. In this
 Figure, the triangles represent the curve binding data obtained for Zn^{2+} , using the Fluo-Zin3 dye monitored at
 516 nm, while the circles represent the data obtained for Ca^{2+} using the Fluo-3 dye monitored at 522 nm. The
 association constants (K_a 's) for zinc and calcium (assuming a single binding site) were determined to be
 0.401 nM and 0.037 nM, respectively. These results indicate that purified MULTIFECT® neutral
 10 metalloprotease bound the zinc ion with approximately 10-fold greater affinity than the calcium ion. Based on
 the weaker binding of calcium, initial protein engineering experiments are designed to involve either (i)
 designing tighter calcium binding site(s) and/or (ii) eliminating the structural stability requirement for
 calcium (*e.g.*, to stabilize the protein to greater than 80 %).

EXAMPLE 5

15 STORAGE STABILITY

In this Example, experiments conducted to assess the storage stability of PMN and recombinant *B.*
amyloliquefaciens neutral metalloprotease expressed in *B. subtilis* are described. Proteolysis of these neutral
 metalloprotease preparations was assessed in the presence of increasing LAS (lauryl sodium sulfate; Sigma)
 solutions (0 % up to an including 10 %). Proteolytic fragments generated from the purified MULTIFECT®
 20 neutral metalloprotease (PMN) were observed using 10 % (w/v) NUPAGE® SDS-PAGE.

The storage stability of the recombinant neutral metalloprotease from *B. amyloliquefaciens*
 expressed in *B. subtilis* produced as described above, was determined in buffer alone (50 mM Tris-HCl
 buffer, pH 8.4) and in the presence of detergent base obtained from Procter & Gamble. The buffer and/or
 detergent base contained zinc ions, calcium ions or a combination thereof. The concentration of both the zinc
 25 and calcium ions was varied from 0 to 25 mM. These results were always compared with those for the neutral
 metalloprotease incubated in buffer alone.

PROTEASE ASSAYS

AZO-CASEIN ASSAY:

The azo-casein endpoint assay was used to assess the amount of proteolysis that occurred under
 30 certain conditions. In these assays, 75 μ L of enzyme were incubated with excess calcium or zinc or both ions
 added to 250 μ L of 1 % (w/v) azo-casein (Sigma). The reaction proceeded at 30 °C for 15 minutes, after which
 10 % (w/v) trichloroacetic acid was added to stop the reaction. The precipitated protein and the unreacted
 azo-casein were removed by centrifugation for 10 minutes at 14 000 rpm. The color of the azo-group was
 developed by addition of 750 μ L 1 M sodium hydroxide. The development of the color proceeded for 5
 35 minutes, after which the reaction was stopped and the absorbance was measured at 440 nm.

SUCCINYLATED-CASEIN AND TNBSA ASSAY:

The activity of the neutral metalloprotease was determined using the QuantiCleave Protease Assay
 Kit™ (Pierce). This assay is based on the digestion of succinylated-casein by the enzyme. The primary amino
 groups formed are then reacted with trinitrobenzene sulfonic acid (TNBSA) and form a colored complex that

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has maximum absorbance at 450 nm. The assay is performed in 96-well microtiter format. The assay requires a 15-minute incubation with the succinylated casein and a 15-minute reaction with the TNBSA. During both incubations, the samples are placed on a shaker. TPCK-trypsin (Pierce) is the general standard used for overall protease activity determinations. However, optimum conditions for activity for specific proteases require the use of the protease of interest. In the case of the assays performed in these experiments, both trypsin and the protease of interest were used, in order to calibrate the assay. The accuracy of the assay requires that the standard dilutions made of 0.5 mg/mL trypsin always result in absorbance values (at 450 nm) below 0.5.

Every sample was measured relative to a control containing no casein. The reported change in absorbance ($\Delta\text{Abs}(450\text{ nm})$) accounts for the interference from the amino groups of casein. Further, any possible interference from primary amino groups in the buffer and/or other components of the detergent was/were also corrected for in this manner. The activity of all samples was determined relative to detergent with no added neutral metalloprotease, as well as for enzyme incubated in BupH™ borate buffer supplied with the kit, for the same length of time and at the same temperature.

This test is an end-point assay, in which 50 mM borate buffer, pH 8.5, was used at 32 °C. The protease assays were typically performed in duplicate. In most experiments to determine stability measurements, the protein and detergent were diluted using the above-mentioned buffer by 1:1000, although in some experiments dilutions of were also 1:500 or 1: 200, in order to obtain readings where the absorbance of the blanks was less than 0.5. The microtiter spectrophotometer used in these experiments was a SpectraMax250® (Molecular Devices) and all assays were conducted in medium protein-binding 96-well plates (Corning).

The results for the standards protein samples (*e.g.*, trypsin and purified metalloprotease) obtained in these assays indicated that there was a non-linear response (a linear scale may be adequate only in a narrow assay range). Hence, the curve was fitted to a quadratic function where $f = y_0 + ax^2 + bx$; f is fit to y (SigmaPlot® v. 9; SPSS, Inc.). Thus, if a linear equation was used to quantitate the amount of protein, inaccurate data were obtained; the quadratic equation was found to be required in order to obtain accurate results. It is noted that the manufacturer's (Pierce) kit insert indicates that the results may be fitted with "x" being a log scale.

EXAMPLE 6

EFFECT OF PH AND LAS ON NEUTRAL METALLOPROTEASE ACTIVITY

The pH optimum of the activity for 0.36 mg/mL of formulated nprE was also determined. The buffers investigated in this study were 50 mM sodium acetate over the pH range 3.5-5.5 ($\text{pK}_a = 4.76$), 50 mM MES buffer over the pH range 5.5 to 7.0 ($\text{pK}_a = 6.10$), and 50 mM Tris-HCl buffer at pH 8.4. The pH optimum for formulated nprE was determined to be between 5.5 and 6.0.

The effect of the detergent component LAS on the activity of 0.36 mg/ml of formulated nprE was investigated by incubation with 0 to 1 % (w/v) LAS. The results are shown in the graph provided at Figure 2. As these results indicate, the protease is significantly inactivated by the detergent component, thereby necessitating a means to stabilize the protease against this deleterious effect.

In some experiments, the high density liquid detergent (HDL) composition designated as "TIDE® 2005," provided by Procter & Gamble was used. As supplied, this detergent contained all necessary components, except for the neutral metalloprotease of the present invention.

STORAGE STABILITY IN LIQUID DETERGENT BASE AS A FUNCTION OF TIME

- 5 The stability test was performed in a mini-storage manner. The conditions to be varied and the various concentrations of calcium and zinc chloride salts to be added were assessed using a matrix designed using the FusionPro™ (S-Matrix) software. The following table summarizes the conditions tested to ascertain the long-term storage stability of neutral metalloprotease from *B. amyloliquefaciens*.

Table 6. Long-Term Storage Test Conditions		
Condition	[CaCl ₂] (mM)	[ZnCl ₂] (mM)
1	15	-
2	7.5	7.5
3	-	15
4	-	-
5	12	3
6	-	-
7	-	15
8	7.5	7.5
9	15	-
10	15	15
11	12	3

- 10 The final volume of each tested condition was 1 mL. TIDE® 2005 was dosed with 0.36 mg enzyme/mL. Formulated culture fluid and purified recombinant metalloprotease were incubated in the TIDE® 2005 base at 32 °C over a period of approximately 4 weeks. The storage stability of the metalloprotease in detergent was compared to the stability of the neutral metalloprotease in 50 mM MES buffer, pH 5.8.

- 15 Prior to testing, the samples were diluted 5 in 1000 using assay buffer (50 mM borate buffer, pH 8.5). The residual activity was determined and compared relative to the neutral metalloprotease in assay buffer. All measurements were determined in duplicate. Each sample was tested in parallel with appropriate control blanks (*i.e.*, the detergent, buffer and any necessary additives being tested). The samples were then assayed as described in the instructions provided with the QuantiCleave™ Protease Assay Kit (Pierce).

- 20 The results of these stability tests conducted over a 3-4 week period are shown in Figure 22. In TIDE® 2005, the neutral metalloprotease in the absence of ions (*i.e.*, no added salt) rapidly lost all of its proteolytic/hydrolytic activity against casein. Indeed it was determined that less than 20% of the activity remained after less than 1 hour of incubation. In contrast, incubation of nprE in TIDE® 2005 containing zinc

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ions (up to and including 15 mM) stabilized the protease and prevented proteolysis over a 7-day period. Thus, the presence of zinc ions in this formulation functioned well in maintaining at least 60 % of the protease activity. Likewise, a concentration of 7.5 mM zinc ions resulted in a similar stabilization effect. This concentration of zinc ions is exceeding low and is contemplated to find use in a variety of detergent formulations. In these experiments, no added effect was provided by the inclusion of calcium ions. Furthermore, the addition of calcium ions in excess of 15 mM, and up to and including 25 mM, induced precipitation when added to TIDE® 2005 base. Although it is not intended that the present invention be limited to any particular mechanism, it was contemplated that the absence of an effect of added calcium ions on protease stabilization in these experiments was the result of the detergent composition.

For thermolysin, which displays 55 % amino acid sequence identity with neutral metalloprotease from *B. amyloliquefaciens* (sequence alignment performed using CLUSTAL W, v. 1.82), it has been clearly shown that zinc ions are essential for activity, whereas the calcium ions and engineering of the calcium binding sites have been shown to play a stabilization role (See e.g., Mansfield, et al., J. Biol. Chem., 272:11152-11156; and Van den Berg et al., Biotechnol. Appl. Biochem., 30:35-40).

In alternative embodiments, other cations (e.g., Co^{2+} , Mn^{2+} and Fe^{2+}) find use in the present invention for the stabilization of neutral metalloprotease from *B. amyloliquefaciens*. This is in contrast to prior data that has indicated that none of these ions resulted in 100 % restoration of specific activity (Holmquist and Vallee, J. Biol. Chem., 249:4601-4607). It is contemplated that these ions will affect stability by preventing the unfolding and subsequent proteolytic degradation of the metalloprotease. However, it is not intended that the present invention be limited to any particular mechanism of action.

EXAMPLE 7

NPPE PROTEASE PRODUCTION IN *B. SUBTILIS* USING THE NPPE EXPRESSION VECTOR PUBNPPE

In this Example, experiments conducted to produce NprE protease in *B. subtilis*, in particular, the methods used in the transformation of plasmid pUBnprE into *B. subtilis* are described. Transformation was performed as known in the art (See e.g., WO 02/14490). The DNA sequence (nprE leader, nprE pro and nprE mature DNA sequence from *B. amyloliquefaciens*) provided below, encodes the NprE precursor protein:

GTGGGTTTAGGTAAGAAATTGTCTGTTGCTGTCGCCGCTTCCTTTATGAGTTTAAC
CATCAGTCTGCCGGGTGTTTCAGGCCGCTGAGAATCCTCAGCTTAAAGAAAACCTG
ACGAATTTTGTACCGAAGCATTCTTTGGTGCAATCAGAATTGCCTTCTGTCTCAGTGA

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CAAAGCTATCAAGCAATACTTGAAACAAAAACGGCAAAGTCTTTAAAGGCAATCCT
TCTGAAAGATTGAAGCTGATTGACCAAACGACCGATGATCTCGGCTACAAGCACT
TCCGTTATGTGCCTGTCGTAAACGGTGTGCCTGTGAAAGACTCTCAAGTCATTATT
CACGTCGATAAATCCAACAACGTCTATGCGATTAAACGGTGAATTAACAACGATG
TTTCCGCCAAAACGGCAAACAGCAAAAAATTATCTGCAAATCAGGCGCTGGATCA
TGCTTATAAAGCGATCGGCAAATCACCTGAAGCCGTTTCTAACGGAACCGTTGCA
AACAAAAACAAAGCCGAGCTGAAAGCAGCAGCCACAAAAAGACGGCAAATACCG
CCTCGCCTATGATGTAACCATCCGCTACATCGAACCAGAACCTGCAAACCTGGGAA
GTAACCGTTGATGCGGAAACAGGAAAAATCCTGAAAAAGCAAACAAAGTGGAG
CATGCCGCCACAACCGGAACAGGTACGACTCTTAAAGGAAAAACGGTCTCAT
TAAATATTTCTTCTGAAAGCGGCAAATATGTGCTGCGCGATCTTTCTAAACCT
ACCGGAACACAAATTATTACGTACGATCTGCAAAACCGCGAGTATAACCTGC
CGGGCACACTCGTATCCAGCACCAAAACCAGTTTACAACCTTCTTCTCAGCG
CGCTGCCGTTGATGCGCATTACAACCTCGGCAAAGTGTATGATTATTTCTAT
CAGAAGTTTAATCGCAACAGCTACGACAATAAAGGCGGCAAGATCGTATCCT
CCGTTCAATACGGCAGCAGATACAATAACGCAGCCTGGATCGGCGACCAAAT
GATTTACGGTGACGGCGACGGTTTCATTCTTCTCACCTCTTCCGGTTCAATG
GACGTAACCGCTCATGAAATGACACATGGCGTTACACAGGAAACAGCCAACC
TGAACTACGAAAAATCAGCCGGGCGCTTTAAACGAATCCTTCTCTGATGTATT
CGGGTACTTCAACGATACTGAGGACTGGGATATCGGTGAAGATATTACGGTC
AGCCAGCCGGCTCTCCGCAGCTTATCCAATCCGACAAAATACGGACAGCCTG
ATAATTTCAAAAATTACAAAAACCTTCCGAACACTGATGCCGGCGACTACGG
CGGCGTGATACAAACAGCGGAATCCCGAACAAGCCGCTTACAATACGATT
ACAAAAATCGGCGTGAACAAAGCGGAGCAGATTTACTATCGTGCTCTGACGG
TATACCTCACTCCGTCATCAACTTTTAAAGATGCAAAAGCCGCTTTGATTCAA
TCTGCGCGGGACCTTTACGGCTCTCAAGATGCTGCAAGCGTAGAAGCTGCCT
GGAATGCAGTCGGATTGTAA (SEQ ID NO:12)

- In the above sequence, bold indicates the DNA that encodes the mature NprE protease, standard font indicates the leader sequence (nprE leader), and underlined indicates the pro sequences (nprE pro). The amino acid sequence (NprE leader, NprE pro and NprE mature DNA sequence) (SEQ ID NO:13) provided below,
- 5 encodes the NprE precursor protein. In this sequence, underlined indicates the pro sequence and bold indicates the mature NprE protease. SEQ ID NO:17 provides the NprE pro-sequence separately from the mature NprE sequence and SEQ ID NO:18 provides the mature NprE sequence. This sequence was used as the basis for making the variant libraries described herein.

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MGLGKKLSVAVAASFMSLTISLPGVQAAENPQLKENLTNFVPKHSLVQSELPSVSDK
AIKQYLKQNGKVFKGNPSERLKLIDQTTDDLGYKHFRYVPVNVGVPVKDSQVHHVD
KSNNVYAINGELNNDVSAKTANSKKLSANQALDHAYKAIGKSPEAVSNGTVANKNK
AELKAAATKDGKYRLAYDVTIRYIEPEPANWEVTVDAETGKILKKQNKVEHAATTG
TGTTLKGKTVSLNISSESGKYVLRDLSKPTGTQIITYDLQNREYNLPGTLVSSTTN
QFTTSSQRAAVDAHYNLGKVYDYFYQKFNRNSYDNKGGKIVSSVHYGSRYNNA
AWIGDQMIYGDGDGSFFSPLSGSMDVTAHEMTHGVTQETANLNYENQPGALNE
SFSDVFGYFNDTEDWDIGEDITVSQPALRSLSNPTKYGQPDNFKNYKNLPNTDAG
DYGGVHTNSGIPNKAAYNTITKIGVNKAEQIYYRALTVYLTSPSTFKDAKAALIQ
SARDLYGSQDAASVEAAWNAVGL (SEQ ID NO:13)

AENPQLKENLTNFVPKHSLVQSELPSVSDKAIKQYLKQNGKVFKGNPSERLKLIDQTT
 DDLGYKHFRYVPVNVGVPVKDSQVHHVDKSNNVYAINGELNNDVSAKTANSKKLS
 ANQALDHAYKAIGKSPEAVSNGTVANKNKAELKAAATKDGKYRLAYDVTIRYIEPE
 PANWEVTVDAETGKILKKQNKVEH (SEQ ID NO:17)

AATTGTGTTLKGKTVSLNISSESGKYVLRDLSKPTGTQIITYDLQNREYNLPGTLVSST
 TNQFTTSSQRAAVDAHYNLGKVYDYFYQKFNRNSYDNKGGKIVSSVHYGSRYNNA
 AWIGDQMIYGDGDGSFFSPLSGSMDVTAHEMTHGVTQETANLNYENQPGALNESFS
 DVFGYFNDTEDWDIGEDITVSQPALRSLSNPTKYGQPDNFKNYKNLPNTDAGDYGG
 VHTNSGIPNKAAYNTITKIGVNKAEQIYYRALTVYLTSPSTFKDAKAALIQSARDLYG
 SQDAASVEAAWNAVGL (SEQ ID NO:18)

- 5 The pUBnprE expression vector was constructed by amplifying the nprE gene from the
 chromosomal DNA of *B. amyloliquefaciens* by PCR using two specific primers:

Oligo AB 1740: CTGCAGGAATTCAGATCTTAACATTTTCCCCTATCATTTTCCCG (SEQ
 ID NO:19)

Oligo AB 1741: GGATCCAAGCTTCCCGGAAAAGACATATATGATCATGGTGAAGCC
 (SEQ ID NO:20)

- 10 PCR was performed on a thermocycler with Phusion High Fidelity DNA polymerase (Finnzymes).
 The PCR mixture contained 10 µl 5x buffer (Finnzymes Phusion), 1µl 10mM dNTP's, 1.5µl DMSO, 1µl of
 each primer, 1 µl Finnzymes Phusion DNA polymerase, 1 µl chromosomal DNA solution 50ng/µl, 34.5 µl
 MilliQ water. The following protocol was used:

PCR PROTOCOL:

- 15 1) 30 sec 98°C;
 2) 10 sec 98°C;
 3) 20 sec 55°C;
 4) 1 min 72°C;
 5) 25 cycles of steps 2 to 4; and
 20 6) 5 min 72°C.

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This resulted in a 1.9 kb DNA fragment which was digested using *Bgl*II and *Bcl*II DNA restriction enzymes. The multicopy *Bacillus* vector pUB 110 (See e.g., Gryczan, J. Bacteriol., 134:318-329) was digested with *Bam*HI. The PCR fragment x *Bgl*II x *Bcl*II was then ligated in the pUB 110 x *Bam*HI vector to form pUBnprE expression vector (See, Figure 14).

5 pUBnprE was transformed to a *B. subtilis* (*AprE*, *AnprE*, *oppA*, *AspoIIIE*, *degUHy32*, *ΔamyE::(xylR,pxyIA-comK)* strain. Transformation into *B. subtilis* was performed as described in WO 02/14490. Selective growth of *B. subtilis* transformants harboring the pUBnprE vector was performed in shake flasks containing 25 ml MBD medium (a MOPS based defined medium), with 20 mg/L neomycin. MBD medium was made essentially as known in the art (See, Neidhardt et al., J. Bacteriol., 119: 736-747),
 10 except that NH_4Cl_2 , FeSO_4 , and CaCl_2 were left out of the base medium, 3 mM K_2HPO_4 was used, and the base medium was supplemented with 60 mM urea, 75 g/L glucose, and 1 % soytone. Also, the micronutrients were made up as a 100 X stock containing in one liter, 400 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 100 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 100 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 100 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mg $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 100 mg $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 10 ml of 1M CaCl_2 , and 10 ml of 0.5 M sodium citrate. The culture was incubated for three days at 37°C in an
 15 incubator/shaker (Infors). This culture resulted in the production of secreted NprE protease with proteolytic activity as demonstrated by protease assays. Gel analysis was performed using NuPage Novex 10% Bis-Tris gels (Invitrogen, Cat.No. NP0301BOX). To prepare samples for analysis, 2 volumes of supernatant were mixed with 1 volume 1M HCl, 1 volume 4xLDS sample buffer (Invitrogen, Cat.No. NP0007), and 1% PMSF (20 mg/ml) and subsequently heated for 10 minutes at 70°C. Then, 25 μL of each sample were loaded onto
 20 the gel, together with 10 μL of SeeBlue plus 2 pre-stained protein standards (Invitrogen, Cat.No.LC5925). The results clearly demonstrated that the nprE cloning strategy described in this example yield active NprE produced by *B. subtilis*.

EXAMPLE 8

GENERATION OF NPRESITE EVALUATION LIBRARIES (SELS)

25 In this Example, methods used in the construction of nprE SELs are described. The pUBnprE vector, containing the nprE expression cassette described above, served as template DNA. This vector contains a unique *Bgl*II restriction site, which was utilized in the site evaluation library construction.

The pUBnprE expression vector, primers, synthesized at Invitrogen (desalted, 50 nmol scale) were used to generate the libraries. The sequences of the primers are provided in Table 8-1.

30 To construct a nprE site evaluation library, three PCR reactions were performed, including two mutagenesis PCRs to introduce the mutated codon of interest in the mature nprE DNA sequence and a third PCR used to fuse the two mutagenesis PCRs in order to construct the pUBnprE expression vector including the desired mutated codon in the mature nprE sequence.

35 The method of mutagenesis was based on the codon-specific mutation approach, in which the creation of all possible mutations at a time in a specific DNA triplet was performed using a forward and reverse oligonucleotide primer with a length of 25 to 45 nucleotides enclosing a specific designed triple DNA sequence NNS ((A,C,T or G), (A,C,T or G), (C or G)) that corresponded with the sequence of the codon to be mutated and guaranteed random incorporation of nucleotides at that specific nprE mature codon. The number listed in the primer names (See, Table 8-1) corresponds with the specific nprE mature codon position.

Two additional primers used to construct the site evaluation libraries contained the *Bgl*III restriction site together with a part of the pUBnprE DNA sequence flanking the *Bgl*III restriction site. These primers were produced by Invitrogen (50nmole scale, desalted) and are listed in Table 8-1.

Table 8-1. Primer Sequences	
Primer Name	Primer Sequence and SEQ ID NO:
pUB-BglIII-FW	GTCAGTCAGATCTTCCTTCAGGTTATGACC (SEQ ID NO:21)
pUB-BglIII-RV	GTCTCGAAGATCTGATTGCTTAACTGCTTC (SEQ ID NO:22)
Specific nprE Forward Mutagenesis Primers	
nprE4F	GTGGAGCATGCCGCCACANNSGGAACAGGTACGACTCTTAA (SEQ ID NO:23)
nprE12F	CAGGTACGACTCTTAAANNSAAAACGGTCTCATTAATAT (SEQ ID NO:24)
nprE13F	GTACGACTCTTAAAGGANNNSACGGTCTCATTAATATTTC (SEQ ID NO:25)
nprE14F	CGACTCTTAAAGGAAAAANNSGTCTCATTAATATTTC (SEQ ID NO:26)
nprE23F	CATTAATATTTCTTCTGAANNSGGCAAATATGTGCTGCG (SEQ ID NO:27)
nprE24F	TAAATATTTCTTCTGAAAGCNSAAATATGTGCTGCGCGATC (SEQ ID NO:28)
nprE33F	GTGCTGCGCGATCTTTCTNNSCCTACCGGAACACAAATTAT (SEQ ID NO:29)
nprE45F	AAATTATTACGTACGATCTGNNSAACCGCGAGTATAACCTG (SEQ ID NO:30)
nprE46F	TTATTACGTACGATCTGCAANNSCGCGAGTATAACCTGCC (SEQ ID NO:31)
nprE47F	CGTACGATCTGCAAAACNNSGAGTATAACCTGCCGGG (SEQ ID NO:32)
nprE49F	GATCTGCAAAACCGCGAGNNSAACCTGCCGGGCACACTC (SEQ ID NO:33)
nprE50F	CTGCAAAACCGCGAGTATNNSCTGCCGGGCACACTCGTATC (SEQ ID NO:34)
nprE54F	GAGTATAACCTGCCGGGCNNSCTCGTATCCAGACCAC (SEQ ID NO:35)
nprE58F	CGGGCACACTCGTATCCNNSACCACAAACCAGTTTAC (SEQ ID NO:36)
nprE59F	GCACACTCGTATCCAGCNNSACAAACCAGTTTACAAC (SEQ ID NO:37)
nprE60F	CACTCGTATCCAGCACCNNSAACAGTTTACAACCTC (SEQ ID NO:38)
nprE65F	CCACAAACCAGTTTACANNSTCTTCTCAGCGCGCTGC (SEQ ID NO:39)
nprE66F	CAAACCAGTTTACAACNNSTCTCAGCGCGCTGCCGTTG (SEQ ID NO:40)
nprE87F	GTGTATGATTATTTCTATNNSAAGTTTAATCGCAACAG (SEQ ID NO:41)
nprE90F	ATTATTTCTATCAGAAGTTTNNSCGCAACAGCTACGACAATAA (SEQ ID NO:42)
Primer Name	Primer Sequence and SEQ ID NO:
nprE96F	TTAATCGCAACAGCTACGACNNSAAAGGCGGCAAGATCGTATC (SEQ ID NO:43)

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nprE97F	GCAACAGCTACGACAATNNSGGCGGCAAGATCGTATC (SEQ ID NO:44)
nprE100F	CTACGACAATAAAGGCGGCNNSATCGTATCCTCCGTTTCATTA (SEQ ID NO:45)
nprE186F	GAGGACTGGGATATCGGTNNSGATATTACGGTCAGCCAG (SEQ ID NO:46)
nprE196F	GTCAGCCAGCCGGCTCTCNNSAGCTTATCCAATCCGAC (SEQ ID NO:47)
nprE211F	GACAGCCTGATAATTCNNSAATTACAAAAACCTTCC (SEQ ID NO:48)
nprE214F	GATAATTTCAAAAATTACNNSAACCTTCCGAACACTGATG (SEQ ID NO:49)
nprE228F	GCGACTACGGCGGCGTGNNSACAAACAGCGGAATCCC (SEQ ID NO:50)
nprE280F	CTTTGATTCAATCTGCGNNSGACCTTTACGGCTCTCAAG (SEQ ID NO:51)
Specific nprE Reverse Mutagenesis Primers	
nprE4R	TTAAGAGTCGTACCTGTTCCSNNTGTGGCGGCATGCTCCAC (SEQ ID NO:52)
nprE12R	ATATTTAATGAGACCGTTTTSNNTTTAAGAGTCGTACCTG (SEQ ID NO:53)
nprE13R	GAAATATTTAATGAGACCGTSNNTCCTTTAAGAGTCGTAC (SEQ ID NO:54)
nprE14R	GAAATATTTAATGAGACSNNTTTTCCTTTAAGAGTCG (SEQ ID NO:55)
nprE23R	CGCAGCACATATTTGCCSNNTTCAGAAGAAATATTTAATG (SEQ ID NO:56)
nprE24R	GATCGCGCAGCACATATTTSNNGCTTTCAGAAGAAATATTTA (SEQ ID NO:57)
nprE33R	ATAATTTGTGTTCCGGTAGGSNNAGAAAGATCGCGCAGCAC (SEQ ID NO:58)
nprE45R	CAGGTTATACTCGCGGTTSNNCAGATCGTACGTAATAATTT (SEQ ID NO:59)
nprE46R	GGCAGGTTATACTCGGSNNTTGCAGATCGTACGTAATAA (SEQ ID NO:60)
nprE47R	CCCGGCAGGTTATACTCSNNGTTTTGCAGATCGTACG (SEQ ID NO:61)
nprE49R	GAGTGTGCCCCGGCAGGTTSNNTCGCGGTTTTGCAGATC (SEQ ID NO:62)
nprE50R	GATACGAGTGTGCCCCGCAGSNNATACTCGCGGTTTTGCAG (SEQ ID NO:63)
Primer Name	Primer Sequence and SEQ ID NO:
nprE54R	GTGGTGCTGGATACGAGSNNGCCCGCAGGTTATACTC (SEQ ID NO:64)
nprE58R	GTAAACTGGTTTGTGGTSNNGGATACGAGTGTGCCCCG (SEQ ID NO:65)
nprE59R	GTTGTAAACTGGTTTGTSNNGCTGGATACGAGTGTGC (SEQ ID NO:66)
nprE60R	GAAGTTGTAAACTGGTTSNNGGTGCTGGATACGAGTG (SEQ ID NO:67)
nprE65R	GCAGCGCGCTGAGAAGASNNTGTAAACTGGTTTGTGG (SEQ ID NO:68)
nprE66R	CAACGGCAGCGCGCTGAGASNAGTTGTAAACTGGTTTG (SEQ ID NO:69)
nprE87R	CTGTTGCGATTAACTTSNNATAGAAATAATCATACAC (SEQ ID NO:70)
nprE90R	TTATTGTCGTAGCTGTTGCGSNNAACCTTCTGATAGAAATAAT (SEQ ID NO:71)

nprE96R	GATACGATCTTGCCGCCTTTSNNGTCGTAGCTGTTGCGATTAA (SEQ ID NO:72)
nprE97R	GATACGATCTTGCCGCCSNNATTGTCGTAGCTGTTGC (SEQ ID NO:73)
nprE100R	TAATGAACGGAGGATACGATSNNGCCGCCTTTATTGTCGTAG (SEQ ID NO:74)
nprE186R	CTGGCTGACCGTAATATCSNNACCGATATCCCAGTCCTC (SEQ ID NO:75)
nprE196R	GTCGGATTGGATAAGCTSNNAGAGCCGGCTGGCTGAC (SEQ ID NO:76)
nprE211R	GGAAGGTTTTTGTAATTSNNGAAATTATCAGGCTGTC (SEQ ID NO:77)
nprE214R	CATCAGTGTTTCGGAAGGTTSNNGTAATTTTTGAAATTATC (SEQ ID NO:78)
nprE228R	GGGATTCCGCTGTTTGTSNNCACGCCCGCTAGTCGC (SEQ ID NO:79)
nprE280R	CTTGAGAGCCGTAAAGGTCSNNCGCAGATTGAATCAAAG (SEQ ID NO:80)

Construction of each site evaluation library started with two primary PCR amplifications using the pUB-BglII-FW primer and a specific nprE reverse mutagenesis primer. For the second PCR, the pUB-BglII -RV primer and a specific nprE forward mutagenesis primer (equal nprE mature codon positions for the forward and reverse mutagenesis primers) were used.

- 5 The introduction of the mutations in the mature nprE sequence was performed using Phusion High-Fidelity DNA Polymerase (Finnzymes; Cat. no. F-530L). All PCRs were performed according to the Finnzymes protocol supplied with the polymerase. The PCR conditions for the primary PCRs were:

FOR PRIMARY PCR 1:

pUB-BglII-FW primer and a specific NPPE reverse mutagenesis primer - both 1 μ L (10 μ M);

- 10 FOR PRIMARY PCR 2:

pUB-BglII -RV primer and a specific NPPE forward mutagenesis primer - both 1 μ L (10 μ M) ;

together with

5 x Phusion HF buffer	10 μ L
10 mM dNTP mixture	1 μ L
Phusion DNA polymerase	0.75 μ L (2 units/ μ L)
DMSO, 100%	1 μ L
pUBnprE template DNA	1 μ L (0.1 - 1 ng/ μ L)
Distilled, autoclaved water	up to 50 μ L

- 15 The PCR program was: 30 seconds 98°C, 30x (10 seconds 98°C, 20 seconds 55°C, 1,5 minute 72°C) and 5 min 72°C., performed in a PTC-200 Peltier thermal cycle (MJ Research). The PCR experiments result in two fragments of approximately 2 to 3 kB, which had about 30 nucleotide base overlap around the NPPE mature codon of interest. Fragments were fused in a third PCR reaction using these two aforementioned fragments and the forward and reverse *BglII* primers. The fusion PCR reaction was carried out in the following solution:

pUB-BglII-FW primer and pUB-BglII-RV primer- both 1 μ L (10 μ M)

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5 x Phusion HF buffer	10 µL
10 mM dNTP mixture	1 µL
Phusion DNA polymerase	0.75 µL (2 units/ µL)
DMSO, 100%	1 µL
primary PCR 1 reaction mix	1 µL
primary PCR 2 reaction mix	1 µL
Distilled, autoclaved water	up to 50 µL

The PCR fusion program was as follows: 30 seconds 98°C, 30x (10 seconds 98°C, 20 seconds 55°C, 2:40 minute 72°C) and 5 min 72°C, in a PTC-200 Peltier thermal cycler (MJ Research).

The amplified linear 6.5 Kb fragment was purified using the Qiaquick PCR purification kit (Qiagen, Cat. no. 28106) and digested with *Bgl*III restriction enzyme to create cohesive ends on both sides of the fusion

5 fragment:

- 35 µL purified linear DNA fragment
- 4 µL REACT® 3 buffer (Invitrogen)
- 1 µL *Bgl*III, 10 units/ml (Invitrogen)

Reaction conditions: 1 hour, 30°C.

10 Ligation of the *Bgl*III digested and purified using Qiaquick PCR purification kit (Qiagen, Cat. no. 28106) fragment results in circular and multimeric DNA containing the desired mutation:

- 30 µL of purified *Bgl*III digested DNA fragment
- 8 µL T4 DNA Ligase buffer (Invitrogen® Cat. no. 46300-018)
- 1 µL T4 DNA Ligase, 1 unit/µL (Invitrogen® Cat. no. 15224-017)

15 Reaction conditions: 16-20 hours, 16°

Subsequently, the ligation mixture was transformed to a *B. subtilis* ($\Delta aprE$, $\Delta nprE$, *oppA*, $\Delta spoIIE$, *degUHy32*, $\Delta amyE::(xylR,pxyIA-comK)$) strain. Transformation to *B. subtilis* was performed as described in WO 02/14490. For each library, 96 single colonies were picked and grown in MOPS media with neomycin and 1.25 g/L yeast extract for sequence analysis (BaseClear) and screening purposes. Each library included a

20 maximum of 19 nprE site-specific variants.

The variants were produced by growing the *B. subtilis* SEL transformants in 96 well MTP at 37°C for 68 hours in MBD medium with 20 mg/L neomycin and 1.25 g/L yeast extract (*See*, above).

EXAMPLE 9

GENERATION OF NP RE COMBINATORIAL LIBRARIES (RCLS)

25 In this Example, methods used to generate nprE combinatorial libraries are described. For this enzyme, one property was chosen as the property that needed to be changed the most. This property is defined herein as the "primary property." All other properties were "secondary properties" for the purpose of combinatorial library design. The basic strategy for improving a protein as used herein, was to combine mutations that improve the primary property and also maintain or improve the secondary properties. The site

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evaluation data were used to identify those mutations which improved the primary property while maintaining or improving the secondary properties. Mutations that were to be combined were identified by their Performance Index (PI or Pi) and associated $\Delta\Delta G_{app}$ values.

The "Apparent Free Energy Change" ($\Delta\Delta G_{app}$) as used herein is defined as:

$$\Delta\Delta G_{app} = -RT \ln (P_{variant}/P_{parent})$$

where $P_{variant}$ is the performance value for the variant and P_{parent} is the performance value for the parent enzyme under the same conditions. The ratio $P_{variant}/P_{parent}$ is defined as the performance index (Pi) for the property. The $\Delta\Delta G_{app}$ values were expected to behave in a similar fashion to actual $\Delta\Delta G$ values for data distributions and additivity. However, since $\Delta\Delta G$ represents the maximum amount of work that can be carried out by the variant compared to the parent enzyme, the quantity $\Delta\Delta G_{app}$ generally underestimates the $\Delta\Delta G$ and may lead to results that appear synergistic in that the properties of two additive positions may be greater than the value predicted by adding their $\Delta\Delta G_{app}$ values together.

For example, when TIDE® stability is the primary property and BMI activity is the secondary property, mutations that have $\Delta\Delta G_{app}$ values < 0 ($P_i > 1$) and BMI $\Delta\Delta G_{app}$ values < 0.06 ($P_i > 0.9$) may be chosen for combination. Indeed, these relationships were explored in these experiments.

To produce the variants used in these experiments, synthetic nprE library fragments, containing multiple mutations at multiple nprE mature DNA positions, were produced by GeneArt (Geneart). These 1.5 kB nprE library fragments were digested with DNA restriction enzymes *PvuI* and *AvaI*, purified and ligated in the 5 kB pUB vector fragment (also digested with DNA restriction enzymes *PvuI* and *AvaI*) by a ligase reaction using T4 DNA Ligase (Invitrogen® Cat. no. 15224-017).

To transform the ligation reaction mix directly into *Bacillus* cells, the library DNA (nprE library fragment mix ligated in pUB vector fragment) was amplified using the TempliPhi kit (Amersham cat. #25-6400). For this purpose, 1 µL of the ligation reaction mix was mixed with 5 µL of sample buffer from the TempliPhi kit and heated for 3 minutes at 95°C to denature the DNA. The reaction was placed on ice to cool for 2 minutes and then spun down briefly. Next, 5 µL of reaction buffer and 0.2 µL of phi29 polymerase from the TempliPhi kit were added, and the reactions were incubated at 30°C in an MJ Research PCR machine for 4 hours. The phi29 enzyme was heat inactivated in the reactions by incubation at 65°C for 10 min in the PCR machine.

For transformation of the libraries into *Bacillus*, 0.1 µL of the TempliPhi amplification reaction product was mixed with 500 µL of competent *B. subtilis* cells ($\Delta aprE$, $\Delta nprE$, $oppA$, $\Delta spoIIE$, $degUHy32$, $\Delta amyE::(xylR,pxylA-comK)$) followed by vigorous shaking at 37°C for 1 hour. Then, 100 and 500 µL were plated on HI-agar plates containing 20 mg/L neomycin and 0.5% skim milk. In general, transformation to *B. subtilis* was performed as described in WO 02/14490. *B. subtilis* nprE combinatorial libraries, constructed by this method are contemplated to contain wild type amino acids at one or more of the positions targeted for mutagenesis.

The variants obtained in these libraries were then tested for their stability in TIDE® and their performance in BMI wash performance tests as described herein. Table 9 provides performance indices for the variants tested in the BMI assay. In this Table, "Pos." indicates the position in the NprE amino acid sequence that was changed, and "AA" indicates the amino acid substitution made for each variant.

Table 9. Results (Performance Indices) for Tested Variants								
Pos.	Variant	AA	TIDE (-)	TIDE(-) $\Delta\Delta G$	TIDE(+)	TIDE(+) $\Delta\Delta G$	BMI Pi	BMI $\Delta\Delta G$
4	T004L	L	0.80	0.13	1.13	-0.07	1.01	0.00
23	S023Y	Y	1.08	-0.05	1.13	-0.07	1.02	-0.01
23	S023W	W	1.12	-0.07	1.13	-0.07	1.29	-0.15
23	S023N	N	1.33	-0.17	1.10	-0.06	0.95	0.03
23	S023T	T	0.88	0.07	1.06	-0.03	0.91	0.05
23	S023G	G	1.29	-0.15	1.06	-0.03	0.92	0.05
23	S023R	R	0.98	0.01	1.06	-0.03	1.46	-0.22
23	S023L	L	0.90	0.06	1.03	-0.02	1.24	-0.13
23	S023M	M	1.04	-0.02	1.03	-0.02	1.09	-0.05
23	S023V	V	0.82	0.12	1.02	-0.01	0.93	0.04
23	S023K	K	1.01	-0.01	1.02	-0.01	1.50	-0.24
24	G024Y	Y	0.60	0.30	1.11	-0.06	1.10	-0.06
24	G024W	W	0.36	0.60	1.10	-0.06	1.20	-0.11
24	G024M	M	0.71	0.20	1.09	-0.05	1.12	-0.07
24	G024F	F	0.50	0.41	1.08	-0.04	1.19	-0.10
24	G024L	L	0.49	0.42	1.07	-0.04	1.22	-0.12
24	G024H	H	0.80	0.13	1.05	-0.03	1.17	-0.09
24	G024K	K	0.55	0.35	1.04	-0.02	1.55	-0.26
24	G024T	T	0.57	0.33	1.03	-0.02	0.94	0.04
24	G024R	R	0.56	0.34	1.02	-0.01	1.47	-0.23
46	N046Q	Q	0.88	0.08	1.07	-0.04	1.22	-0.12
47	R047K	K	1.12	-0.07	1.09	-0.05	1.15	-0.08
50	N050F	F	1.07	-0.04	1.07	-0.04	1.38	-0.19
50	N050Y	Y	1.00	0.00	1.04	-0.02	1.27	-0.14
50	N050W	W	1.01	-0.01	1.04	-0.02	1.46	-0.22
50	N050P	P	1.23	-0.12	1.03	-0.02	1.12	-0.07
54	T054H	H	1.08	-0.04	1.11	-0.06	1.17	-0.09
54	T054K	K	1.03	-0.02	1.11	-0.06	1.47	-0.23

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54	T054L	L	1.09	-0.05	1.08	-0.05	1.26	-0.14
54	T054N	N	0.97	0.02	1.07	-0.04	1.25	-0.13
54	T054Y	Y	1.14	-0.08	1.07	-0.04	1.08	-0.04
54	T054W	W	1.02	-0.01	1.07	-0.04	1.22	-0.12
54	T054S	S	0.99	0.01	1.05	-0.03	1.03	-0.02
54	T054I	I	1.09	-0.05	1.04	-0.02	1.34	-0.17
54	T054R	R	0.96	0.02	1.04	-0.02	1.46	-0.22
54	T054Q	Q	1.09	-0.05	1.03	-0.02	1.23	-0.12
54	T054F	F	0.98	0.01	1.03	-0.02	1.16	-0.09
54	T054V	V	1.14	-0.08	1.01	-0.01	1.11	-0.06
59	T059R	R	0.76	0.16	1.28	-0.14	1.56	-0.26
59	T059W	W	0.56	0.34	1.26	-0.14	1.32	-0.16
59	T059K	K	0.99	0.00	1.16	-0.09	1.60	-0.28
59	T059N	N	0.98	0.01	1.15	-0.08	1.16	-0.09
59	T059G	G	0.94	0.04	1.13	-0.07	1.11	-0.06
59	T059P	P	1.18	-0.10	1.12	-0.07	1.19	-0.10
59	T059M	M	1.04	-0.02	1.10	-0.06	1.10	-0.05
59	T059H	H	0.98	0.01	1.07	-0.04	1.32	-0.16
59	T059S	s	1.09	-0.05	1.04	-0.03	0.91	0.06
59	T059A	A	1.05	-0.03	1.04	-0.02	0.96	0.03
59	T059Q	Q	1.05	-0.03	1.04	-0.02	1.31	-0.16
59	T059I	I	0.64	0.26	1.01	-0.01	1.43	-0.21
60	T060N	N	0.79	0.14	1.03	-0.02	1.07	-0.04
66	S066Q	Q	0.75	0.17	1.01	-0.01	1.12	-0.07
66	S066N	N	1.08	-0.05	1.01	-0.01	1.00	0.00
110	R110K	K	1.08	-0.04	1.04	-0.02	1.05	-0.03
119	D119H	H	1.03	-0.02	1.15	-0.08	1.16	-0.09
129	S129I	I	2.32	-0.49	1.68	-0.30	0.98	0.01
129	S129V	V	2.34	-0.50	1.55	-0.26	1.01	0.00
129	S129Q	Q	1.86	-0.37	1.44	-0.21	0.99	0.00

129	S129T	T	1.59	-0.27	1.36	-0.18	1.04	-0.02
129	S129L	L	1.70	-0.31	1.35	-0.18	1.01	-0.01
129	S129H	H	1.60	-0.28	1.30	-0.15	1.17	-0.09
129	S129Y	Y	1.28	-0.14	1.06	-0.04	1.25	-0.13
129	S129A	A	1.13	-0.07	1.06	-0.03	1.12	-0.07
129	S129K	K	1.18	-0.10	1.05	-0.03	1.33	-0.17
130	F130L	L	1.29	-0.15	1.52	-0.25	0.91	0.05
130	F130I	I	1.18	-0.10	1.14	-0.08	1.03	-0.02
130	F130V	V	1.05	-0.03	1.06	-0.03	0.99	0.00
130	F130K	K	0.99	0.00	1.04	-0.02	1.26	-0.14
138	M138L	L	1.11	-0.06	1.43	-0.21	0.95	0.03
152	E152H	H	1.53	-0.25	1.36	-0.18	1.15	-0.08
152	E152W	w	1.32	-0.16	1.31	-0.16	1.06	-0.03
152	E152F	F	1.32	-0.16	1.15	-0.08	1.09	-0.05
179	T179P	P	1.33	-0.17	1.50	-0.24	1.04	-0.03
190	V190I	I	1.37	-0.18	1.68	-0.30	1.16	-0.09
220	D220P	P	2.24	-0.47	2.66	-0.57	1.05	-0.03
220	D220E	E	2.23	-0.47	2.44	-0.52	1.05	-0.03
243	T243I	I	1.13	-0.07	1.17	-0.09	1.06	-0.03
263	T263W	w	1.37	-0.18	1.40	-0.20	0.92	0.05
263	T263H	H	1.03	-0.02	1.01	-0.01	1.05	-0.01
273	A273H	H	1.10	-0.06	1.14	-0.08	0.98	0.01
282	L282M	M	1.03	-0.01	1.16	-0.09	1.01	-0.01
282	L282F	F	0.91	0.05	1.06	-0.04	1.09	-0.05
282	L282Y	Y	0.83	0.11	1.04	-0.02	0.92	-0.05
285	S285R	R	1.08	-0.04	1.38	-0.19	1.23	-0.12
285	S285P	P	1.11	-0.06	1.30	-0.16	0.98	0.01
285	S285W	W	1.08	-0.05	1.28	-0.14	0.95	0.03
285	S285Q	Q	1.06	-0.03	1.10	-0.05	0.98	0.01
285	S285K	K	0.89	0.07	1.00	0.00	1.20	-0.10

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286	Q286R	R	0.95	0.03	1.18	-0.10	1.14	-0.08
286	Q286P	P	0.98	0.01	1.15	-0.08	0.97	0.02
286	Q286K	K	0.93	0.04	1.09	-0.05	1.22	-0.12

EXAMPLE 10

ALTERNATIVE METHOD GENERATE NPRED SITE EVALUATION LIBRARIES (SELS) VIA QUIKCHANGE® MUTAGENESIS

In this Example, alternative methods to generate nprE SELs are described. As in Example 8, above, the pUBnprE vector served as the template DNA source for the generation of nprE SELs. The major difference between the two methods is that this method requires amplification of the entire vector using complementary site-directed mutagenic primers.

MATERIALS:

- Bacillus* strain containing the pUBnprE vector
- Qiagen Plasmid Midi Kit (Qiagen cat # 12143)
- Ready-Lyse Lysozyme (Epicentre cat # R1802M)
- dam Methylase Kit (New England Biolabs cat # M0222L)
- Zymoclean Gel DNA Recovery Kit (Zymo Research cat # D4001)
- nprE site-directed mutagenic primers, 100nmole scale, 5' Phosphorylated, PAGE purified (Integrated DNA Technologies, Inc.)
- QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene cat # 200514)
- MJ Research PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories)
- 1.2% agarose E-gels (Invitrogen cat # G5018-01)
- TempliPhi Amplification Kit (GE Healthcare cat # 25-6400-10)
- Competent *B. subtilis* cells ($\Delta aprE$, $\Delta nprE$, $oppA$, $\Delta spoIIE$, $degUH_32$, $\Delta amyE::(xylR,pxyA-comK)$)

METHODS:

To obtain the pUBnprE vector, a single colony of a *Bacillus* strain containing the pUBnprE vector was used to inoculate a 5ml LB + 10ppm neomycin tube. This was the starter culture used in these methods. The culture was grown at 37°C, with shaking at 225 rpm for 6 hours. Then, 100 ml of fresh LB + 10ppm neomycin were inoculated with 1ml of the starter culture. This culture was grown overnight at 37°C, with shaking at 225 rpm. Following this incubation, the cell pellet was harvested by sufficient centrifugation to provide a cell pellet. The cell pellet was resuspended in 10 ml Buffer P1 (Qiagen Plasmid Midi Kit). Then, 10ul of Ready-Lyse Lysozyme was added to the resuspended cell pellet and incubated at 37°C for 30 min. Then, the Qiagen Plasmid Midi Kit protocol was continued (using 10ml of Buffer P2 and P3 to account for the increased volume of cell culture). After isolation of pUBnprE vector from *Bacillus*, the concentration of pUBnprE vector was quantitated. The vector was then *dam* methylated using the *dam* Methylase Kit (New England Biolabs), using the methods set forth in the kit protocols, to methylate approximately 2 ug of pUBnprE vector per tube. The Zymoclean Gel DNA recovery kit was used to purify and concentrate the *dam*-methylated pUBnprE vector. The *dam*-methylated pUBnprE vector was quantitated and then diluted to a

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working concentration of 50ng/ul. Complementary site-directed mutagenic primers (1 ul of each primer at 10uM) (*See*, Table 10-1), were used in a PCR reaction in the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene), following the manufacturer's protocol (*e.g.*, 1ul dam methylated pUBnprE vector (50ng/ul), 1ul nprE site-directed Forward mutagenic primer (10uM), 1ul nprE site-directed Forward mutagenic primer (10uM), 2.5ul 10x QuikChange Multi Reaction buffer, 1ul dNTP Mix, 1ul QuikChange Multi enzyme blend (2.5U/ul), and 17.5ul distilled, autoclaved water, to provide a 25ul total reaction mix). The nprE site evaluation libraries were amplified using the following conditions: 95°C, for 1 min. (1st cycle only), followed by 95°C for 1 min., 55°C for 1 min, 65°C for 13 ½ min., and repeat cycling 23 times. The reaction product was stored at 4°C overnight. Then, the reaction mixture underwent *DpnI* digest treatment (supplied with QuikChange Multi Site-Directed Mutagenesis Kit) to digest parental pUBnprE vector, using the manufacturer's protocol (*i.e.*, 1.5ul *DpnI* restriction enzyme was added to each tube and incubated at 37°C for 3 hours; 2ul of *DpnI*-digested PCR reaction was then analyzed on a 1.2% E-gel for each nprE SEL to ensure PCR reaction worked and that parental template was degraded). TempliPhi rolling circle amplification was then used to generate large amounts of DNA for increasing library size of each nprE SEL, using the manufacturer's protocol (*i.e.*, 1ul *DpnI* treated QuikChange Multi Site-Directed Mutagenesis PCR, 5ul TempliPhi Sample Buffer, 5ul TempliPhi Reaction Buffer, and 0.2ul TempliPhi Enzyme Mix, for an ~11ul total reaction; incubated at 30°C for 3 hours; the TempliPhi reaction was diluted by adding 200ul distilled, autoclaved water and briefly vortexed. Then, 1.5ul of diluted TempliPhi material was transformed into competent *B. subtilis* cells, and nprE SELs were selected for using LA + 10ppm Neomycin + 1.6% skim milk plates. Table 10-1 provides the names, sequences and SEQ ID NOS for the primers used in these experiments. All of the primers were synthesized by Integrated DNA Technologies, on 100nmole scale, 5'-phosphorylated, and PAGE purified.

Table 10-1. Primers	
PRIMER	SEQUENCE
rprE-14F	ETGAGCATGCCGCCACANNSSGAACAGGTACGACTCTTAAAGS (SEQ ID NO:81)
rprE-G12F	CCGGACAGCTACGACTCTTAAANNSSAAACGGTCTCATTAATAATTTCTTCTGAAACC (SEQ ID NO:82)
rprE-Q45F	CGSAACACAAATTATTAGGTACGATCTGNNSAACGCGAGTATAACCTGCC (SEQ ID NO:83)
rprE-Y48F	ACCGGAGNNSAACCTGCGGGCACACTCGTATCC (SEQ ID NO:84)
rprE-N50F	GTACGATCTGCAAAACCGGAGTATNNSCTGCCGGGCACACTCGTATCCAG (SEQ ID NO:85)
rprE-185F	CCAGCCACAAACCCAGTTTACANNSTTCTCAGCCGCTGCCGTTG (SEQ ID NO:86)
rprE-D119F	GCAGATACAATAACGACGCTGGATCGGONNSOAAAATGATTTACGGTACGCGGAC (SEQ ID NO:87)
rprE-Q128F	CCAAATGATTTACGGTGACGGCGGACNNSCTATCTTCTCACTCTTTCCGGTTC (SEQ ID NO:88)
rprE-F130F	GGTGACGGGACGGTTCANNSTTCTCACTCTTTCCGGTTC (SEQ ID NO:89)
rprE-Q151F	CATGAAATCACACATGCGGTTACANNSSGAACAGCCCAACCTGAAC1AC (SEQ ID NO:90)
rprE-E152F	CATCAAA1GACACATGCGGTTACACAGNNSACAGCCCAACCTGAAC1ACG (SEQ ID NO:91)
rprE-N155F	CATCGCGTTACACAGGAACAGCCNNSCTGAAC1ACGAAATCAGCCG (SEQ ID NO:92)
rprE-1179F	CTGATG1ATTTCGGGTACTTCAACGATNNSGAGGACTCGGATATCGG1TG(SEQ ID NO:93)
rprE-Y204F	GCAGCTATCCAA1CCGACAAANNSSGGACAGCC1GATAATTTCAAAAAT1AC (SEQ ID NO:94)
rprE-G205F	GCAGCTATCCAA1CCGACAAAA1ACNNSAGCC1GATAATTTCAAAAAT1ACAAAACC (SEQ ID NO:95)
rprE-Y224F	GAACACTGATSCCGGCGACNNSGGGGGGTGCATACAAAC (SEQ ID NO:96)
rprE-1243F	GAACAAAGCCGCTTACAATACGATTNNSAAAATCGCGGTGAACAAAGCG (SEQ ID NO:97)
rprE-V260F	GCAGATTTACTATG1GC1CTGACGNNSCTCACTCGGTCATCAACTTTTAAAG (SEQ ID NO:98)
rprE-Y261F	GATTTACTATG1GC1CTGACCGTANNSTCACTCCGTCATCAACTTTTAAAG (SEQ ID NO:99)
rprE-1263F	GTGCTCTGACGCTATACCTCNNSCGCTCATCAACTTTTAAAGATGC (SEQ ID NO:100)
rprE-A273F	CCGTCATCAACTTTTAAAGATGCAAAANNSSGCTTTGATTCATCTCGCGGG (SEQ ID NO:101)
rprE-L282F	GATTTCAA1CTCGCGGGGACNNS1ACGCGCTCTCAAGATGCTGC (SEQ ID NO:102)
rprE-S285F	CGCGGGACCTTTACGGGONNSCAAGATGCTGCAAGCGTAG (SEQ ID NO:103)
rprE-A288F	CCTTTACGGCTCTCAAGATGCTNNSAGCGTAGAAGCTGCCCTGGAA1G (SEQ ID NO:104)
rprE-A293F	CTCAAGATGCTGCAAGCGTAGAANNSSGCTTGGAA1GCGAGTCCGGA1TG (SEQ ID NO:105)

(continued)

Table 10-1. Primers	
PRIMER	SEQUENCE
rprE-N296F	GCAAGCGTAGAAGCTGCTCCCTGCGNNSGAGTGGGATTGTAAACAAGAAAG (SEQ ID NO:106)
rprE-G298F	GAAGCTGCTTGGAAATCCAGTCTNNSTTGTAAACAAGAAAGAACCCGAAATCC (SEQ ID NO:107)
rprE-T80F	CACACTGCTATCCAGCACCCNNSAACCAAGTTTACAACCTTCTTCTCAG (SEQ ID NO:108)
rprE-R110F	CTCCGTTTCATTACGGCAGCGNNSACAAATAACGAGCCCTGGATC (SEQ ID NO:109)
rprE-D139F	CTCACCTCTTTCCGGTTCAATGNNSGTAACCGCTCATGAAATGACAC (SEQ ID NO:110)
rprE-T14R	OCCTTAAGAGTCGTACCTGTTCCSNNGTGTCGCGCATCTCCAC (SEQ ID NO:111)
rprE-G12R	GCCTTCAGAAGAAATATTTAATGAGACCGTTTSMKTTTAAGAGTCGTACCTGTTCGGG (SEQ ID NO:112)
rprE-Q45R	GGCAGGTTATACTCGCGGTTGNNCAGATCGTACGTAATAATTGTGTTCGG (SEQ ID NO:113)
rprE-Y49R	GGATACGAGTGTCCCGCCAGGTTGNNCTGCGGTTTCCAGATCGTAC (SEQ ID NO:114)
rprE-N50R	CTGATACGAGTGTGCCCCGCGCAGGAGGNNATACGCGGTTTTCAGATCTGAC (SEQ ID NO:115)
rprE-T65R	CAACGCGACCGCGCTGAGAAGASNNGTGTAACCTGTTTGTGCTGG (SEQ ID NO:116)
rprE-D119R	GTCCCGTCAACCGTAAATCATTTGSMKCCGATCCAGGCTGCGTTATGTATCTGC (SEQ ID NO:117)
rprE-G128R	GAACCGGAAAGAGTGCAGAAATGASNNGTGCGCGTCAACCTAAATCATTTGG (SEQ ID NO:118)
rprE-F130R	GGACCGGAAAGAGGTCAGAAASNNTGAACCGTCCCGCTCACC (SEQ ID NO:119)
rprE-Q151R	GTAGTTCAAGTTGGCTGTTTCSNNGTGTAACGCCATGTGTATTTTCATG (SEQ ID NO:120)
rprE-E152R	CGTAGTTCAGGTTGGCTGTSNNCTGTGTACGGCATGTGTATTTTCATG (SEQ ID NO:121)
rprE-N155R	CGGCTGATTTTCTGTAGTTCAGSNNGGCTGTTTCTGTGTAAACGCCCATG (SEQ ID NO:122)
rprE-T179R	CACCGATATCCAGTCTCTCSNNATCGTTGAAGTACCCGAATACATCAG (SEQ ID NO:123)
rprE-Y204R	GTAATTTTGAATATACGGCTGTCCSNNTTTGTCCGATTTGGATTAAGCTGC (SEQ ID NO:124)
rprE-G205R	GCCTTTTGTAAATTTTGAATATACGGCTGSMNGTATTTTGTCCGATTTGGATTAAGCTGC (SEQ ID NO:125)
rprE-Y224R	GTTTTGATGACGCCCGCCSMNGTCCCGGCAATCAGTGTTC (SEQ ID NO:126)
rprE-T243R	CGCTTTGTTACGCCGGAATTTSMMAATCGTATTTGAAGCGGCTTGTTC (SEQ ID NO:127)
rprE-V260R	CTTTAAAAGTTGATGACCGAGTGAGGTSNNCGTCAGACCCAGTAAATCTGC (SEQ ID NO:128)
rprE-Y261R	CTTTAAAAGTTGATGACCGAGTGAGSNNTACCGTCAGAGCCAGTAAATCTGC (SEQ ID NO:129)

(continued)

Table 10-1. Primers	
PRIMER	SEQUENCE
np1E-T263R	GCATCTTTAAAGTTGATGACGGSNMGAGGTATACCGTCAGAGCAC (SEQ ID NO:130)
np1E-A273R	CCGGCAGATTGAATCAAGCSNNTTTGCACTCTTTAAAGTTGATGACGG (SEQ ID NO:131)
np1E-L282R	GCAGCATCTTGAGAGCGGTASNNGTCCGCGCCAGATTGAATC (SEQ ID NO:132)
np1E-S285R	CTACGCTTGCAGCATCTTGSNNGCCGTAAAGTCCCGCG (SEQ ID NO:133)
np1E-A289R	CATTOAGGCAGCTTCTACGCTSNNAQCATCTTGAGAGCCGTAAGG (SEQ ID NO:134)
np1E-A293R	CAATCCGACTGCATTCAGGCSNNTTCTACGCTTGACSCATCTTCAG (SEQ ID NO:135)
np1E-N296R	CTTTCTTGTTTACAAATCCGACTGCSNNGCAGGCAGCTTCTACGCTTGC (SEQ ID NO:136)
np1E-G299R	CGATTTCCGGTCTCTTTCTTGTTTACAAASNNAGCTGCATTCAGGCGAGCTTC (SEQ ID NO:137)
np1E-T300R	CTGAGAGAAGTTGTAAACTGGTTSMNGGTCGCTGATACGAGTGTG (SEQ ID NO:138)
np1E-R110R	GATCCAGGCTGGGTTATTTASNNNGCTCCCGTAAATGAACGGAG (SEQ ID NO:139)
np1E-D139R	GTGTCAATTCATGAGCGGTTACSNNCATTCGAACCGGAAAGAGG1GAG (SEQ ID NO:140)
np1E-S135F	GGCAGCGTTCAATCTTCTCACTCTTNNSGTTCAATGAGCTTAACGCTC (SEQ ID NO:141)
np1E-G136F	GGGACGGTTCAATCTTCTCACTCTTTCGNNSTCAATGAGCTTAACGCTCATG (SEQ ID NO:142)
np1E-S137F	CTTCTCAGCTCTTCCGGTNNATGGACGTAACCGCTCATG (SEQ ID NO:143)
np1E-V140F	CGTCTTTCCSGTTCAATGGACNNSACCGCTCATGAAATGACAC (SEQ ID NO:144)
np1E-S137F	CAGCCAGCGGCTCTCCGNNSTTATCCAAATCCGACAAATACGGACAG (SEQ ID NO:145)
np1E-L198F	CAGCCAGCGGCTCTCCGACGNNSTCCAAATCCGACAAATACGGACAG (SEQ ID NO:146)
np1E-S192F	CAGCCAGCGGCTCTCCGACGTTANNSAATCCGACAAATACGGACAGCC (SEQ ID NO:147)
np1E-L218F	CAGCCTGATAATTTCAAAAATACAAAACNNSCCGAACACTGATGCCGGCGAC (SEQ ID NO:148)
np1E-P217F	CAGCCTGATAATTTCAAAAATACAAAACCTTNNSAACACTGATCCGGCGAC (SEQ ID NO:149)
np1E-N218F	CAGCCTGATAATTTCAAAAATACAAAACCTTCCGNNAGCTGATGCCGGCGACTAC (SEQ ID NO:150)
np1E-T219F	CAGCCTGATAATTTCAAAAATACAAAACCTTCCGAACNNSGATCCGGCGACTACGG (SEQ ID NO:151)
np1E-D220F	CAGCCTGATAATTTCAAAAATACAAAACCTTCCGAACACTNNSGCCGCGACTACGGCGCG (SEQ ID NO:152)
np1E-A221F	CAGCCTGATAATTTCAAAAATACAAAACCTTCCGAACACTGATNNSGCGACTACGGCGCGCTG (SEQ ID NO:153)

(continued)

Table 10-1. Primers	
PRIMER	SEQUENCE
rprE-G222F	CCTTCGGAACACTGATGCCNNSGACTACGGCGCGTGCAIAC (SEQ ID NO:154)
rprE-Q285F	CGCGACCTTTACGGCTCTNNSGATGCTGCAAGCGTAGAAGCTG (SEQ ID NO:155)
rprE-A297F	GCGTAGAAGCTGCTGGATNNSGTCCGATTGTAACAAGAAAAGAGACCGG (SEQ ID NO:156)
rprE-S135R	GAGCGGTTACGTCCATTGAACCCSNNAAGAGGTGAGAAGATGAACCGTCGC (SEQ ID NO:157)
rprE-G136R	CATGAGCGGTTACGTCCATTGASNNGGAAAGAGGTGAGAAGATGAACCGTCGC (SEQ ID NO:158)
rprE-S137R	CATGAGCGGTTACGTCCATSNNAACGGGAAAGAGGTGAGAAG (SEQ ID NO:159)
rprE-V140R	GTGTCAATTTCAATGAGCGGTSNNGTCCATTGAACCGGAAAGAGG (SEQ ID NO:160)
rprE-S197R	CTGTCCGTATTTTGTCCGATTGGA TAASNNGGGAGAGCCCGCTGGCTG (SEQ ID NO:161)
rprE-L198R	CTGTCCGTATTTTGTCCGATTGCA SNNGCTGCCGAGAGCCCGCTGGCTG (SEQ ID NO:162)
rprE-S199R	GGCTGTCCGTATTTTGTCCGATTSNNTAAGCTGCCGAGAGCCCGCTGGCTG (SEQ ID NO:163)
rprE-L216R	GTCCCGCGCATCAGTGTTCGGSNNGTTTTTGTAATTTTGAAATTATCAGGCTG (SEQ ID NO:164)
rprE-P217R	GTCCCGCGCATCAGTGTTSNNAAGGTTTTTGTAATTTTGAAATTATCAGGCTG (SEQ ID NO:165)
rprE-N218R	GTAGTCCCGCGCATCAGTSNNGGGAAGGTTTTTGTAATTTTGAAATTATCAGGCTG (SEQ ID NO:166)
rprE-T219R	CCGTAGTCCCGCGCATCSNNGTTCGGAAGGTTTTTGTAATTTTGAAATTATCAGGCTG (SEQ ID NO:167)
rprE-D220R	CGCCGCGGTAGTCCCGGCSNNAAGTGTTCGGAAGGTTTTTGTAATTTTGAAATTATCAGGCTG (SEQ ID NO:168)
rprE-A221R	CACGCGCGCGTAGTCCGCSNNAATCAGTGTTCGGAAGGTTTTTGTAATTTTGAAATTATCAGGCTG (SEQ ID NO:169)
rprE-G222R	GTATGCACGCGCGGTAGTCSNNGGCATCAGTGTTCGGAAGG (SEQ ID NO:170)
rprE-Q285R	CAGCTTCTACGCTTCACGCATCSNNAAGACCGTAACGTCGCCG (SEQ ID NO:171)
rprE-A297R	CCGGTCTCTTTTCTTGTATTACAAATCCGACSNNAATCCAGGCAGCTCTACGC (SEQ ID NO:172)

EXAMPLE 11

IDENTIFICATION OF NPPE HOMOLOGUES

In this Example, experiments conducted to identify npr homologues are described. In particular, in this Example, experiments were conducted to clone neutral protease (npr) homologs from different and closely related *Bacillus* species. The different species were chosen in order to explore the diversity and properties from which these different species are isolated.

The various npr homologs explored included:

B. caldolyticus npr (P23384)

B. cereus nprC (P05806)

10 *B. cereus* E33L npr (AAY60523)

B. stearothermophilus nprT

B. subtilis nprB

B. subtilis nprE

B. thasringiensis nprB (AAK00602)

15 *S. aureus* aur (P81177)

Figure 3 provides a sequence alignment of these homologs (SEQ ID NOS:173-181) and Figure 4 (SEQ ID NOS:182-191) provides another sequence alignment of various other homologs.

In these experiments, the materials included:

Chromosomal DNA of *B. subtilis* strain I168

20 The following DNA plasmids were synthesized at DNA2.0 with *B. subtilis* codon optimization:

pJ4:G01905 (*B. thuringiensis* nprB) (See, Figure 6)

pJ4:G01906 (*B. cereus* E33L npr) (See, Figure 7)

pJ4:G01907 (*B. cereus* nprC) (See, Figure 8)

pJ4:G01908 (*B. caldolyticus* npr) (See, Figure 9)

25 pJ4:G01909 (*S. aureus* aur) (See, Figure 10)

pJ4:G01938 (*S. stearothermophilus* nprT) (See, Figure 11)

pJHT vector (See, Figure 12)

pAC vector (See, Figure 13)

MJ Research PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories)

30 Primers (Operon Inc)

PfuUltra II Fusion HS DNA Polymerase (Stratagene)

Restriction endonucleases (Roche)

TOP10 chemically competent *E. coli* cells (Invitrogen)

B. subtilis competent cells ((Δ aprE, Δ nprE, oppA, Δ spoIIE, degUHy32, Δ amyE::(*xylR*,*pxylA*-comK)

Table 11. Primers	
Primer Name	Primer Sequence and SEQ ID NO:
EL-689	CGTCTTCAACAATTGTCCATTTTCTTCTGC (SEQ ID NO:196)
EL-693	CAGACAATTTCTTACCTAAACCCACTCTTACCCTCTCCTTTTAAAAAAATTC (SEQ ID NO:197)

Table 11. Primers	
Primer Name	Primer Sequence and SEQ ID NO:
EL-694	GAATTTTAAAAAGGAGAGGGTAAAGAGTGGGTTTAGGTAAGAAATTGTCTG (SEQ ID NO:198)
EL-695	GCTTATGGATCCCGTCGTTTCAGCTGAGAGAG (SEQ ID NO:199)
EL-696	GATGTCTTGGTCAAGTTGCGCACTCTTACCCTCTCCTTTTAAAAAAATTC (SEQ ID NO:200)
EL-697	GAATTTTAAAAAGGAGAGGGTAAAGAGTGCGCAACTTGACCAAGACATC (SEQ ID NO:201)
EL-698	GCCGGTTTTTATGTAAGCTTATAGAATGCCGACAGCCTCATACG (SEQ ID NO:202)
EL-699	CGTATGAGGCTGTGCGCATTCTATAAGCTTACATAAAAAACCGGCCTTGG (SEQ ID NO:203)
EL-700	AATGGTGCATGCAAGGAGATGGCG (SEQ ID NO:204)
EL-755	CGTCTTCAAGAATTCCTCCATTTTCTTCTGC (SEQ ID NO:205)
EL-733	GCACCCAACATTGCACGTTTATTCACTCTTACCCTCTCCTTTTAAAAAAATTC (SEQ ID NO:206)
EL-734	GAATTTTAAAAAGGAGAGGGTAAAGAGTGAATAAACGTGCAATGTTGGGTGC (SEQ ID NO:207)
EL-735	GCTTATAAGCTTAATATACTCCAACCGCGTTG (SEQ ID NO:208)
EL-739	CCAGCATAGCGCGTTTGTTCACCTCTTACCCTCTCCTTTTAAAAAAATTC (SEQ ID NO:209)
EL-740	GAATTTTAAAAAGGAGAGGGTAAAGAGTGAACAAACGCGCTATGCTGG (SEQ ID NO:210)
EL-741	GCTTATAAGCTTAATAGACACCCACGGCATTAAACGCC (SEQ ID NO:211)
EL-742	CAGGACAAGAGCTAAGGACTTTTTTTCACCTCTTACCCTCTCCTTTTAAAAAATTC (SEQ ID NO:212)
EL-743	GAATTTTAAAAAGGAGAGGGTAAAGAGTGAAAAAAAGTCCTTAGCTCTTGTCCTG (SEQ ID NO:213)
EL-744	GCTTATAAGCTTAATTAATGCCGACGGCAC (SEQ ID NO:214)

A. Cloning of *B. subtilis* nprE

- To construct the *B. subtilis* nprE plasmid, the amplified aprE promoter fragment (from pJHT vector) and *B. subtilis* nprE gene with terminator fragment (from *B. subtilis* strain I168) were separately prepared. Figure 15 provides a schematic, illustrating the amplification of the individual DNA fragments.

- PCR Splice Overlap Extension (SOE) reaction was used to join the 2 separate DNA fragments together. In this reaction, the following reagents were combined: 1ul aprE promoter DNA fragment, 1ul *B. subtilis* nprE gene + Terminator fragment, 1ul Primer EL-689 (25uM), 1ul Primer EL-695 (25uM), 5ul 10x PfuUltra II Fusion HS DNA polymerase buffer, 1ul dNTP (10mM), 1ul PfuUltra II Fusion HS DNA polymerase, and 39ul distilled, autoclaved water to provide a total reaction volume of 50 ul. The PCR cycles

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were: 95°C for 2 minutes (1st cycle only), followed by 28 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 0:45 seconds.

The PCR fusion fragment of aprE promoter-*B. subtilis* nprE gene + Terminator was digested with *Mfe*I and *Bam*HI restriction endonucleases. The pJHT vector was digested with *Eco*RI and *Bam*HI restriction endonucleases. The restriction endonuclease digested aprE promoter-*B. subtilis* nprE gene + Terminator DNA fragment was then ligated with the restriction endonuclease digested pJHT vector. The ligation mixture was then transformed into TOP10 chemically competent *E. coli* cells for selection on LA + 50ppm carbenicillin. After identification of plasmids containing the correct DNA construct sequence for plasmid pEL501 (See, Figure 16), transformed into competent *B. subtilis* cells for integration into aprE promoter locus. Transformants were selected for protease activity (*i.e.* skim milk clearing) on LA + 5ppm chloramphenicol + 1.6% skim milk plates. Amplified strains were then transferred to LA + 25ppm chloramphenicol + 1.6% skim milk plates. Strains were then transferred to LA + 25ppm chloramphenicol + 1.6% skim milk plates for amplification.

B. CLONING OF *B. SUBTILIS* NPRB

To construct the *B. subtilis* nprB plasmid, amplified the aprE promoter fragment (from pJHT vector), *B. subtilis* nprB gene fragment (from *B. subtilis* strain I168), and Terminator fragment (from pJHT vector) were separately prepared. Figure 17 provides a schematic diagram of the amplification of the individual DNA fragments.

PCR Splice Overlap Extension (SOE) reaction was used to join the three separate DNA fragments together. In this reaction, the following reagents were combined: 1ul aprE promoter DNA fragment, 1ul *B. subtilis* nprB gene + Terminator fragment, 1ul Terminator DNA fragment, 1ul Primer EL-689 (25uM), 1ul Primer EL-700 (25uM), 5ul 10x PfuUltra II Fusion HS DNA polymerase buffer, 1ul dNTP (10mM), 1ul PfuUltra II Fusion HS DNA Polymerase, and 38ul distilled, autoclaved water, for a 50 ul total reaction volume. The PCR cycles were: 95°C for 2 minutes (1st cycle only), followed by 28 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 0:45 seconds.

The PCR fusion fragment of aprE promoter-*B. subtilis* nprB gene + Terminator was digested with *Mfe*I and *Sph*I restriction endonucleases. The pJHT vector was digested with *Eco*RI and *Sph*I restriction endonucleases. The restriction endonuclease digested aprE promoter-*B. subtilis* nprB gene + Terminator DNA fragment was then ligated with the restriction endonuclease digested pJHT vector. The ligation mixture was then transformed into TOP10 chemically competent *E. coli* cells for selection on LA + 50ppm carbenicillin. After identification of plasmids containing the correct DNA construct sequence for plasmid pEL508 (See, Figure 18), transformed into competent *B. subtilis* cells for integration into aprE promoter locus. Transformants were selected for protease activity (*i.e.* skim milk clearing) on LA + 5ppm chloramphenicol + 1.6% skim milk plates. Amplified strains were then transferred to LA + 25ppm chloramphenicol + 1.6% skim milk plates. Strains were then transferred to LA + 25ppm chloramphenicol + 1.6% skim milk plates for amplification.

C. CLONING OF *B. STEAROTHERMOPHILUS* NPRT

To construct the *B. stearothermophilus* nprT plasmid, the amplified aprE promoter fragment (from pJHT vector) and *B. stearothermophilus* nprT fragment (from plasmid pJ4:G01938) were separately prepared. Figure 19 provides a schematic diagram of the amplification of the individual DNA fragments.

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PCR Splice Overlap Extension (SOE) reaction was used to join the 2 separate DNA fragments together. In this reaction, the following reagents were combined: 1ul aprE promoter DNA fragment, 1ul *B. stearothermophilus* nprT gene fragment, 1ul Primer EL-755 (25uM), 1ul Primer EL-735 (25uM), 5ul 10x PfuUltra II Fusion HS DNA Polymerase buffer, 1ul dNTP (10mM), 1ul PfuUltra II Fusion HS DNA Polymerase, and 39ul distilled, autoclaved water, to provide a total reaction volume of 50 ul. The PCR cycles were: 95°C for 2 minutes (1st cycle only), followed by 28 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 0:45 seconds.

The PCR fusion fragment of aprE promoter-*B. stearothermophilus* nprT gene + Terminator was digested with *Eco*RI and *Hind*III restriction endonucleases. The pAC vector was digested with *Eco*RI and *Hind*III restriction endonucleases. The restriction endonuclease digested aprE promoter-*B. stearothermophilus* nprT DNA fragment was then ligated with the restriction endonuclease digested pAC vector. TempliPhi rolling circle amplification was then used to generate large amounts of the ligated aprE promoter-*B. stearothermophilus* nprT pAC DNA molecule, using the manufacturer's protocol (*i.e.*, 1ul aprE promoter-*B. stearothermophilus* nprT pAC ligation reaction, 5ul TempliPhi Sample Buffer, 5ul TempliPhi Reaction Buffer, and 0.2ul TempliPhi Enzyme Mix, for an ~11ul total reaction; incubated at 30°C for 3 hours). The TempliPhi reaction was then transformed directly into competent *B. subtilis* cells for integration into aprE promoter locus, thereby generating *Bacillus* strain EL560, confirmed by DNA sequencing analysis (*See*, Figure 20). Transformants were selected for protease activity (*i.e.* skim milk clearing) on LA + 5ppm chloramphenicol + 1.6% skim milk plates. Strains were then transferred to LA + 25ppm chloramphenicol + 1.6% skim milk plates for amplification.

D. CLONING OF *B. CALDOLYTICUS* NPR

To construct the *B. caldolyticus* npr plasmid, the amplified aprE promoter fragment (from pJHT vector) and *B. caldolyticus* npr fragment (from plasmid pJ4:G01908) were separately prepared. Figure 21 provides a schematic diagram of the amplification of the individual DNA fragments.

PCR Splice Overlap Extension (SOE) reaction was used to join the 2 separate DNA fragments together. In this reaction, the following reagents were combined: 1ul aprE promoter DNA fragment, 1ul *B. caldolyticus* npr gene fragment, 1ul Primer EL-755 (25uM), 1ul Primer EL-741 (25uM), 5ul 10x PfuUltra II Fusion HS DNA Polymerase buffer, 1ul dNTP (10mM), 1ul PfuUltra II Fusion HS DNA Polymerase, and 39ul distilled, autoclaved water, to provide a total reaction volume of 50 ul. The PCR cycles were: 95°C for 2 minutes (1st cycle only), followed by 28 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 0:45 seconds.

The PCR fusion fragment of aprE promoter-*B. caldolyticus* npr gene + Terminator was digested with *Eco*RI and *Hind*III restriction endonucleases. The pAC vector was digested with *Eco*RI and *Hind*III restriction endonucleases. The restriction endonuclease digested aprE promoter-*B. caldolyticus* npr DNA fragment was then ligated with the restriction endonuclease digested pAC vector. TempliPhi rolling circle amplification was then used to generate large amounts of the ligated aprE promoter-*B. caldolyticus* npr pAC DNA molecule, using the manufacturer's protocol (*i.e.*, 1ul aprE promoter-*B. caldolyticus* npr pAC ligation reaction, 5ul TempliPhi Sample Buffer, 5ul TempliPhi Reaction Buffer, and 0.2ul TempliPhi Enzyme Mix, for an ~1 ul total reaction; incubated at 30°C for 3 hours). The TempliPhi reaction was then transformed directly into competent *B. subtilis* cells for integration into aprE promoter locus, thereby generating *Bacillus*

strain EL561 (See, Figure 22), confirmed by DNA sequencing analysis. Transformants were selected for protease activity (*i.e.* skim milk clearing) on LA + 5ppm chloramphenicol + 1.6% skim milk plates. Strains were then transferred to LA + 25ppm chloramphenicol + 1.6% skim milk plates for amplification.

E. CLONING OF *B. THURINGIENSIS* NPRB

5 To construct the *B. thuringiensis* nprB plasmid, the amplified aprE promoter fragment (from pJHT vector) and *B. thuringiensis* nprB fragment (from plasmid pJ4:G01905) were separately prepared. Figure 23 provides a schematic showing the amplification of the individual DNA fragments.

PCR Splice Overlap Extension (SOE) reaction was used to join the 2 separate DNA fragments together. In this reaction, the following reagents were combined: 1ul aprE promoter DNA fragment, 1ul *B.*
 10 *thuringiensis* nprB gene fragment, 1ul Primer EL-755 (25uM), 1ul Primer EL-744 (25uM), 5ul 10x PfuUltra II Fusion HS DNA Polymerase buffer, 1ul dNTP (10mM), 1ul PfuUltra II Fusion HS DNA Polymerase, and 39ul distilled, autoclaved water, to provide a total reaction volume of 50 ul. The PCR cycles were: 95°C for 2 minutes (1st cycle only), followed by 28 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 0:45 seconds.

15 The PCR fusion fragment of aprE promoter-*B. thuringiensis* nprB gene + Terminator was digested with *Eco*RI and *Hind*III restriction endonucleases. The pAC vector was digested with *Eco*RI and *Hind*III restriction endonucleases. The restriction endonuclease digested aprE promoter-*B. thuringiensis* nprB DNA fragment was then ligated with the restriction endonuclease digested pAC vector. The ligation mixture was then transformed into TOP10 chemically competent *E. coli* cells. After identification of plasmids containing
 20 the correct DNA construct sequence for plasmid pEL568, transformed into competent *B. subtilis* cells. Transformants were then selected for protease activity (*i.e.* skim milk clearing) on LA + 5ppm chloramphenicol + 1.6% skim milk plates. DNA plasmid preparation shows that plasmid pEL568 is stable in *B. subtilis* and does not integrate into the aprE promoter locus. Figure 24 provides a map of plasmid pEL568.

E. HOMOLOGY MODELING

25 In yet additional embodiments, a homology model of the mature domain of NprE from *Bacillus amyloliquefaciens* is provided. In these experiments, the protein sequence of the mature domain of the NprE sequence (SEQ ID NO:192) was run through the program BlastP (NCBI). This program matches the sequence to other known sequences of varying sequence identity. From the output, sequences for which an X-ray crystal structures are known were identified. These sequences included *S. aureus* Npr (pdb id 1BQB) *P.*
 30 *aeruginosa* Npr (pdb id 1EZM), *B. thermolyticus* thermolysin (pdb id 1KEI) and *B. cereus* Npr (pdb id 1NPC). Figure 5 provides a sequence alignment of the various sequences analyzed. (SEQ ID NOS:192-195)

The sequence of the *B. cereus* Npr was found to be the most identical to NprE. *B. thermolyticus* thermolysin (pdb id 1KEI) was excluded from subsequent steps as it is very similar to *B. cereus* Npr. A homology model was then prepared as detailed below, and all calculation were made using the program MOE
 35 (Chemical Computing).

First, the *S. aureus* Npr (pdb id 1BQB; SEQ ID NO:193) *P. aeruginosa* Npr (pdb id 1EZM)(SEQ ID NO:194), and *B. cereus* Npr (pdb id 1NPC)(SEQ ID NO:195) sequences were aligned using known structure, in order to obtain the most accurate sequence alignment (*i.e.*, a structure based sequence alignment was produced). Next, the NprE mature sequence was aligned to this structure based sequence alignment. Then, an
 40 initial homology model of NprE was produced using the *B. cereus* Npr (pdb id 1NPC) structure as a template,

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and using the sequence alignment of NprE to *B. cereus* Npr produced above. It was clear from inspection of this alignment that whereas *B. cereus* Npr contains four Ca^{2+} ion binding sites, while NprE only contains two Ca^{2+} ion binding sites.

- 5 Finally, after the initial homology model was built, the metal ions (*i.e.*, Zn^{2+} , and two Ca^{2+}) were computationally fixed, as were their respective protein ligands. The rest of the model structure was computationally minimized using the CHARMM22 parameter set, resulting in the final homology model.

EXAMPLE 12

WASH PERFORMANCE TESTS

- 10 In this Example, experiments conducted to determine the wash performance of the metalloprotease of the present invention are described. All wash performance tests were performed under American wash conditions, as indicated below:

LAUNDRY WASH PERFORMANCE TESTS

Equipment:	Terg-O-Tometer (US Testing) 6 pot bench top model
Temperature:	15°C / 60°F
Wash Time:	12 minutes
Agitation:	100 rpm
Rinse Time:	3 minutes
Water Hardness:	6 grains per gallon / 105 ppm as CaCO_3 (3/1 Ca^{+2} / Mg^{+2})
Sud concentration:	1.6 g/l TIDE@ 2005 liquid detergent base
Enzyme dosage:	0.00 - 0.55 - 2.75 - 5.50 mg active protein/l wash solution
Swatches:	EMPA 116 Fixed, fixated at 20°C: Blood, milk, ink on cotton (10 x 7.5 cm)
	EMPA 116 Unfixed: Blood, milk, ink on cotton (10 x 7.5 cm)
	Equest grass: Grass Medium scrubbed on cotton (10 x 7.5 cm)
	CFT C-10: Pigment, oil ,milk on cotton (10 x 7.5 cm)
	EMPA 221: Unsoiled cotton used as ballast (10 x 10 cm)
	6 EMPA 116 fixed + 2 EMPA 221 were put in one vessel
	6 EMPA 116 unfixed + 2 EMPA 221 were put in one vessel
	6 Equest grass + 2 EMPA 221 were put in one vessel
	6 CFT C-10 + 2 EMPA 221 were put in one vessel
Drying conditions:	Spin-drier, Grass stains were dried to the air, covered with dark clothes. The other stains were ironed.
Measuring swatches:	Tristimulus Minolta Meter CR-300 with equation L (L^*a^*b), D65 Std. Illuminate, on a white background. Expressed on Delta % Soil Removal.

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3 readings per swatch (before and after washing)

$$\% \text{ Stain Removal} = (L \text{ value after washing} - L \text{ value before washing}) / (L_{\text{white cotton}} - L \text{ value before washing}) \times 100\%$$

All experiments were done in quadruplicate

The proteases were tested in a specially developed washing test, using three different cotton swatches, soiled with:

- (a) milk, blood and ink (10.0 x 7.5 cm; EMPA), designated with the numbers 116 unfixed and fixed (the stains were fixed at 20°C);
- (b) grass medium (10.0 x 7.5 cm; Equest); and
- (c) pigment, oil and milk (10.0 x 7.5 cm designated with the numbers C-10 CFT).

These experiments are described in greater detail below. The washing tests were performed in a bench top model Terg-O-Tometer (US Testing), equipped with six stainless steel test vessels. The stainless steel test vessels each contained 1.6 g of TIDE® 2005 liquid detergent base, dissolved in 1000 ml water of 105 ppm/6 grains per gallon, and were each loaded with six of the same soiled cotton swatches and two extra ballast cotton swatches (EMPA 221). A selected protease (e.g., neutral metalloprotease or another protease) was added to each vessel in a concentration from 0.00 to 5.50 mg active protein per liter suds.

The tests were carried out for 12 minutes at 15 °C/60 °F, with an agitation of 100 rpm. After washing, the swatches were rinsed for 3 minutes under cold tap water and placed in a spin-drier. The grass swatches were air-dried and covered with dark clothing to limit the sensitivity of the grass stains to light. All other swatches were ironed. All experiments were performed in quadruplicate.

The reflectance of the tested swatches was measured with a Tristimulus Minolta Meter CR-300 using the equation $L (L^*a^*b)$. Wash performance values were calculated using the following relationship:

$$\% \text{ Stain Removal} = (L \text{ value after washing} - L \text{ value before washing}) / (L_{\text{white cotton}} - L \text{ value before washing}) \times 100\%$$

The results of the Terg-O-Tometer (TOM) assay for purified MULTIFECT® are shown in Figure 26, and compared with those of subtilisin (BPN' Y217L), a serine alkaline protease. The TOM provided a fully operational and valid means for discriminating between the different wash performances of various proteases (e.g., serine proteases, neutral metalloprotease, and variants thereof). The TOM tests were performed on BMI and Equest medium-soiled grass surface with TIDE® 2005 as the base detergent.

As indicated in Figure 26, it was apparent that the purified neutral metalloprotease clearly performed better in the wash test than the serine protease (BPN' Y217L). In particular, 2.75 ppm of purified neutral metalloprotease was required to show a delta soil removal of ~ 10 % compared to only 0.55 ppm of the serine protease (BPN' Y217L) on the Equest grass stain. The wash performance of the neutral metalloprotease was also tested at low temperature and found to perform very well medium solid Equest stain fabric. Similar results were obtained with purified commercially available MULTIFECT® neutral protease as with the recombinant nprE produced as described above.

EXAMPLE 13

PERFORMANCE OF NPPE VARIANTS IN BMI-TIDE® 2X PERFORMANCE ASSAY

In this Example, experiments conducted to assess the performance of various nprE variants in the BMI assay outlined above are described. The methods provided prior to Example 1 were used (*See*,
5 "Microswatch Assay for Testing Protease Performance"). The results for multiply-substituted variants with Performance Indices greater than one ($PI > 1$) and those with Performance Indices less than one ($PI < 1$) are provided in the Tables below.

In Table 13.1, data obtained for selected single-substitution variants in the BMI-TIDE® 2X performance assay are provided. The Table provides performance indices, which were calculated as
10 described above for the variants, which show improved performance compared to the WT enzyme. Those variants, which have a performance index greater than 1, have an improved performance.

In Table 13.2, data obtained for selected multiple-substitution variants in the BMI-TIDE® 2X performance assay are provided. The Table provides performance indices, which were calculated as
15 described above for the variants, which show improved performance compared to the WT enzyme. Those variants, which have a performance index greater than 1, have an improved performance.

Table 13.1 Performance Assay Results for All Variants with Performance Index >1

Variant Code	BMI Tide 2X Liquid Detergent [Perf. Index]
T004H	1.07
T004I	1.25
T004K	1.62
T004L	1.01
T004M	1.05
T004N	1.03
T004P	1.18
T004R	1.65
T004V	1.18
T004W	1.21
T004Y	1.32
G012I	1.24
G012K	1.64
G012L	1.25
G012M	1.11
G012Q	1.09
G012R	1.54
G012T	1.38
G012V	1.18
G012W	1.46
T014F	1.17
T014G	1.17
T014I	1.28
T014K	1.53
T014L	1.19
T014M	1.11
T014P	1.04
T014Q	1.24
T014R	1.48
T014S	1.07
T014V	1.14
T014W	1.17
T014Y	1.11
E022K	1.79
S023F	1.30
S023I	1.20
S023K	1.67

Table 13.1 Performance Assay Results for All Variants with Performance Index >1

Variant Code	BMI Tide 2X Liquid Detergent [Perf. Index]
S023L	1.27
S023M	1.04
S023P	1.23
S023Q	1.22
S023R	1.75
S023V	1.09
S023W	1.41
S023Y	1.06
G024F	1.26
G024H	1.33
G024I	1.24
G024K	1.70
G024L	1.23
G024M	1.14
G024N	1.28
G024P	1.18
G024R	1.67
G024T	1.07
G024V	1.12
G024W	1.42
G024Y	1.12
K033H	1.01
Q045F	1.25
Q045H	1.25
Q045I	1.40
Q045K	1.64
Q045L	1.24
Q045N	1.07
Q045R	1.68
Q045T	1.09
Q045W	1.60
N046A	1.06
N046F	1.11
N046G	1.07
N046H	1.32
N046K	1.61
N046L	1.14

Table 13.1 Performance Assay Results for All Variants with Performance Index >1

Variant Code	BMI Tide 2X Liquid Detergent [Perf. Index]
N046M	1.13
N046Q	1.22
N046R	1.19
N046S	1.02
N046T	1.20
N046W	1.24
N046Y	1.21
R047K	1.15
Y049F	1.06
Y049I	1.08
Y049K	1.10
Y049L	1.06
Y049R	1.54
Y049W	1.34
N050F	1.38
N050H	1.13
N050I	1.36
N050K	1.65
N050L	1.35
N050M	1.05
N050P	1.12
N050Q	1.16
N050R	1.81
N050W	1.46
N050Y	1.27
T054F	1.16
T054G	1.12
T054H	1.17
T054I	1.34
T054K	1.47
T054L	1.26
T054N	1.25
T054Q	1.23
T054R	1.46
T054S	1.03
T054V	1.11
T054W	1.22

Table 13.1 Performance Assay Results for All Variants with Performance Index >1	
Variant Code	BMI Tide 2X Liquid Detergent [Perf. Index]
T054Y	1.08
S058H	1.03
S058N	1.12
S058Q	1.08
T059G	1.11
T059H	1.32
T059I	1.43
T059K	1.60
T059L	1.31
T059M	1.10
T059N	1.16
T059P	1.19
T059Q	1.31
T059R	1.56
T059V	1.13
T059W	1.32
T060F	1.07
T060I	1.09
T060K	1.49
T060L	1.13
T060N	1.07
T060Q	1.10
T060R	1.42
T060V	1.13
T060W	1.23
T060Y	1.07
T065F	1.06
T065H	1.07
T065I	1.12
T065K	1.32
T065L	1.10
T065M	1.09
T065P	1.11
T065Q	1.01
T065R	1.28
T065V	1.15
T065Y	1.09

Table 13.1 Performance Assay Results for All Variants with Performance Index >1	
Variant Code	BMI Tide 2X Liquid Detergent [Perf. Index]
S066F	1.05
S066H	1.06
S066I	1.24
S066K	1.44
S066L	1.09
S066N	1.00
S066Q	1.12
S066R	1.47
S066V	1.19
S066W	1.21
S066Y	1.06
Q087H	1.06
Q087I	1.17
Q087K	1.30
Q087L	1.07
Q087M	1.00
Q087N	1.06
Q087R	1.35
Q087T	1.08
Q087V	1.04
Q087W	1.15
N090F	1.05
N090H	1.09
N090K	1.37
N090L	1.18
N090R	1.37
N096G	1.00
N096H	1.04
N096K	1.54
N096R	1.06
K097H	1.03
K097Q	1.05
K097W	1.02
K100R	1.26
R110K	1.05
D119E	1.05
D119H	1.16

Table 13.1 Performance Assay Results for All Variants with Performance Index >1	
Variant Code	BMI Tide 2X Liquid Detergent [Perf. Index]
D119I	1.09
D119L	1.21
D119Q	1.17
D119R	1.14
D119S	1.10
D119T	1.23
D119V	1.24
D119W	1.09
G128F	1.10
G128H	1.27
G128K	1.90
G128L	1.20
G128M	1.11
G128N	1.23
G128Q	1.22
G128R	1.94
G128W	1.48
G128Y	1.42
S129A	1.12
S129F	1.11
S129G	1.03
S129H	1.17
S129K	1.33
S129L	1.01
S129R	1.37
S129T	1.04
S129V	1.01
S129W	1.28
S129Y	1.25
F130I	1.03
F130K	1.26
F130R	1.37
F130Y	1.31
S135P	1.03
M138K	1.36
M138Q	1.03
M138V	1.10

Table 13.1 Performance Assay Results for All Variants with Performance Index >1	
Variant Code	BMI Tide 2X Liquid Detergent [Perf. Index]
V140C	1.03
Q151I	1.02
E152A	1.14
E152C	1.15
E152D	1.14
E152F	1.09
E152G	1.03
E152H	1.15
E152L	1.15
E152M	1.12
E152N	1.11
E152R	1.19
E152S	1.02
E152W	1.06
N155D	1.13
N155K	1.05
N155R	1.14
T179A	1.03
T179F	1.15
T179H	1.20
T179I	1.21
T179K	1.62
T179L	1.20
T179M	1.12
T179N	1.04
T179P	1.04
T179Q	1.23
T179R	1.49
T179S	1.02
T179V	1.12
T179W	1.05
T179Y	1.07
V190H	1.02
V190I	1.16
V190K	1.75
V190Q	1.23
V190R	1.67

Table 13.1 Performance Assay Results for All Variants with Performance Index >1	
Variant Code	BMI Tide 2X Liquid Detergent [Perf. Index]
S191F	1.18
S191G	1.03
S191H	1.29
S191I	1.12
S191K	1.58
S191L	1.07
S191N	1.13
S191Q	1.13
S191R	1.74
S191W	1.16
L198M	1.19
L198V	1.05
S199F	1.10
S199I	1.08
S199K	1.64
S199L	1.15
S199N	1.14
S199Q	1.14
S199R	1.68
S199V	1.06
Y204H	1.03
G205F	1.13
G205H	1.61
G205L	1.14
G205M	1.14
G205N	1.39
G205R	2.07
G205S	1.25
G205Y	1.21
K211R	1.23
K214R	1.19
L216F	1.13
L216H	1.05
L216Q	1.05
L216R	1.64
L216Y	1.02
N218K	1.57

Table 13.1 Performance Assay Results for All Variants with Performance Index >1	
Variant Code	BMI Tide 2X Liquid Detergent [Perf. Index]
N218P	1.27
D220E	1.05
D220H	1.00
D220N	1.04
D220P	1.05
A221F	1.13
A221I	1.14
A221K	1.49
A221L	1.05
A221M	1.01
A221N	1.05
A221V	1.14
A221Y	1.17
G222H	1.01
G222N	1.01
G222R	1.04
Y224F	1.05
Y224H	1.29
Y224N	1.23
Y224R	1.12
T243G	1.13
T243H	1.48
T243I	1.06
T243K	1.87
T243L	1.11
T243Q	1.36
T243R	1.62
T243W	1.25
T243Y	1.14
V260A	1.69
V260D	1.59
V260E	1.17
V260G	2.00
V260H	1.36
V260I	2.09
V260K	1.45
V260L	1.18

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Table 13.1 Performance Assay Results for All Variants with Performance Index >1	
Variant Code	BMI Tide 2X Liquid Detergent [Perf. Index]
V260M	1.41
V260P	1.45
V260Q	1.73
V260R	1.47
V260S	1.59
V260T	1.66
V260W	1.83
V260Y	1.20
T263H	1.06
S265K	1.30
S265N	1.04
S265R	1.28
S265W	1.00
A273I	1.19
A273K	1.47
A273L	1.14
A273N	1.10
A273Q	1.00
A273R	1.78
A273Y	1.07
L282F	1.09
L282G	1.14
L282H	1.17
L282I	1.23
L282K	1.67
L282M	1.01
L282N	1.08
L282Q	1.17
L282R	1.41
L282V	1.22
S285K	1.20
S285R	1.23
Q286K	1.22
Q286R	1.14
A289K	1.23
A289R	1.32
A293R	1.36

Table 13.1 Performance Assay Results for All Variants with Performance Index >1	
Variant Code	BMI Tide 2X Liquid Detergent [Perf. Index]
N296K	1.28
N296R	1.42
A297K	1.56
A297N	1.02
A297Q	1.02
A297R	1.50
G299N	1.02

Table 13.2 BMI Performance Assay Results for All Variants with Performance Index > 1	
Variant Code	BMI TIDE® 2X Liquid Detergent [Perf. Index]
S023W-G024M	2.36
T004V-S023W-G024W	2.25
S023W-G024Y-A288V	2.14
T004L-S023W-G024Y	2.09
N046Q-N050F-T054L	2.03
N050Y-T059R-S129Q	1.97
S023W-G024W	1.97
A273H-S285P-E292G	1.94
S023Y-G024Y	1.93
S023Y-G024W	1.92
T004S-S023Y-G024W	1.91
N046Q-T054K	1.90
S023W-G024Y	1.90
T004V-S023W	1.89
T059K-S066N	1.88
N046Q-N050W-T054H-T153A	1.87
T004V-S023W-G024Y	1.85
L282M-Q286P-A289R	1.83
N046Q-R047K-N050Y-T054K	1.82
L044Q-T263W-S285R	1.81
T004L-S023W-G024W	1.79
R047K-N050F-T054K	1.78
A273H-S285R	1.78
N050Y-T059K-S066Q	1.78
T054K-Q192K	1.76
N046Q-N050W	1.75
L282M-Q286K	1.75
T059K-S066Q	1.74
T004S-S023W	1.74
L282M-Q286R-A289R-K11N	1.73
L282M-A289R	1.73
N046Q-N050W-T054H	1.73
T059K-S129Q	1.72

Table 13.2 BMI Performance Assay Results for All Variants with Performance Index > 1	
Variant Code	BMI TIDE® 2X Liquid
T004S-S023N-G024Y-F210L	1.71
T004V-S023W-G024M-A289V	1.70
L282M-Q286K-A289R-S132T	1.70
N050W-T054H	1.70
L282M-Q286R	1.69
L282F-Q286K-A289R	1.69
T059R-S066Q	1.68
R047K-N050W-T054H	1.68
S265P-L282M-Q286K-A289R	1.66
L282M-Q286R-T229S	1.66
L282F-Q286K	1.66
T263W-S285R	1.65
S265P-L282M-Q286K	1.65
T263H-A273H-S285R	1.65
T059R-S129V	1.64
S032T-T263H-A273H-S285R	1.64
T059R-S066Q-S129Q	1.64
T004S-G024W	1.64
T004V-S023W-G024M	1.64
T059K-S066Q-S129Q	1.63
L282M-Q286K-A289R-I253V	1.63
T004V-S023Y-G024W	1.63
T059R-S066N-S129Q	1.62
N050F-T054L	1.62
T004S-S023N-G024W	1.62
T059R-S066N	1.62
T059R-S066N-S129V	1.60
Q286R-A289R	1.60
N046Q-R047K-N050F-T054K	1.60
S265P-L282M-Q286R-A289R	1.57

Table 13.2 BMI Performance Assay Results for All Variants with Performance Index > 1	
Variant Code	BMI TIDE® 2X Liquid
S265P-L282M-Q286P-A289R	1.68
Q062K-S066Q-S129I	1.59
S023N-G024W	1.59
N046Q-R047K-N050W-T054H	1.58
R047K-T054K	1.58
T004L-G024W	1.58
T014M-T059R-S129V	1.58
T059R-S066Q-N092S-S129I	1.58
R047K-N050W-T054K	1.58
T004V-G024W	1.58
N047K-N050F-T054K	1.57
S265P-L282F-Q286K-N061Y	1.57
L282F-Q286K-E159V	1.57
T004V-S023Y-G024M	1.57
S265P-L282F-A289R-T065S	1.55
T059K-F063L-S066N-S129V	1.55
T004L-S023W	1.55
N050F-T054H	1.55
T059R-S066Q-S129V	1.54
V190I-D220E-S265W-L282F	1.54
T004S-S023Y-G024M	1.53
T004L-S023N-G024Y	1.53
T059K-S066N-S129I	1.53
T059R-S066N-S129I	1.53
L282M-Q286R-A289R-P162S	1.52
N046Q-N050F-T179N	1.52
T059K-Y082C-S129V	1.52
T059K-S129I	1.52
N050Y-T054K	1.51
T059K-S066Q-V102A-	1.51

Table 13.2 BMI Performance Assay Results for All Variants with Performance Index > 1	
Variant Code	BMI TIDE® 2X Liquid
S129Q	
T059R-S066Q-S129I	1.51
T059W-S066N-S129V-S290R	1.51
T059R-S129I	1.50
T059K-S066Q-S129I	1.50
T059K-S066Q-S129V	1.50
S265P-L282M-Q286R-A289R-T202S-K203N	1.49
T004V-S023N-G024W	1.49
S265P-Q286K	1.49
S265P-L282F-A289R	1.49
D220P-S265W	1.49
L055F-T059W-S129V	1.49
T059R-S129Q-S191R	1.49
N050W-T054K	1.49
T004S-S023W-G024M	1.49
R047K-N050F-T054H	1.48
T059K-S066N-K088E	1.48
T059K-S066Q-S129I-V291L	1.48
L282M-Q286R-A289R	1.48
T059R-S066N-F085S-S129I	1.47
L282F-Q286P-A289R	1.45
L282F-Q286R-A289R	1.47
G099D-S265P-L282F-Q286K-A289R	1.46
N046Q-N050F	1.46
N050Y-T059W-S066N-S129V	1.45
T009I-D220P-S265N	1.45
V190F-D220P-S265W	1.45
N157Y-T263W-A273H-S285R	1.44
T263W-A273H-S285R	1.44
T263W-S285W	1.44
T004V-S023Y	1.43

**Table 13.2 BMI Performance Assay
Results for All Variants with
Performance Index > 1**

Variant Code	BMI TIDE® 2X Liquid
N046Q-R047K-N050W	1.42
N050W-T054L	1.42
N200Y-S265P-L282F- Q286P-A289R	1.42
T059R-S066Q-P264Q	1.42
T004V-G024Y	1.40
T004L-G024Y	1.40
N050Y-S191I	1.39
N050Y-T054L	1.39
T004L-S023W-G024Y- N155K	1.39
F169I-L282F-Q286R- A289R	1.39
L282M-Q286K-A289R	1.38
F130L-M138L-E152W- D183N	1.38
N046Q-R047K-N050Y- T054H	1.38
T004V-G024M	1.38
N050Y-T059W-S066Q- S129V	1.37
S023N-G024Y	1.37
T054H-P162Q	1.37
T004S-S023W-G024Y	1.37
N050Y-T054H	1.36
L282F-Q286R-A289R- F169I	1.35
R047K-N050W	1.35
V190F-D220P	1.35
L282M-F173Y	1.34
T004L-S023Y	1.33
N050W-A288D	1.33
V190I-D220P-S265Q	1.33
S265P-L282F-Q286P- A289R	1.24
S265P-L282F-Q286R- A289R	1.39
N046Q-N050Y-T054K	1.33

**Table 13.2 BMI Performance Assay
Results for All Variants with
Performance Index > 1**

Variant Code	BMI TIDE® 2X Liquid
T059W-S066Q	1.31
T263W-A273H-S285R	1.44
T263W-A273H-S285W	1.27
S023Y-G024M	1.30
T004L-S023N-G024W	1.30
T004V-S023N-G024Y	1.30
T059W-S066N-S129Q	1.30
T004S-S023Y	1.29
T004S-S023N-G024M	1.29
T059W-S066N-A070T	1.29
T059W-S066Q-S129Q	1.29
T263W-A273H	1.29
A273H-S285P	1.28
N046Q-R047K-N050Y- T054L	1.28
N046Q-R047K-N050Y	1.28
R047K-N050Y	1.27
T263H-S285W	1.26
R047K-N050F	1.25
N046Q-R047K-N050F- T054H	1.25
S023N-G024M	1.25
T004S-G024Y	1.24
R047K-N050Y-T054H	1.24
T059W-S066N-S129I	1.22
R047K-T054L	1.21
T004S-S023W-G024W	1.21
M138L-E152F-T146S	1.21
D220P-S265N	1.21
T004S-G024M	1.20
T004V-S023N	1.20
N046Q-N050F-T054K	1.19
N046Q-N050Y-T054H	1.19
Q062H-S066Q-S129Q	1.19
T059W-S129Q	1.19
T059W-S129V	1.19
N050F-T054K	1.18
R047K-N050F-T054L	1.18

**Table 13.2 BMI Performance Assay
Results for All Variants with
Performance Index > 1**

Variant Code	BMI TIDE® 2X Liquid
V190I-D220P-S265W	1.18
N112I-T263H-A273H-S285R	1.17
T059W-S066N-S129V	1.17
T059W-S066Q-S129I	1.17
T059W-S129I	1.17
T263W-S285P	1.17
V190I-D220P	1.16
A289V-T263H-A273H	1.16
T263H-A273H-S285P	1.16
N90S-A273H-S285P	1.15
R047K-N050Y-T054L	1.15
T004S-S023N	1.15
T059R-S129Q	1.14
N046Q-R047K-T054H	1.14
T059W-S066Q-S129V	1.13
E152W-T179P	1.13
N050Y-S066Q-S129V	1.13
T202S-T263W-A273H	1.13
T263W-A273H-S285P	1.13
M138L-E152W-T179P	1.11
N046Q-R047K	1.10
N046Q-T054H-F176L	1.10
T004L-G024M	1.10
T004S-L282M	1.10
T263H-A273H	1.10
T263H-A273H-S285W	1.10
T004L-S023Y-G024M	1.09
L282F-Q286P	1.09
T004V-S023Y-G024Y	1.09
V190F-S265W	1.09
M138L-E152F	1.08
V190F-D220E-S265W	1.07
N046Q-N050F-T054H	1.06
N157Y-S285W	1.06
T004F-S023Y-G024M	1.06
T004V-S023N-G024M	1.06
L198I-D220E-S265Q	1.05

**Table 13.2 BMI Performance Assay
Results for All Variants with
Performance Index > 1**

Variant Code	BMI TIDE® 2X Liquid
N046Q-N050Y-T054K-A154T	1.05
S016L-D220E-S265W	1.05
D220E-S265W	1.04
D220E-A237S-S265W	1.04
S066Q-S129Q	1.04
V190F-D220E-S265Q-T267I	1.04
L282M-F173Y-T219S	1.04
E152F-T179P	1.04
V190I-S265W	1.03
M138L-S066Q	1.01
M138L-E152W	1.01
T059W-S066Q-A070T-S129I	1.01
V190F-D220E-S265N	1.01
V190F-S265N	1.01
N046Q-N050Y	1.01
M138L-E152F-T179P	1.00

EXAMPLE 14

STABILITY OF NPfE VARIANTS

In this Example, experiments conducted to determine the stability of nprE variants are described. In these experiments, the methods describe prior to Example 1 were used to determine the performance indices (See, "NprE stability assays in the presence of detergent" above). The following tables provide the results for those variants with Performance Indices greater than one (PI>1) tested with and without DTPA.

The stability was measured by determining AGLA activity before and after incubation at elevated temperature. The table contains the relative stability values compared to WT under these conditions. It is the quotient of (Variant residual activity/WT residual activity). A value greater than one indicates higher stability

in the presence of detergent. In Tables 14.1 and 14.2, data are provided showing the relative stability data of single-substitution variants of NprE relative to the stability of the WT NprE stability under these conditions, with and without DTPA.

In Tables 14.3 and 14.4, data are provided showing the relative stability data of multiple-substitution variants of NprE relative to the stability of the WT NprE stability under these conditions, with and without DTPA.

Table 14.1 Stability Results in the Presence of 25% TIDE® 2X with DTPA	
Variant Code	Stability in the presence of 25% Tide 2x with DTPA
T004C	1.19
T004E	1.05
T004L	1.13
T004S	1.00
G012D	1.06
G012E	1.06
K013A	1.39
K013C	1.57
K013D	1.09
K013F	1.30
K013G	1.41
K013H	1.34

Table 14.1 Stability Results in the Presence of 25% TIDE® 2X with DTPA	
Variant Code	Stability in the presence of 25% Tide 2x with DTPA
K013I	1.33
K013L	1.56
K013M	1.28
K013N	1.39
K013Q	1.34
K013S	1.35
K013T	1.22
K013V	1.40
K013Y	1.34
S023A	1.01
S023D	1.08
S023F	1.05

Table 14.1 Stability Results in the Presence of 25% TIDE® 2X with DTPA	
Variant Code	Stability in the presence of 25% Tide 2x with DTPA
S023G	1.11
S023I	1.05
S023K	1.07
S023L	1.04
S023M	1.11
S023N	1.09
S023Q	1.03
S023R	1.10
S023S	1.45
S023T	1.06
S023V	1.05
S023W	1.08

Table 14.1 Stability Results in the Presence of 25% TIDE® 2X with DTPA	
Variant Code	Stability in the presence of 25% Tide 2x with DTPA
S023Y	1.15
G024A	1.01
G024D	1.05
G024F	1.08
G024G	1.46
G024H	1.05
G024K	1.08
G024L	1.06
G024M	1.10
G024N	1.11
G024R	1.07
G024S	1.02
G024S	1.02
G024T	1.04
G024W	1.11
G024Y	1.08
Q045D	1.02
Q045E	1.28
N046C	1.29
N046E	1.35
N046Q	1.07
R047K	1.09
R047L	1.13
R047M	1.00
R047S	1.21
N050D	1.04
N050F	1.07
N050P	1.03
N050W	1.04
N050Y	1.04
T054C	1.04
T054D	1.04
T054E	1.03
T054F	1.03

Table 14.1 Stability Results in the Presence of 25% TIDE® 2X with DTPA	
Variant Code	Stability in the presence of 25% Tide 2x with DTPA
T054H	1.11
T054I	1.04
T054K	1.11
T054L	1.08
T054M	1.06
T054N	1.07
T054Q	1.03
T054R	1.04
T054S	1.05
T054V	1.01
T054W	1.07
T054Y	1.07
T059A	1.04
T059C	1.04
T059E	1.02
T059G	1.13
T059H	1.07
T059I	1.01
T059K	1.16
T059M	1.10
T059N	1.15
T059P	1.12
T059Q	1.04
T059R	1.28
T059S	1.04
T059W	1.26
T060N	1.03
T065E	1.01
S066C	1.36
S066D	1.42
S066E	1.58
S066N	1.01
S066Q	1.01
Q087D	1.25

Table 14.1 Stability Results in the Presence of 25% TIDE® 2X with DTPA	
Variant Code	Stability in the presence of 25% Tide 2x with DTPA
Q087E	1.32
N090C	1.10
N090D	1.01
K100H	1.09
K100P	1.01
R110A	1.17
R110C	1.28
R110E	1.20
R110H	1.12
R110K	1.04
R110L	1.23
R110M	1.23
R110N	1.11
R110Q	1.28
R110S	1.10
R110Y	1.12
D119H	1.15
G128C	1.00
S129A	1.06
S129C	1.38
S129D	1.23
S129H	1.30
S129I	1.68
S129K	1.05
S129L	1.35
S129M	1.33
S129Q	1.44
S129T	1.36
S129V	1.55
S129Y	1.06
F130I	1.14
F130K	1.04
F130L	1.52
F130M	1.66

**Table 14.1 Stability Results
in the Presence of 25%
TIDE® 2X with DTPA**

Variant Code	Stability in the presence of 25% Tide 2x with DTPA
F130Q	1.10
F130T	1.41
F130V	1.06
S137A	1.46
M138L	1.43
E152F	1.15
E152H	1.36
E152W	1.31
T179P	1.50
V190I	1.68
V190L	1.93
S199C	1.27
S199E	1.95
Y204T	1.03
K211A	1.96
K211C	1.30
K211D	1.89
K211M	1.20
K211N	1.29
K211Q	2.00
K211S	1.43
K211T	1.18
K211V	1.52
K214A	1.74
K214C	1.62
K214I	1.17
K214M	1.27
K214N	1.35
K214Q	2.09
K214V	2.00
L216C	1.35
T219D	1.05
D220A	1.11
D220E	2.44

**Table 14.1 Stability Results
in the Presence of 25%
TIDE® 2X with DTPA**

Variant Code	Stability in the presence of 25% Tide 2x with DTPA
D220P	2.66
A221D	1.04
A221E	1.57
G222C	1.72
T243C	1.30
T243I	1.17
K244A	1.61
K244C	1.75
K244D	2.00
K244E	1.77
K244F	1.27
K244G	1.23
K244L	1.55
K244M	1.79
K244N	1.25
K244Q	1.82
K244S	1.87
K244T	1.65
K244V	1.82
K244W	1.01
K244Y	1.45
V260E	1.07
V260K	1.17
V260L	1.28
V260M	1.21
V260P	1.22
V260S	1.00
V260T	1.03
V260W	1.02
Y261C	1.28
Y261F	1.07
Y261I	1.20
Y261L	1.14
T263E	1.12

**Table 14.1 Stability Results
in the Presence of 25%
TIDE® 2X with DTPA**

Variant Code	Stability in the presence of 25% Tide 2x with DTPA
T263F	1.19
T263H	1.01
T263L	1.02
T263Q	1.12
T263V	1.25
T263W	1.40
T263Y	1.06
S265A	1.04
S265C	1.11
S265D	1.11
S265E	1.34
S265P	1.72
S265Q	1.00
S265T	1.15
S265V	1.17
K269E	1.61
K269F	1.21
K269G	1.32
K269H	1.63
K269I	1.73
K269L	1.53
K269M	1.52
K269N	1.60
K269P	1.47
K269Q	1.55
K269S	1.51
K269T	1.89
K269V	1.43
K269W	1.00
K269Y	1.38
A273C	1.19
A273D	1.29
A273H	1.14
R280A	1.33

**Table 14.1 Stability Results
in the Presence of 25%
TIDE® 2X with DTPA**

Variant Code	Stability in the presence of 25% Tide 2x with DTPA
R280C	1.96
R280D	1.82
R280E	1.77
R280F	1.46
R280G	1.21
R280H	1.52
R280K	1.14
R280L	1.78
R280M	1.78
R280S	1.46
R280T	1.35
R280W	1.51
R280Y	1.56
L282F	1.06
L282M	1.16
L282Y	1.04
S285A	1.16
S285C	1.27
S285D	1.39
S285E	1.59
S285K	1.00
S285P	1.30
S285Q	1.10
S285R	1.38
S285W	1.28
Q286A	1.04
Q286D	1.08
Q286E	1.31
Q286K	1.09
Q286P	1.15
Q286R	1.18
A289C	1.24
A289D	1.04
A289E	1.15

**Table 14.1 Stability Results
in the Presence of 25%
TIDE® 2X with DTPA**

Variant Code	Stability in the presence of 25% Tide 2x with DTPA
A289L	1.05
A293C	1.11
N296D	1.11
N296E	1.87
N296V	1.37
A297C	1.07

Table 14.2 Stability of Variants in Tide @ 2X Without DTPA	
Variant Code	Stability in the presence of Tide 2x without DTPA
T004C	1.16
T004V	1.04
K013A	1.52
K013C	1.83
K013D	1.47
K013F	1.02
K013G	1.61
K013H	1.62
K013I	1.19
K013L	1.54
K013M	1.48
K013N	1.70
K013Q	1.55
K013S	1.56
K013T	1.39
K013V	1.49
K013Y	1.39
S023A	1.03
S023D	1.23
S023G	1.25
S023M	1.05
S023N	1.25
S023Q	1.10
S023S	1.50
S023W	1.02
S023Y	1.07
G024D	1.05
G024G	1.41
Q045C	1.01
Q045D	1.02
Q045E	1.41
Q045M	1.01
N046C	1.53
N046E	1.41
R047K	1.12
R047L	1.20
R047M	1.08

Table 14.2 Stability of Variants in Tide @ 2X Without DTPA	
Variant Code	Stability in the presence of Tide 2x without DTPA
R047Q	1.13
R047S	1.25
Y049D	1.16
Y049H	1.02
Y049N	1.07
Y049S	1.01
N050D	1.08
N050F	1.07
N050G	1.02
N050P	1.23
N050W	1.01
T054C	1.07
T054D	1.01
T054E	1.08
T054H	1.08
T054I	1.09
T054K	1.03
T054L	1.09
T054Q	1.09
T054V	1.14
T054W	1.02
T054Y	1.14
T059A	1.05
T059C	1.07
T059E	1.25
T059M	1.04
T059P	1.18
T059Q	1.05
T059S	1.09
T065C	1.04
T065E	1.07
S066C	1.61
S066D	1.61
S066E	1.80
S066N	1.08
Q087D	1.27
Q087E	1.30

Table 14.2 Stability of Variants in Tide @ 2X Without DTPA	
Variant Code	Stability in the presence of Tide 2x without DTPA
N090C	1.09
N090D	1.00
N090E	1.03
K100A	1.00
K100D	1.07
K100E	1.03
K100F	1.07
K100H	1.16
K100N	1.06
K100P	1.06
K100Q	1.06
K100S	1.05
K100Y	1.10
R110A	1.11
R110C	1.24
R110E	1.19
R110H	1.09
R110K	1.08
R110L	1.11
R110M	1.12
R110N	1.18
R110Q	1.25
R110S	1.09
R110Y	1.16
D119H	1.03
G128C	1.15
S129A	1.13
S129C	1.86
S129D	1.52
S129H	1.60
S129I	2.32
S129K	1.18
S129L	1.70
S129M	1.64
S129Q	1.86
S129T	1.59
S129V	2.34

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Table 14.2 Stability of Variants in Tide @ 2X Without DTPA	
Variant Code	Stability in the presence of Tide 2x without DTPA
S129Y	1.28
F130I	1.18
F130L	1.29
F130M	1.44
F130Q	1.17
F130T	1.32
F130V	1.05
S137A	1.37
M138L	1.11
E152A	1.01
E152C	1.16
E152F	1.32
E152H	1.53
E152N	1.12
E152W	1.32
N155Q	1.07
T179P	1.33
V190I	1.37
V190L	1.40
S199C	1.18
S199D	1.11
S199E	1.71
K211A	1.77
K211C	1.18
K211D	1.67
K211G	1.06
K211M	1.17
K211N	1.44
K211Q	1.51
K211S	1.44
K211T	1.17
K211V	1.26
K214A	1.47
K214C	1.54
K214E	1.42
K214I	1.14
K214M	1.19
K214N	1.15

Table 14.2 Stability of Variants in Tide @ 2X Without DTPA	
Variant Code	Stability in the presence of Tide 2x without DTPA
K214Q	1.84
K214V	1.79
L216C	1.31
D220A	1.07
D220E	2.23
D220P	2.24
A221D	1.15
A221E	1.47
G222C	1.89
T243C	1.34
T243I	1.13
K244A	1.57
K244C	1.40
K244D	1.58
K244E	1.56
K244F	1.05
K244G	1.01
K244L	1.38
K244M	1.37
K244N	1.18
K244Q	1.42
K244S	1.55
K244T	1.51
K244V	1.42
K244Y	1.19
V260K	1.09
V260L	1.08
V260P	1.12
V260Y	1.02
Y261I	1.19
Y261L	1.11
T263F	1.11
T263H	1.03
T263M	1.08
T263Q	1.04
T263V	1.22
T263W	1.37
T263Y	1.05

Table 14.2 Stability of Variants in Tide @ 2X Without DTPA	
Variant Code	Stability in the presence of Tide 2x without DTPA
S265C	1.03
S265D	1.02
S265E	1.22
S265N	1.07
S265P	1.43
S265T	1.10
S265V	1.09
K269E	1.33
K269F	1.10
K269G	1.17
K269H	1.52
K269I	1.34
K269L	1.34
K269M	1.34
K269N	1.25
K269P	1.26
K269Q	1.39
K269S	1.50
K269T	1.32
K269V	1.39
K269Y	1.38
A273C	1.12
A273D	1.16
A273H	1.10
R280A	1.32
R280C	1.77
R280D	1.52
R280E	1.67
R280F	1.37
R280G	1.16
R280H	1.31
R280K	1.07
R280L	1.64
R280M	1.60
R280S	1.46
R280T	1.28
R280V	1.10
R280W	1.42

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Table 14.2 Stability of Variants in Tide @ 2X Without DTPA	
Variant Code	Stability in the presence of Tide 2x without DTPA
R280Y	1.49
L282M	1.03
S285A	1.03
S285C	1.10
S285D	1.25
S285E	1.36
S285P	1.14
S285Q	1.05
S285R	1.10
S285W	1.12
Q286D	1.05
Q286E	1.17
Q286P	1.04
Q286R	1.02
A289C	1.05
A289E	1.13
A289L	1.06
N296C	1.01
N296D	1.02
N296E	1.67
N296V	1.32
A297C	1.02

Table 14.3 Stability Assay Results in the Presence of 25% TIDE® 2X With DTPA

Variant Code	Stability in the presence of TIDE® 2X with DTPA [Perf. Index]
V190L-D220E-S265Q	2.43
V190I-D220E-S265W	2.38
V190L-D220E-S265N	2.34
T059R-S066Q-S129I	2.33
V190I-D220E-S265N	2.32
V190L-D220E-S265W	2.30
V190I-D220E	2.29
T059W-S066N-S129V	2.28
T059K-S066Q-S129V	2.27
T059K-Y082C-S129V	2.27
T059R-S066N-S129I	2.27
S066Q-S129V	2.25
T059R-S066Q-S129V	2.25
T059R-S129I	2.24
N050Y-T059W-S066N-S129V	2.21
D220P-S265N	2.21
S066Q-S129I	2.21
T059W-S066Q-S129V	2.20
T059K-S066Q-S129I	2.20
T059R-S129V	2.19
N050Y-S066Q-S129V	2.19

Table 14.3 Stability Assay Results in the Presence of 25% TIDE® 2X With DTPA

Variant Code	Stability in the presence of TIDE® 2X with DTPA [Perf. Index]
T059W-S066Q-S129I	2.19
N050Y-T059W-S066Q-S129V	2.18
T059K-S129I	2.17
D220P-S265W	2.17
F130L-M138L-T179P	2.16
S066N-S129I	2.15
T059R-S066N-S129V	2.15
F130L-M138L-T179P	2.14
T059R-S066Q-N092S-S129I	2.13
S066N-S129V	2.11
D220E-S265Q	2.11
F130L-M138L-E152W-T179P	2.10
T059W-S129V	2.10
S265P-L282M-Q286R-A289R	2.09
S265P-L282F-Q286R-A289R	2.09
T059W-S066N-S129I	2.08
V190I-D220P-S265W	2.08
F130L-E152W-T179P	2.06
F130L-M138L-E152F-T179P	2.06
Q062K-S066Q-S129I	2.04
T059K-S066N-S129I	2.04
E152H-T179P	2.03

Table 14.3 Stability Assay Results in the Presence of 25% TIDE® 2X With DTPA

Variant Code	Stability in the presence of TIDE® 2X with DTPA [Perf. Index]
S265P-L282M-Q286K-A289R	2.03
F130L-M138L-E152H-T179P	2.02
T263W-A273H-S285R	2.00
D220E-S265N	1.99
F130I-M138L-E152H-T179P	1.99
F130V-M138L-E152W-T179P	1.99
F130I-M138L-E152W-T179P	1.99
T059W-S129I	1.97
D220E-S265W	1.97
F130V-M138L-T179P	1.96
F130L-E152V-T179P	1.96
T059R-S129Q	1.95
T263W-S285P	1.94
F130I-M138L-E152F-T179P	1.93
E152W-T179P	1.93
V190L-S265Q	1.93
F130L-E152F-T179P	1.92
L282M-Q286R-A289R-P162S	1.91
D220P-S265Q	1.91
M138L-E152F-T179P	1.91
F130I-E152H-T179P	1.91
M138L-E152W-T179P	1.91
F130L-T179P	1.90

Table 14.3 Stability Assay Results in the Presence of 25% TIDE® 2X With DTPA

Variant Code	Stability in the presence of TIDE® 2X with DTPA [Perf. Index]
F130L-M138L-E152W-T179P-Q286H	1.90
F130L-M138L-E152H	1.89
T263W-A273H-S285W	1.89
S265P-Q286K	1.88
T059W-S066Q-S129Q	1.87
T263W-S285R	1.85
T059W-S066N-S129Q	1.83
T263W-S285W	1.83
T059R-S066N-S129Q	1.83
S265P-L282M-Q286R-A289R-T202S-K203N	1.81
T059W-S129Q	1.81
Q062H-S066Q-S129Q	1.81
L282M-Q286R-A289R	1.80
V190L-D220E-S265N-V291I	1.80
V190L-S265N	1.80
F130L-M138L-E152W	1.79
N050Y-T059R-S129Q	1.79
F130I-T179P	1.78
T059K-S066Q-S129Q	1.78
T059K-S129Q	1.78
S265P-L282M-Q286P-A289R	1.77

Table 14.3 Stability Assay Results in the Presence of 25% TIDE® 2X With DTPA

Variant Code	Stability in the presence of TIDE® 2X with DTPA [Perf. Index]
S265P-L282F-Q286P-A289R	1.77
T263W-A273H-S285P	1.77
S265P-L282M-Q286K	1.76
S016L-D220E-S265W	1.76
S066Q-S129Q	1.76
S265P-L282M-Q286P	1.75
L282F-Q286R-A289R	1.75
F130V-E152W-T179P	1.74
L044Q-T263W-S285R	1.74
L055F-T059W-S129V	1.74
V190L-S265W	1.74
Q286R-A289R	1.74
G99D-S265P-L282F-Q286K-A289R	1.73
F130L-M138L-E152F	1.73
T059R-S066Q-S129Q	1.72
F130L-E152H	1.71
S066N-S129Q	1.71
T004S-S023N-G024M-K269N	1.71
S265P-L282M	1.71
E152F-T179P	1.71
T059W-S066N-S129V-S290R	1.68

Table 14.3 Stability Assay Results in the Presence of 25% TIDE® 2X With DTPA

Variant Code	Stability in the presence of TIDE® 2X with DTPA [Perf. Index]
L282F-Q286K-A289R	1.67
F130L-M138L	1.66
F130I-M138L-E152W	1.65
S265P-L282F	1.65
F130I-M138L-E152H	1.65
F130V-M138L-E152H	1.64
V190I-S265Q	1.64
M138L-E152M	1.61
S265P-L282F-Q286P	1.59
M138L-E152H	1.59
T059K-S066N-K088E	1.59
V190I-S265W	1.59
F130L-E152W	1.59
L282M-Q286K-A289R	1.58
L282M-Q286K-A289R-I253V	1.57
T263W-A273H	1.56
V190I-S265N	1.55
M138L-E152W	1.55
A273H-S285R	1.52
F130I-M138L	1.51
F130L-E152F	1.50
F130V-M138L-E152W	1.50
T059K-S066Q-V102A-S129Q	1.48
F130V-E152H-T179P	1.47
F130I-M138L-E152F	1.47

Table 14.3 Stability Assay Results in the Presence of 25% TIDE® 2X With DTPA

Variant Code	Stability in the presence of TIDE® 2X with DTPA [Perf. Index]
F130V-M138L-E152F	1.44
M138L-E152F	1.44
L282M-Q286R	1.43
F130I-E152H	1.43
S265P-L282F-A289R-T065S	1.43
T263H-A273H-S285R	1.43
F130V-M138L	1.42
T014M-T059R-S129V	1.42
L282M-Q286R-A289R-K11N	1.41
A273H-S285P	1.41
L282M-Q286K-A289R-S132T	1.40
T263H-A273H-S285W	1.39
F130V-E152W	1.38
S265P-L282F-Q286K-N061Y	1.37
F130I-E152W	1.36
L198I-D220E-S265Q	1.36
V190I-S265L	1.36
T263H-S285W	1.35
S265P-L282F-A289R	1.34
M138L-S066Q	1.32
F130I-E152F	1.32
N90S-A273H-S285P	1.31
S032T-T263H-A273H-S285R	1.31
L282F-Q286P-A289R	1.28

Table 14.3 Stability Assay Results in the Presence of 25% TIDE® 2X With DTPA

Variant Code	Stability in the presence of TIDE® 2X with DTPA [Perf. Index]
N157Y-T263W-A273H-S285R	1.27
V105A-S129V	1.26
T263H-A273H-S285P	1.25
S129Q-L282H	1.23
T059W-S066Q	1.23
F130V-E152H	1.21
S023W-G024Y	1.21
T004V-S023N	1.21
T059R-S066Q	1.21
N050W-T054L	1.20
L282M-Q286P-A289R	1.20
A115V-V190L-S265W	1.19
L282M-Q286K	1.19
T059R-S066N	1.18
L282F-Q286P	1.15
T004V-S023W-G024M	1.15
S265P-L282F-Q286R-L78H	1.15
L282F-Q286K	1.14
T004V-S023W-G024Y	1.14
S023W-G024M	1.13
T059R-R256S	1.13
F130V-E152F	1.12
T004V-G024W	1.12
N050W-T054K	1.11
S023Y-G024M	1.11
T004V-S023Y	1.11
T004V-S023Y-G024M	1.11
N050Y-T054H	1.10

Table 14.3 Stability Assay Results in the Presence of 25% TIDE® 2X With DTPA

Variant Code	Stability in the presence of TIDE® 2X with DTPA [Perf. Index]
S023W-G024W	1.10
T004V-S023Y-G024Y	1.10
T004V-S023N-G024W	1.09
F130L-M138L-E152F-T179P-V291I	1.09
N050Y-T059K-S066Q	1.09
T004V-S023Y-G024W	1.09
T059K-S066N	1.09
T004V-S023N-G024Y	1.09
S023Y-G024W	1.09
N050F-T054L	1.08
R047K-T054K	1.08
S023N-G024W	1.07
L282M-A289R	1.07
S023Y-G024Y	1.07
T004V-G024M	1.07
L282F	1.06
R047K-N050F-T054K	1.06
N050F-T054K	1.05
T059K-S066Q	1.05
S023N-G024M	1.05
S023N-G024Y	1.04
T004L-S023N	1.04
R047K-N050W-T054H	1.04
T004L-S023W-G024Y	1.04
T004S-S023W	1.03
N046Q-N050W-T054H-A142T	1.03

Table 14.3 Stability Assay Results in the Presence of 25% TIDE® 2X With DTPA

Variant Code	Stability in the presence of TIDE® 2X with DTPA [Perf. Index]
T004L-S023Y	1.03
T004V-S023W	1.03
N050W-T054H	1.02
T004S-S023N	1.02
T004S-L282M	1.02
T004L-S023W	1.02
N050F-T054H	1.01
N050Y-T054L	1.00
R047K-N050W-T054K	1.00

Table 14.4 Stability Assay Results in the Presence of 25% TIDE® 2X Without DTPA	
Variant Code	Stability Assay Results in the presence of TIDE® 2X without DTPA [Perf. Index]
S066Q-S129V	2.24
S066Q-S129I	2.19
N050Y-S066Q-S129V	2.12
S066N-S129I	2.08
T059K-S066Q-S129V	2.06
S066N-S129V	2.05
F130L-E152W-T179P	1.98
S265P-L282M-Q286R-A289R	1.96
F130L-E152V-T179P	1.96
T059K-S066Q-S129I	1.91
T263W-S285P	1.85
T059K-S066N-S129I	1.84
T263W-A273H-S285P	1.83
S265P-L282F-Q286R-A289R	1.83
F130V-E152W-T179P	1.83
T263W-A273H-S285R	1.82
V190I-D220P-S265W	1.79
F130L-E152H	1.78
S066N-S129Q	1.77
S265P-L282M-Q286K-A289R	1.77
V190I-D220E	1.76
T059R-S066N-S129I	1.76

Table 14.4 Stability Assay Results in the Presence of 25% TIDE® 2X Without DTPA	
Variant Code	Stability Assay Results in the presence of TIDE® 2X without DTPA [Perf. Index]
V190I-D220E-S265W	1.75
T059K-S129I	1.75
T059R-S066Q-S129I	1.75
F130I-M138L-E152H-T179P	1.74
F130I-T179P	1.74
T263W-A273H-S285W	1.73
S016L-D220E-S265W	1.72
S066Q-S129Q	1.72
V190I-D220E-S265Q	1.72
T059R-S066Q-S129V	1.71
D220E-S265N	1.69
V190L-D220E	1.69
D220E-S265W	1.68
V190I-D220P	1.68
V190L-D220E-S265N	1.68
L044Q-T263W-S285R	1.67
S265P-L282M-Q286P-A289R	1.67
F130L-M138L-E152H-T179P	1.66
T263W-S285R	1.66
L282M-Q286R-A289R	1.65
T263W-S285W	1.65
F130I-E152H-T179P	1.65

Table 14.4 Stability Assay Results in the Presence of 25% TIDE® 2X Without DTPA	
Variant Code	Stability Assay Results in the presence of TIDE® 2X without DTPA [Perf. Index]
V190I-D220E-S265N	1.64
V190L-D220E-S265W	1.63
V190I-D220P-S265Q	1.63
T059R-S066N-S129V	1.62
V190L-D220E-S265Q	1.62
E152H-T179P	1.62
F130L-M138L-E152F-T179P	1.61
Q062H-S066Q-S129Q	1.59
T059R-S129V	1.58
V190I-D220E-S265W-L282F	1.58
V190I-S265Q	1.58
F130L-E152F-T179P	1.58
D220E-S265Q	1.57
E152W-T179P	1.56
T059K-S066Q-S129Q	1.56
F130L-M138L-T179P	1.55
F130I-M138L-E152F-T179P	1.55
F130L-M138L-E152W-T179P	1.54
N050Y-T059W-S066Q-S129V	1.54
S265P-L282M-Q286K	1.54
T059R-S129I	1.53

Table 14.4 Stability Assay Results in the Presence of 25% TIDE® 2X Without DTPA	
Variant Code	Stability Assay Results in the presence of TIDE® 2X without DTPA [Perf. Index]
F130V-E152H-T179P	1.53
D220P-S265N	1.52
S265P-L282M-Q286P	1.51
F130I-E152H	1.51
T059R-S066Q-N092S-S129I	1.51
F130L-T179P	1.49
G99D-S265P-L282F-Q286K-A289R	1.48
T263W-A273H	1.48
V190I-S265N	1.48
D220P-S265W	1.47
F130L-E152W	1.47
F130L-M138L-E152H	1.46
S265P-L282M	1.45
V190I-S265Q	1.45
F130L-E152F	1.45
T059K-S129Q	1.45
Q286R-A289R	1.45
M138L-E152W-T179P	1.44
F130I-M138L-E152H	1.43
D220P-S265Q	1.42
V190L-S265N	1.42
F130I-M138L-E152W	1.42
S265P-Q286K	1.41
V190L-S265Q	1.41
V190I-S265W	1.40

**Table 14.4 Stability Assay Results
in the Presence of 25% TIDE® 2X
Without DTPA**

Variant Code	Stability Assay Results in the presence of TIDE® 2X without DTPA [Perf. Index]
F130L-M138L-E152F	1.40
F130V-E152H	1.40
E152F-T179P	1.39
N050Y-T059W-S066N-S129V	1.38
T059R-S066N-S129Q	1.38
F130I-E152W	1.37
F130V-E152W	1.37
T059R-S066Q-S129Q	1.37
T263H-A273H-S285P	1.36
N90S-A273H-S285P	1.36
V190L-D220E-S265N-V291I	1.36
T059R-S129Q	1.35
A273H-S285P	1.34
F130I-M138L-E152W-T179P	1.34
F130V-M138L-E152F	1.34
N050Y-T059R-S129Q	1.34
T059W-S066Q-S129I	1.34
F130V-M138L-T179P	1.34
F130V-M138L-E152W-T179P	1.33
V190L-S265W	1.33
F130V-M138L-E152W	1.32

**Table 14.4 Stability Assay Results
in the Presence of 25% TIDE® 2X
Without DTPA**

Variant Code	Stability Assay Results in the presence of TIDE® 2X without DTPA [Perf. Index]
T059W-S066Q-S129V	1.32
V190I-S265Q	1.32
F130V-M138L-E152H	1.32
F130I-E152F	1.31
N157Y-T263W-A273H-S285R	1.31
T263H-S285W	1.30
M138L-E152F-T179P	1.30
A115V-V190L-S265W	1.29
M138L-E152M	1.29
T263H-A273H-S285W	1.29
F130L-M138L-E152W	1.28
T059K-S066N-K088E	1.28
F130I-M138L-E152F	1.27
F130I-M138L-T179P	1.27
T004V-S023N	1.26
T059K-S066Q-V102A-S129Q	1.26
F130L-M138L	1.26
N047K-N050F-T054K	1.24
T263H-A273H-S285R	1.24
F130L-M138L-E152W-T179P-Q286H	1.23
M138L-E152H	1.22

Table 14.4 Stability Assay Results in the Presence of 25% TIDE® 2X Without DTPA	
Variant Code	Stability Assay Results in the presence of TIDE® 2X without DTPA [Perf. Index]
M138L-S066Q	1.22
L282M-Q286R-A289R-P162S	1.21
L282F-Q286R-A289R	1.21
Q062K-S066Q-S129I	1.21
A273H-S285R	1.20
S265P-L282F-Q286P	1.20
S265P-L282F-Q286P-A289R	1.20
S265P-L282M-Q286R-A289R-T202S-K203N	1.19
T059W-S066N-S129I	1.19
V190I-S265L	1.18
T059W-S066N-S129V	1.18
F130I-M138L	1.16
L282M-Q286K-A289R-I253V	1.16
R047K-N050F-T054K	1.15
M138L-E152F	1.15
N050W-T054K	1.15
L198I-D220E-S265Q	1.13
L282F-Q286K-A289R	1.13
N050F-T054K	1.13
L282M-Q286R	1.13
M138L-E152W	1.13
S265P-L282F	1.12

Table 14.4 Stability Assay Results in the Presence of 25% TIDE® 2X Without DTPA	
Variant Code	Stability Assay Results in the presence of TIDE® 2X without DTPA [Perf. Index]
F130V-E152F	1.12
T059W-S066N-S129Q	1.10
F130V-M138L	1.09
T263H-A273H	1.09
L282M-Q286K-A289R	1.07
N046Q-N050W-T054H-A142T	1.07
T059W-S066Q-S129Q	1.07
S265P-L282F-A289R-T065S	1.07
N050F-T054H	1.07
S129Q-L282H	1.06
L282M-Q286K-A289R-S132T	1.03
L282M-Q286R-A289R-K11N	1.03
T059K-S066N	1.02
R047K-N050W-T054K	1.01
T059K-S066Q	1.01
T004V-S023Y	1.01
T059W-S066N-S129V-S290R	1.00
N050Y-T059K-S066Q	1.00
R047K-N050Y	1.00

The data in the following table (Table 14.5) represent the relative stability data of variants of NprE relative to the stability of the WT NprE stability in the citrate stability assay. The stability was measured by determining casein activity by determining AGLA activity before and after incubation at elevated temperature (See, "Citrate Stability Assay" above). The table contains the relative stability values compared to WT under these conditions. It is presented as the quotient of (Variant residual activity/WT residual activity). A value greater than one indicates higher stability in the presence of detergent.

Table 14.5. Citrate Stability Assay Results	
Variant Code	Citrate Stability Relative
K013C	1.22
K013D	1.32
K013E	1.07
K013H	1.50
K013Q	1.38
K013S	1.11
T014G	1.31
T014H	1.75
T014K	1.62
T014M	1.09
T014P	1.07
T014Q	2.01
T014R	1.32
T014V	1.03
S023A	1.12
S023G	1.13
S023I	1.13
S023K	1.39
S023M	1.00
S023N	1.42
S023T	1.15
S023V	1.20
S023W	1.22
G024D	1.38
G024F	1.90
G024H	1.09
G024M	1.23

Table 14.5. Citrate Stability Assay Results	
Variant Code	Citrate Stability Relative
G024R	1.03
G024S	1.11
G024T	1.03
G024W	1.03
Q045D	1.07
Q045E	1.12
Q045M	1.02
Q045N	1.16
Q045P	1.44
N046G	1.10
N046H	1.05
N046I	1.46
N046P	1.47
N046V	1.11
N046Y	1.01
R047E	1.09
R047T	1.07
Y049A	1.02
Y049C	1.03
Y049D	1.01
Y049E	1.04
Y049I	1.08
Y049K	1.04
Y049T	1.16
Y049V	1.19
Y049W	1.00
T054D	1.01

Table 14.5. Citrate Stability Assay Results	
Variant Code	Citrate Stability Relative
T054H	1.09
T054K	1.02
T054L	1.06
T054P	1.63
T054Q	1.17
T054R	1.11
T054S	1.09
T054W	1.02
S058I	1.23
S058L	1.71
S058N	1.08
S058P	2.53
T059E	1.08
T059H	1.19
T059I	1.02
T059K	1.21
T059L	1.16
T059M	1.04
T059S	1.07
S066D	1.03
S066E	1.03
S066P	1.13
S066Q	1.05
S066T	1.17
S066V	1.00
Q087A	1.05
Q087L	1.08

Table 14.5. Citrate Stability Assay Results	
Variant Code	Citrate Stability Relative
Q087S	1.15
Q087T	1.19
N090D	1.17
N090F	1.02
N090G	1.04
N090L	1.25
N090T	1.02
N096G	1.02
K100D	1.30
K100N	1.28
K100P	1.04
K100V	1.01
D119H	1.05
D119T	1.03
D119W	1.00
G136I	1.10
G136L	1.20
G136P	2.19
G136V	2.03
G136W	2.23
G136Y	1.56
M138L	1.48
D139A	2.52
D139C	2.22
D139E	1.51
D139G	2.54
D139H	1.88
D139I	2.40
D139K	2.27
D139L	1.53
D139M	2.49
D139P	2.21
D139R	2.54
D139S	2.22
D139V	1.51
D139W	1.94
D139Y	2.54
E152C	1.17
E152F	1.21

Table 14.5. Citrate Stability Assay Results	
Variant Code	Citrate Stability Relative
E152G	1.09
E152H	1.29
E152R	1.12
E152S	1.17
E152W	1.21
D178A	2.07
D178C	1.79
D178G	2.35
D178H	2.07
D178K	1.73
D178L	1.74
D178M	2.40
D178N	2.34
D178P	1.83
D178Q	1.22
D178R	2.00
D178S	2.58
D178T	1.75
D178V	1.73
D178W	1.02
D178Y	1.78
E186A	2.31
E186C	2.42
E186D	2.03
E186G	2.09
E186H	1.87
E186K	2.69
E186L	1.75
E186M	2.62
E186N	1.72
E186P	2.60
E186Q	1.92
E186R	2.69
E186S	2.57
E186T	2.69
E186V	2.10
E186W	2.47
E186Y	2.48
V190I	1.38

Table 14.5. Citrate Stability Assay Results	
Variant Code	Citrate Stability Relative
V190L	1.41
K211A	1.33
K211M	1.26
K211Q	1.16
K211S	1.28
K214A	1.38
K214C	1.12
K214E	1.08
K214I	1.30
K214L	1.14
K214M	1.03
K214Q	1.47
K214R	1.12
K214S	1.05
K214V	1.49
L216A	1.05
L216C	1.04
L216S	1.19
D220E	1.69
D220H	1.17
D220K	1.17
D220N	1.01
D220P	1.20
A221D	1.11
A221S	1.05
G222C	1.01

EXAMPLE 15

PH PERFORMANCE PROFILE OF NPPE COMPARED TO BPN[®] 217L

- In this Example, experiments conducted to evaluate the comparative performance of nprE and BPN[®] Y217L are described. In these experiments, EMPA 116 (BMI) and Equest grass stains were used.

MATERIALS:

NprE, 8 mg/mL

BPN[®] Y217L, 25.6 mg/mL

EMPA 116 soil cloth, 3" x 4.5" (Testfabrics)

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Equest grass (med.), 3" x 4" (Warwick Equest)

EMPA 221 white cotton swatches, 3" x 4.5"

Minolta Chromameter CR200

TIDE® 2005 (provided by Procter & Gamble)

5 Water hardness concentrate: 15,000 grains per gallon (gpg), 3:1 Ca:Mg 1 M Bis-TRIS-propane buffer

Conc. sulfuric acid

50 L mix tank with spigot and agitator

Terg-O-Tometer

10 DI Water

The swatches were prepared for treatment. Three replicates per treatment were conducted, with 18 swatches used per treatment. The grass swatches were prepared in a dark room to prevent fading. The reflectance values of about 18 soiled swatches were obtained using a Minolta Chromameter. Three readings were obtained per swatch. The L values, average L value and standard deviation were recorded. This is the

15 L_{initial} value.

The detergent solution was prepared as follows (for 40 L total). It was preferred to prepare this solution the night before testing. The solution was stored in the cold over night. The solution was prepared by adding 39.724 Kg of DI water to 50 L mix tank, starting the agitator, mixing in 60 grams of TIDE® liquid detergent, mixing in 16 mL of water hardness solution, and 200 mL of 1 M Bis-TRIS-propane. The pH was

20 adjusted using concentrated sulfuric acid (adjusted to 0.2 pH units below desired pH, if solution was stored overnight). as pH creeps up overnight). The final concentrations were: TIDE® = 1.5 g/L; water hardness = 6 gpg; and Bis-TRIS-propane = 5 mM.

For testing in the Terg-O-Tometer, 1 L of detergent solution was added to each Terg pot and allowed to come to temperature. Enzyme was added to the pots at varying concentrations. For BMI tests, the enzyme

25 concentrations used were 0 mg/L, 0.275 mg/L, 0.55 mg/ml, 1.65 mg/L, 2.65 mg/L, and 5.50 mg/L. For grass stains, the nprE concentrations used were 0 mg/L, 0.1925 mg/L, 0.385 mg/L, 1.155 mg/L, 1.925 mg/L, and 3.85 mg/L (the concentrations of BPN' Y217L were the same as those used in the BMI tests). Agitation was started and the swatches were added. All replicates were run side-by-side in the same Terg-O-Tometer (*e.g.*, 0X & ½ X in the 1st run, 1X & 3X in the 2nd run, and 5X & 10X in the 3rd run). The temperature was 15°C,

30 the agitation speed was 100 cpm, and the wash time was 12 minutes. The treated swatches were rinsed three times in 4 L tap water (~6 gpg). The swatches were air-dried overnight on paper towels. The grass swatches were covered with paper towels and allowed to dry in a darkened room. The reflectance values of the dried swatches were determined as described above. Three readings were obtained per swatch. The L values, average L value and standard deviation were recorded. This is the L_{final} value.

35 The percentage of soil removal (%SR) was determined for each testing condition and both enzymes using the equation below:

$$\%SR = \frac{(L_{\text{final}} - L_{\text{initial}})}{(L_0 - L_{\text{initial}})} \times 100\%$$

Where: L_0 = reflectance of unsoiled swatches

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L_{initial} = reflectance of soiled swatches

L_{final} = reflectance of washed swatches

The delta %SR over no enzyme control was determined using the following formula:

$$\Delta\%SR = \%SR_{\text{treatment}} - \%SR_{\text{no enzyme control}}$$

- 5 BPN' Y217L was compared to nprE on EMPA 116 (BMI), at pH values of 6.7, 7.5, 8.5, and 9.5. The performance of nprE on EMPA 116 appeared to peak at about pH 8, while the performance of BPN' Y217L peaked at about pH 8.8. The results showed that nprE performed better than BPN' Y217L at pH 7.5 and 8.5, although it does not perform as well as BPN' Y217L at pH 6.7. The performance of these enzymes was equal at pH 9.5. In addition, there was no difference in the performance of these enzymes on Equest grass (med) at
10 pH 7.8-8.4.

EXAMPLE 16

COMPARISON OF PMN AND NPPE ENZYMES IN LIQUID DETERGENT

- This Example describes cleaning experiments to determine the cleaning performance of PMN and nprE. The cleaning performance of PMN and nprE enzymes were tested in Liquid TIDE® detergent in
15 comparison with a benchmark serine protease (Protease A) on protease sensitive stains. As shown in the table below, PMN and nprE remove stains much better than protease A, even at low enzyme levels. In the following Tables, the higher SRI values indicate a better cleaning performance.

Table 16.1. Comparison of Cleaning Performance of PMN vs. Protease A in Liquid TIDE® (in full size washing machine)

Active Enzyme Protein in the wash solution	0.55 ppm Protease A	0.55 ppm PMN	5.50 ppm Protease A	5.50 ppm PMN
SRI on Lightly Soiled Grass Stains	53.1	60.8	60.7	67.2
SRI on Medium Soiled Grass Stains	46.5	55.0	54.2	59.8
SRI on Heavily Soiled Grass Stains	39.1	45.8	44.5	51.6

- 20 Table 16.2. Comparison of Cleaning Performance of PMP vs. Protease A in Liquid TIDE® (in mini size washing machine)

Active Enzyme Protein in the wash solution	0.55 ppm Protease A	0.55 ppm PMN
SRI on Lightly Soiled Grass Stains	28.1	52.8
SRI on Medium Soiled Grass Stains	22.8	33.1
SRI on Heavily Soiled Grass Stains	19.9	24.2

Table 16.3. Comparison of Cleaning Performance of nprE vs. Protease A in Liquid TIDE® (in mini-size washing machine)

Active Enzyme Protein in the wash solution	0.55 ppm Protease A	2.75 ppm Protease A	5.50 ppm Protease A	0.28 ppm nprE
SRI on Lightly Soiled Grass Stains	26.3	30.8	30.7	31.5

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SRI on BMI Stains	19.4	24.9	21.4	25.0
Baby Food Beef Stains	63.2	68.8	69.4	71.1

EXAMPLE 17

THERMOSTABILITY OF NPRE AND NPRE VARIANTS

In this Example, experiments conducted to determine the thermostability of NprE and NprE variants are described. The enzymes were produced and purified as described above. The purified proteins were judged to be sufficiently homogenous, with greater than 95% purity as determined using 10% SDS-PAGE, as only one major protein was observed in the gel. This protein was approximately 32 kDa, which is the molecular weight of the mature nprE sequence. The protein was formulated for storage using the 25 mM MES buffer, pH 5.8, containing 1 mM zinc chloride, 4 mM calcium chloride, and 40 % propylene glycol. The assays used in these experiments were the protease assay using fluorescence AGLA activity described above and differential scanning calorimetry (DSC), described below.

DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Excessive heat capacity curves were measured using an ultrasensitive scanning high throughput microcalorimeter VP-Cap DSC (Microcal). The standard procedure for DSC measurements and the theory of the technique is well known to those of skill in the art (*See e.g.*, Freire, 1995) *Meth. Mol. Biol.*, 41, 191-218). Briefly, approx. 500 uL of 200-400 ppm pure or ultrafiltrate concentrate (UFC) protein samples were needed. Typically, 400 ppm of NprE and the variant proteins (in the absence and presence 130 mM citrate) were scanned over 20-100 °C temperature range using a scan rate of 200 °C/hr in 5 mM HEPES, pH 8.0 buffer. The same sample was then rescanned to check the reversibility of the process. For NprE, the thermal unfolding process was irreversible. Scan rate dependence data of the thermal melting for NprE was assessed over a scan rate of 25 to 200°C/hr. The effect of various additives (e.g., primary and secondary alcohols, salts, cyclodextrin, PEG, sorbitol, glycerol) on the thermal melting point of NprE was also assessed.

RESULTS

The thermal stability of wild-type NprE was determined at two different concentrations, in order to show the effect of protein concentration on the thermal melting point. The T_m values for 220 ppm and 440 ppm were determined to be 67.6 ± 0.5 and 69.2 ± 0.5 °C, respectively. The protein concentration effect highlights a second-order event. It is contemplated that this is either aggregation or autolysis. However, it is not intended that the present invention be limited to any particular mechanism. Nonetheless, these results indicate that for an accurate determination and any comparison of thermal melting points for NprE require that the protein concentrations be well matched. The effect of the scan rate on the thermal melting point also showed a dependence where the T_m was dependent on the scan rate up to 150 °C/hr, and then leveled off between 150-200 °C/hr. Based on these results, 200 °C/hr was selected as the upper scan rate for all studies to minimize the dependence of the T_m on scan rate.

All data collected for NprE and variants are shown in Table 4. Table 4 also includes the DSC thermal melting points obtained for NprE and variants in the presence of 130 mM citrate. In most cases, two protein concentrations were scanned. As indicated in this Table, in the case of the scans in the presence of 130 mM citrate not all proteins showed a thermal unfolding profile.

Table 17. DSC Results			
Enzyme Tested	DSC Thermal Concentration		
	Concentration		
	220 ppm Protein	440 ppm Protein	440 ppm Protein with 130 mM citrate
Wild-type NprE	67.6 +/- 0.5	69.2 +/-0.5	No transition
Thermolysin	87.0000		52.1000
<i>B.subtilis</i> NprE	68.0000		55.0000
FNA	64.9000		51.7000
T14R	57.0000		51.7000
S23K	67.8000		None
S23R	67.8000		53.5000
G24R	63.7000		50.7
Q45E	70.6000	70.7000	53.0000
N46K	63.8000		50.7
S58D	63.3000		50.5000
T59P	68.8000		49.1000
S58D,T60D	59.0000		No transition
T60D	66.2000		No transition
S66E	70.3000	71.6000	
S129I	70.2000	70.7000	50.3000
S 129V	69.9000	70.3000	No transition
F130L		69.8000	48.5000
M138I	69.2000		52.5000
M138L	67.8000		
V190I	69.0000	69.4000	51.5
L198M	68.2000	68.5000	53.3000
S199E	70.3000	70.3000	49.1000
D220P	69.3000	69.9000	49.4000
D220E	69.4000	69.8000	50.5000
K211V		69.8000	

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K214Q		68.9000	
A221S	59.1000		52.5000
G222C	69.5000		No transition
K244S		67.6000	
K269T		69.5000	51.5
R280D	67.4000	67.9000	49.2000
N296E	60.5000	69.8000	49.5000
N50W, N296E		62.4000	47.2000
GSC, N61C	67.8000		48.4
Q45K, S199E		67.7000	51.3000
F130L, D220P	62.7000	70.3000	50.8000
M138L, D220P	63.2000	68.2000	50.8000
S129I, V190I		70.3000	55.8000
S129V, V190I		69.9000	55.3000
S129V, D220P		70.6000	55.7000
S129I, D220P		70.7000	53.5000
S129V, R280L		69.5000	54.9000
V190I, D220P		69.8000	52.8000
Q45K, S199E		67.7000	51.3000
N50W, N296E		62.4000	47.2000
G24K K269T D220E		65.0000	51.5000
S129I, F130L, D220P		68.9000	56.6000
nprE-T004S-S023N-G024M(+K269N)		64.6000	None
nprE-T004V-S023N		71.2000	49.0000
nprE-5023 W-G024Y		64.0000	None
nprE-T004V-S023 W-G024M		65.5000	49.3000
nprE-T059K-S66Q-S129I		70.5000	49.3000
nprE-T059R-S66N-S129I		70.2000	54.0000
nprE-T059R-S129I		69.4000	54.0000

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nprE-T059K-S66Q-S129V		70.3000	56.0000
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A representative Figure of the thermal unfolding profiles (DSC scans) for wild type and various mutants of NprE are shown in Figure 27. The unfolding profiles indicate the wild-type midpoint and show selective mutants that display increased thermal melting points relative to wild-type and those that display decreased melting points relative to wild-type. This Figure clearly highlights that the DSC distinguished between stable and less stable NprE variants, and is useful as a secondary screen. A general trend is observed between the thermal melting points of the variants and their stability in detergent. For example, the variants S66E, S 199E, D220P/E, S129I/N are all winners in TIDE® and show an approximate 1 °C increase in thermal melting point relative to wild type NprE. This 1 °C increase in thermal melting point is small yet significant, as thermal stability typically requires multiple amino acid substitutions.

Figure 28 shows the thermal unfolding of NprE variants that display a thermal unfolding profile in the presence of 130 mM citrate. Citrate is a detergent component that rapidly causes the autolysis of NprE, in the absence of calcium. For wild-type NprE, there is no thermal unfolding profile in the presence of citrate, which is consistent with a protein that is already unfolded or lacks a well-formed hydrophobic core. Mutants that display a thermal unfolding profile in the presence of citrate are included in Table 17. These variants have thermal melting points in the range of 47- 56 °C. The DSC scans in the presence of 130 mM citrate indicated variants that are more stable than wild-type NprE to citrate. For example, citrate-stable variants are show to contain either S129I or S 129V and combinatorials containing either of these substitutions show a + 5°C increase in thermal melting point.

EFFECT OF ADDITIVES ON THE THERMAL MELTING POINTS OF NPRE:

Figure 29 shows the results of experiments including various additives. The buffer was 5 mM HEPES, pH 8.0. The samples were scanned from 20 - 100 °C using a scan rate of 200°C/hr. In this Figure, the horizontal line represents the Tm for wild-type NprE with no additive. In these experiments, the data showed little or no effect on the thermal melting point (Tm) of NprE in the presence of these reagents. The inclusion of an inhibitor of NprE activity, namely phosphoramidon, was shown to increase the Tm by approx. 1°C, suggesting that the inhibitor may impart some stabilization to NprE against the thermal unfolding process. None of the conditions above assisted in making the thermal unfolding process reversible. However, it is not intended that the present invention be limited to any particular mechanism.

EXAMPLE 18

NPRE HOMOLOGUE STABILITY IN TIDE® AND HOMOLOG BMI WASH PERFORMANCE

In this Example, experiments conducted to assess the stability of nprE homologs in TIDE®, as well as the wash performance of these homologs are described. Purified NprE ("NprE"), *Bacillus subtilis* NprE (B.S. NprE), *Bacillus thuringiensis* NprB (B.T. NprB) and *Bacillus thermoproteolyticus* thermolysin (TLN) were incubated in 200ul 25% tide in 10mM HEPES, pH8 at 10ug/ml at 25°C for 90 mins. The initial activities and remaining activities were measured using the AGLA assay, as described above. Briefly, 10ul of sample were added into 200ul of AGLA buffer (50mM MES, pH6.5, 0.005% TWEEN®-80, 2.5mM CaCl₂), then 10ul of diluted sample was added into 200ul of AGLA substrate (2.4mM Abz-AGLA-Nba in AGLA buffer). The excitation at 350nm and emission at 415 nm were monitored for first 100 seconds, the initial slope was recorded as enzyme activity. The percent of remaining activity was calculated by dividing the

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remaining activity over initial activity. Figure 30 provides a graph showing the remaining activity after 90 minutes.

To determine the wash performance of these homologues in TIDE®, one pre-washed BMI microswatch was first added into each well of a 96-well plate. Then, 190ul of 1x compact TIDE® (780ug/ml compact TIDE®, 5mM HEPES, pH8, 6gpg water hardness) were added. Then, 10ul of purified NprE, *Bacillus subtilis* NprE, *Bacillus thuringiensis* NprB and *Bacillus thermoproteolyticus* thermolysin were added to the wells to produce a final enzyme concentration is 0.25ug/ml. The plate was incubated at 25°C for 30mins with shaking at 1400rpm on Thermomixer. At the end of incubation, 100ul of supernatant were transferred into a new 96-well plate. The OD at 405nm of supernatant was then measured. The supernatant OD was subtracted with the OD of a blank control without enzyme addition. The performance index was calculated by dividing the OD of each homologue to the OD of NprE. Figure 31 provides a graph showing the BMI was performance of NprE, as well as the nprE homologs described herein.

EXAMPLE 19

METAL ANALYSIS OF WILD-TYPE NPRED AND VARIANTS

In this Example, experiments conducted to determine the zinc and calcium content of nprE and nprE variants are described. In these experiments, total trace metal analysis by inductively coupled plasma - mass spectrometry (ICPMS) and particle induced X-ray emission with a microfocused beam (micro-PIXE) were performed to confirm the zinc and calcium content of NprE. Overall, one zinc and two calcium ions are tightly bound.

All ICPMS and micro-PIXE samples were prepared in metal free buffer to remove any exogenous metal contaminants. Typically, 250 uL of 40 mg/mL NprE samples were buffer exchanged three times with 20 mM HEPES, pH 8.2 using YM-10 microdialysis apparatus. Metal free buffer was generated by passing the buffer through a column packed with Chelax 100 resin. The final protein concentration was determined using Bicinchoninic acid protein determination assay kit (BCA assay) from Sigma. ICPMS samples were analyzed at the West Coast Analytical Services, Inc. Micro-PIXE samples were analyzed at the Ion Beam Analysis Laboratory.

Table 19-1 shows ICPMS metal analysis results for calcium and zinc ions from NprE wild type. Relative to protein concentration, two calcium ions and two zinc ions were found to be present in the sample.

Table 19-1 ICPMS Metal Analysis of Wild-Type NprE		
	Ca (ppm)	Zn (ppm)
ICPMS	73.8	156
Mol w (g/mol)	40.08	65.37
Protein (ppm)	833	833
Ratio/protein	1.4*	1.9

The MicroPIXE elemental composition analysis plot measured the metal contents relative to a protein internal standard. All peaks detected using Micro-PIXE were calculated relative to the sulfur peak

arising from three methionines in the case of NprE. An observed large chloride ion peak was due to the presence of salt in buffer.

Table 18-2 shows the metal content determined by Micro-PIXE, which indicates that in general, NprE contains two tightly bound calcium and one zinc ion per protein molecule. Wild type NprE showed 1 zinc ion with 2 calcium ions. It is contemplated that calcium ions may have shown a low occupancy rate due to preparation of the sample. S58D and T60D showed close to two zinc ions per protein indicating a possible extra zinc ion binding to the site. The double variant has two added cysteines adding the accuracy of the technique. However, it is not intended that the present invention be limited to any particular embodiment with a specific number of ions.

Table 19-2. Micro-PIXE Metal Determination Showing Ca and Zn Contents for NprE Native and Variants						
	#S	Ca/S	Ca/prot	Zn/S	Zn/prot	Ca/Zn
S58D	3	0.72	2.2	0.52	1.6	1.4
T60D	3	0.68	2.0	0.57	1.7	1.2
S58D.T60D	5	0.41	2.1	0.22	1.1	1.9
N46K	3	0.59	1.8	0.42	1.3	1.4
S23K	3	0.62	1.9	0.33	1.0	1.9
A221S	3	0.76	2.3	0.5	1.5	1.5
WT	3	0.54	1.6	0.34	1.0	1.6

Consistent with other well-characterized calcium and zinc dependent neutral proteases such as thermolysin or thermolysin-like proteases (TLPs)(See e.g., Dahlquist et al., Biochem., 15:1103-1111 ; Sprngman et al., (1995) Biochemistry 34, 15713-15720 ; and Willenbrock et al., (1995) FEBS Lett. 358:189-192), NprE was found to contain at least two tightly bound calcium ions and one zinc ion per molecule. A potential third calcium binding site is proposed but expected to be very weak. Since all samples were desalted to remove any exogenous metals, these weakly bounding calcium sites are expected to be unoccupied.

EXAMPLE 20

STABILIZING NPRED WITH CALCIUM FORMATE IN TIDE® COMPACT HDL DETERGENT

In this Example, experiments conducted to develop means to stabilize NprE in TIDE® compact HDL detergent are described. In these experiments, means to stabilize NprE by increasing the calcium formate level at a fixed citrate concentration while lowering DTPA content in experimental TIDE® compact formulation ("TIDE® 2x") were investigated. A statistical design of experiments (DOE) methodology was used in order to simplify the experiments as well as data analyses. It was shown that DTPA present in TIDE® adversely affects NprE stability, while addition of calcium formate helps overcome the detrimental effect in the full strength TIDE® compact formulation.

A full central composite response surface model with duplicate center points was used as a DOE method. A total of 16 unique formulations varying four components were pre-made according to the composition variations listed in Table 19.1. LAS was varied from 0 - 6% (w/w) with DTPA (0 - 0.25%) and

- calcium-formate (0 - 0.2%) at a fixed concentration of citric acid (1.9%). All other components of the TIDE® detergent were held constant. The component concentration boundary conditions were determined based on phase stability of the various mixes. The protein stability tests were conducted with 780 ppm nprE in the full strength (~100%) formulation mixes and incubated at 32 °C. Inactivation was measured up to 24 hours. All assays were done using red fluorescent labeled casein assay kit (Invitrogen) with 0.5 ppm protein concentration. Rates of NprE inactivation were measured in three independent experiments. DOE data were analyzed using DOE Fusion Pro (S-Matrix).

Table 20.1. Composition of the 16 TIDE® Formulations Used for DOE Studies				
	HLAS	Citric acid	DTPA	Ca formate
Form 1	3	1.9	0	0.1
Form 2	3	1.9	0.125	0.1
Form 3	3	1.9	0.25	0.1
Form 4	6	1.9	0.25	0.2
Form 5	0	1.9	0	0.2
Form 6	6	1.9	0	0.2
Form 7	0	1.9	0.25	0.2
Form 8	6	1:9	0.125	0.1
Form 9	6	1.9	0.25	0
Form 10	0	1.9	0.125	0.1
Form 11	6	1.9	0	0
Form 12	3	1.9	0.125	0
Form 13	0	1.9	0.25	0
Form 14	3	1.9	0.125	0.1
Form 15	0	1.9	0	0
Form 16	3	1.9	0.125	0.2

- Table 20.2 and Figure 31 show the results of NprE stability measurements in various formulation mixes. Average rates and the standard deviation were the averaged NprE inactivation rate (hour⁻¹) from three independent measurements. Qualitatively, formulations with low DTPA content with high calcium load tend to be more stable in the full strength compact TIDE®. As an example, Formulation #5, with no addition of DTPA and high calcium formate level showed the lowest inactivation rate, indicating high NprE stability. In contrast, Formulation #9, with high DTPA concentration with no added calcium formate showed lowest stability. In Table 20.2, the ranking is based on measured stability (*i.e.*, averaged rates). Runs are from three independent stability experiments.

Table 20.2. NprE Inactivation Rates in 16 Formulation Mixes

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	Ranking	Run 1	Run 2	Run 3	Average Rate (hour ⁻¹)	Standard Deviation
Form 5	1	0.031	0.053	0.067	0.050	0.019
Form 1	2	0.060	0.044	0.081	0.062	0.019
Form 15	3	0.050	0.079	0.060	0.063	0.015
Form 6	4	0.312	0.057	0.059	0.143	0.147
Form 7	5	0.364	0.254	0.128	0.249	0.118
Form 11	6	0.099	0.288	0.395	0.261	0.150
Form 10	7	0.337	0.238	0.226	0.267	0.061
Form 16	8	0.063	0.593	0.188	0.281	0.277
Form 2	9	0.392	0.372	0.296	0.354	0.051
Form 14	10	0.387	0.451	0.269	0.369	0.093
Form 4	11	0.665	0.333	0.336	0.445	0.191
Form 8	12	0.682	0.554	0.378	0.538	0.153
Form 3	13	0.864	0.440	0.389	0.566	0.261
Form 13	14	1.417	0.931	0.964	1.104	0.272
Form 12	15	1.005	1.620	1.029	1.218	0.349
Form 9	16	0.875	2.099	0.694	1.223	0.764

Figure 33 shows NprE inactivation effects by DTPA at varying levels of fixed calcium formate concentration. Panel A shows rate of NprE inactivation by DTPA without any added calcium formate. The correlation shows that DTPA has significant detrimental effect. Panel B shows some decreased effect of DTPA with 0.1% calcium formate. Panel C shows significantly decreased effect of DTPA with 0.2% calcium formate.

Figure 34 shows DOE analysis software (Fusion Pro) generated prediction profile of DTPA and calcium format composition based on response goal (decay rate) of less than 0.5 hr⁻¹ (Panel A), 0.25 hr⁻¹ (Panel B) and 0.05 hr⁻¹ (Panel C). The shaded areas indicate DTPA and calcium formate composition ratios that are predicted to show stability with decay rate below the set goal. For example, 0.16% calcium formate in the presence of 0.04% DTPA would provide NprE stability with decay rate of less than 0.25 hour⁻¹ as shown in Panel B of Figure 34. On the other hand, 0.08% calcium formate cannot sustain NprE stability with decay rate of at least 0.25 hour⁻¹ in the presence of 0.16% DTPA.

EXAMPLE 21

IDENTIFICATION OF THE CITRATE-INDUCED AUTOLYTIC SITES FOR *B. AMYLOLIQUEFACIENS* NEUTRAL METALLOPROTEASE NPnE

In this Example, methods used to assess the citrate-induced autolysis of wild-type and recombinant variant nprE (e.g., *B. subtilis* variant) are described. In these experiments, autolysis of the neutral

metalloprotease from *B. amyloliquefaciens* (natural and the recombinant variant expressed in *B. subtilis*) was induced using sodium citrate (Sigma). The autolysis process was controlled by performing the reaction at 4°C in 25 mM MES, pH 6.5, buffer. In these experiments, the autolysis of 0.4 mg/ml NprE was optimized by varying either: (a) the time of incubation (10-100 minutes) in 10 mM citrate; or (b) the citrate concentration (10-100 mM) over 100 minutes. A control of neutral metalloprotease diluted in buffer alone (*i.e.*, no citrate) was incubated under similar conditions. The autolytic reactions were terminated by addition of an equal volume of 1N HCl, the samples were precipitated using TCA and the pellet was washed and dried using acetone. The resultant pellet was resuspended in 20 uL buffer, pH 6.5, and 4X LDS sample buffer (NuPage, Invitrogen)

The autolytic fragments were resolved by 10 % (w/v) SDS-PAGE and electroblotted onto a PVDF membrane. The first 10 amino acid residues were sequenced by Edman degradation (Argo Bioanalytica). The partial amino acid sequences of the autolytic fragments were determined using trypsin in-gel digestion and analyzed using LCQ-MS (Agilent). The in-gel digestion process involved macerating the gel piece that contained the protein, removal of the Coomassie blue stain followed by re-hydration of the gel pieces in 25 mM NH₄CO₃ containing 2 M urea. Trypsin was added to the re-hydrated gel pieces for approx. 6 hours at 37 °C. Following the digestion, the peptides were extracted using acetonitrile and TCA. The peptides were separated on a C4-hydrophobic column (Vydac) using an acetonitrile- water gradient. The resultant peptide maps were searched with the REQUEST® database search program against a database containing Genencor enzymes.

The amino acid sequences of the first 10 amino acids of each of the fragments were compared with the known amino acid sequence for *B. amyloliquefaciens* NprE. This enabled the identification of the amino acid at the N-termini and hence the cleavage site(s).

The generation of the citrate-induced fragments and their resolution was shown on 10 % SDS-PAGE. The sizes of the fragments were identified using a standard molecular weight marker from Invitrogen. In the presence of 10 mM citrate, two fragments in addition to remaining intact NprE were observed over the 100 minute time range. The two fragments formed at the low citrate concentration were found to be 24 kDa and 9 kDa in size. The intact nprE is 32 kDa. The 100-minute time range results in a good proportion of cleaved protein (*i.e.*, the primary autolysis fragments). No additional fragments were observed or detected under these conditions. A study over 100 minutes in the presence of increasing citrate was performed to obtain the secondary autolytic fragments. In this experiment, when concentrations between 10 - 30 mM citrate were used, the two fragments described above were observed. At 40 mM citrate, less of the larger 24-kDa fragments were apparent however a 15-kDa fragment was also apparent. Between 50 - 100 mM citrate, the 24 kDa fragment and the 9-kDa fragments were no longer detected but three other fragments, of sizes 21 kDa, 15 kDa and 11 kDa, were observed.

The identity of the N-termini of the 24 kDa, 9 kDa (first two fragments), and the 21 kDa, 15 kDa and 11 kDa (the next autolytic fragments) were determined using Edman degradation (Argo Bioanalytica).

Table 21. N-Terminal Sequence of Fragments

Sample Name	N-terminal Amino acid sequence (5'-3') (SEQ ID NO:)	Corresponding molecule weight on SDS-PAGE (kDa)
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Band A1	AATTGTGTTL (SEQ ID NO:215)	24
Band A2	DAGDYGGVHT (SEQ ID NO:216)	9
	AGDYGGVHTN (SEQ ID NO:217)	
	GDYGGVHTN (SEQ ID NO:218)	
Band A3	AATTGTGTTL (SEQ ID NO:219)	21
Band A4	AATTGTGTTL (SEQ ID NO:220)	15
Band A5	LSNPITYGQP (SEQ ID NO:221)	11

Bands A1, A 3 and A4 have the native N-terminal sequence that matches the N-terminus for the intact NprE. The sequencing report for Band A2 showed three fragments where the least intense sequence appeared to be identical to the more intense sequence, except that it was two residues and one residue shorter than the more intense sequences, respectively. This was consistent with a fraying of that particular protein fragment. The pattern and the sizes of the gel fragments suggest that the 15 kDa (Band A4) may be derived from the 21-kDa fragment (Band A3) and hence the C-terminus is deduced to be at or near position 198. However, it is not intended that the present invention be limited to this particular embodiment.

Figure 35 provides the amino acid sequences for the various fragments (1-5 or A1-5 for N-terminal sequencing purposes). Fragment 1 (A1) has the N-terminal residues equivalent to that for the intact native protein (SEQ ID NO:222), fragment 2 (A2) N-terminus starts at or near D220 (SEQ ID NO:223). The following two amino acid residues (A221 and G222) are also highlighted because this fragment was identified as being frayed. Fragment 3 (A3) (SEQ ID NO:224) and fragment 4 (A4) (SEQ ID NO:225) have the N-terminus of the intact protein, and fragment 5 (A5) (SEQ ID NO:226) starts at L198. The C-terminus of fragment 4 is likely to be at or near M138 (based on the size difference between A3 and A4). The corresponding fragment for A3 was not detected.

Trypsin digestion followed by LCQ-MS of the peptide maps for fragments 1 through 5 positively identified several amino acid peptides within the respective fragments. These are highlighted in Figure 35. The LCQ-MS provided a positive control for the identity of the fragments.

Based on the N-terminal and LCQ-MS analysis of the cleavage fragments, primary cleavage sites were identified at amino acid positions D220, A221, G222, M138, and L198. These sites were targeted using site-directed mutagenesis and site-evaluation libraries of D220, A221, G222, L198, and M138, D139 were created. The mutant proteins were screened for increasing stability in detergent and for BMI-wash performance, as indicated herein. In some instances, the amino acids alongside these sites were also selected for protein engineering, in order to ensure that the clip site was indeed targeted.

The protein engineering results clearly indicated that amino acid substitutions of either Pro or Glu at D220 generated an NprE molecule that is more stable in detergent. In addition, additional stability was afforded to the NprE molecule by replacing G222 with Cys, and M138 with either Ile or Leu. In general, these specific amino acid substitutions provided the NprE with detergent stability advantages without the BMI-wash performance being compromised. Thus, these experiments provide important mapping data for the citrate-induced autolysis sites, facilitating the identification of key amino acid residues that alter and affect the overall stability of NprE. Citrate (a builder added to detergent matrices) destabilizes and autolyzes NprE

and is suggested to do so by chelating the essential calcium-bound atoms. The application of NprE in extreme detergent conditions requires that a more stable NprE molecule be used in these settings. In these experiments, substitutions of one or more of the autolytic sites of NprE have resulted in a more detergent-stable nprE molecule for use in these extreme detergents.

5 EXAMPLE 22

LIQUID LAUNDRY DETERGENT COMPOSITIONS

In this Example, various formulations for liquid laundry detergent compositions are provided. The following liquid laundry detergent compositions of the present invention are prepared:

Compound	Formulations				
	I	II	III	IV	V
LAS	24.0	32.0	6.0	3.0	6.0
NaC ₁₆ -C ₁₇ HSAS	-	-	-	5.0	-
C ₁₂ -C ₁₅ AE _{1.8} S	-	-	8.0	7.0	5.0
C ₈ -C ₁₀ propyl dimethyl amine	2.0	2.0	2.0	2.0	1.0
C ₁₂ -C ₁₄ alkyl dimethyl amine oxide	-	-	-	-	2.0
C ₁₂ -C ₁₅ AS	-	-	17.0	-	8.0
CFAA	-	5.0	4.0	4.0	3.0
C ₁₂ -C ₁₄ Fatty alcohol ethoxylate	12.0	6.0	1.0	1.0	1.0
C ₁₂ -C ₁₈ Fatty acid	3.0	-	4.0	2.0	3.0
Citric acid (anhydrous)	4.5	5.0	3.0	2.0	1.0
DETPMP	-	-	1.0	1.0	0.5
Monoethanolamine	5.0	5.0	5.0	5.0	2.0
Sodium hydroxide	-	-	2.5	1.0	1.5
1 N HCl aqueous solution	#1	#1	-	-	-
Propanediol	12.7	14.5	13.1	10.	8.0
Ethanol	1.8	2.4	4.7	5.4	1.0
DTPA	0.5	0.4	0.3	0.4	0.5
Pectin Lyase	-	-	-	0.005	-
Amylase	0.001	0.002	-		-
Cellulase	-	-	0.0002		0.0001
Lipase	0.1	-	0.1	-	0.1
nprE	0.05	0.3	-	0.5	0.2

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PMN	-	-	0.08	-	-
Protease A	-	-	-	-	0.1
Aldose Oxidase	-	-	0.3	-	0.003
ZnCl ₂	0.1	0.05	0.05	0.05	0.02
Ca formate	0.05	0.07	0.05	0.06	0.07
DETBCHD	-	-	0.02	0.01	-
SRP1	0.5	0.5	-	0.3	0.3
Boric acid	-	-	-	-	2.4
Sodium xylene sulfonate	-	-	3.0	-	-
Sodium cumene sulfonate	-	-	-	0.3	0.5
DC 3225C	1.0	1.0	1.0	1.0	1.0
2-butyl-octanol	0.03	0.04	0.04	0.03	0.03
Brightener 1	0.12	0.10	0.18	0.08	0.10
Balance to 100% perfume/dye and/or water					
#1: Add 1N HCl aq. soln to adjust the neat pH of the formula in the range from about 3 to about, 5.					

The pH of Examples above 22(I)-(II) is about 5 to about 7, and of 22(III)-(V) is about 7.5 to about 8.5.

EXAMPLE 23

HAND DISH LIQUID DETERGENT COMPOSITIONS

- 5 In this Example, various hand dish liquid detergent formulations are provided. The following hand dish liquid detergent compositions of the present invention:

Compound	Formulations					
	I	II	III	IV	V	VI
C ₁₂ -C ₁₅ AE _{1.8} S	30.0	28.0	25.0	-	15.0	10.0
LAS	-	-	-	5.0	15.0	12.0
Paraffin Sulfonate	-	-	-	20.0	-	-
C ₁₀ -C ₁₈ Alkyl Dimethyl Amine Oxide	5.0	3.0	7.0	-	-	-
Betaine	3.0,	-	1.0	3.0	1.0	-
C ₁₂ poly-OH fatty acid amide	-	-	-	3.0	-	1.0
C ₁₄ poly-OH fatty acid amide	-	1.5	-	-	-	-
C ₁₁ E ₉	2.0	-	4.0	-	-	20.0

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DTPA	-	-	-	-	0.2	-
Tri-sodium Citrate dihydrate	0.25	-	-	0.7	-	-
Diamine	1.0	5.0	7.0	1.0	5.0	7.0
MgCl ₂	0.25	-	-	1.0	-	-
nprE	0.02	0.01	-	0.01	-	0.05
PMN	-	-	0.03	-	0.02	-
Protease A	-	0.01	-	-	-	-
Amylase	0.001	-	-	0.002	-	0.001
Aldose Oxidase	0.03	-	0.02	-	0.05	-
Sodium Cumene Sulphonate	-	-	-	2.0	1.5	3.0
PAAC	0.01	0.01	0.02	-	-	-
DETBCHD	-	-	-	0.01	0.02	0.01
Balance to 100% perfume / dye and/or water						

The pH of Examples 23(I)-(VI) is about 8 to about 11

EXAMPLE 24

LIQUID AUTOMATIC DISHWASHING DETERGENT COMPOSITIONS

- In this Example, various liquid automatic dishwashing detergent formulations are provided. The following hand dish liquid detergent compositions of the present invention:

Compound	Formulations				
	I	II	III	IV	V
STPP	16	16	18	16	16
Potassium Sulfate	-	10	8	-	10
1,2 propanediol	6.0	0.5	2.0	6.0	0.5
Boric Acid	-	-	-	4.0	3.0
CaCl ₂ dihydrate	0.04	0.04	0.04	0.04	0.04
Nonionic	0.5	0.5	0.5	0.5	0.5
nprE	0.1	0.03	-	0.03	-
PMN	-	-	0.05	-	0.06
Protease B	-	-	-	0.01	-
Amylase	0.02	-	0.02	0.02	-
Aldose Oxidase	-	0.15	0.02	-	0.01

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Galactose Oxidase	-	-	0.01	-	0.01
PAAC	0.01	-	-	0.01	-
DETBCHD	-	0.01	-	-	0.01
Balance to 100% perfume / dye and/or water					

EXAMPLE 25

GRANULAR AND/OR TABLET LAUNDRY COMPOSITIONS

This Example provides various formulations for granular and/or tablet laundry detergents. The following laundry compositions of present invention, which may be in the form of granules or tablet, are prepared.

5

Compound	Formulations				
	I	II	III	IV	V
Base Product					
C ₁₄ -C ₁₅ AS or TAS	8.0	5.0	3.0	3.0	3.0
LAS	8.0	-	8.0	-	7.0
C ₁₂ -C ₁₅ AE ₃ S	0.5	2.0	1.0	-	-
C ₁₂ -C ₁₅ E ₃ or E ₃	2.0	-	5.0	2.0	2.0
QAS	-	-	-	1.0	1.0
Zeolite A	20.0	18.0	11.0	-	10.0
SKS-6 (dry add)	-	-	9.0	-	-
MA/AA	2.0	2.0	2.0	-	-
AA	-	-	-	-	4.0
3Na Citrate 2H ₂ O	-	2.0	-	-	-
Citric Acid (Anhydrous)	2.0	-	1.5	2.0	-
DTPA	0.2	0.2	-	-	-
EDDS	-	-	0.5	0.1	-
HEDP	-	-	0.2	0.1	-
PB1	3.0	4.8	-	-	4.0
Percarbonate	-	-	3.8	5.2	-
NOBS	1.9	-	-	-	-
NACA OBS	-	-	2.0	-	-
TAED	0.5	2.0	2.0	5.0	1.00

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BB1	0.06	-	0.34	-	0.14
BB2	-	0.14	-	0.20	-
Anhydrous Na Carbonate	15.0	18.0	-	15.0	15.0
Sulfate	5.0	12.0	5.0	17.0	3.0
Silicate	-	1.0	-	-	8.0
nprE	0.03	-	0.1	0.06	-
PMN	-	0.05	-	-	0.1
Protease B	-	0.01	-	-	-
Protease C	-	-	-	0.01	-
Lipase	-	0.008	-	-	-
Amylase	0.001	-	-	-	0.001
Cellulase	-	0.0014	-	-	-
Pectin Lyase	0.001	0.001	0.001	0.001	0.001
Aldose Oxidase	0.03	-	0.05	-	-
PAAC	-	0.01	-	-	0.05
Balance to 100% Moisture and/or Minors*					
* Perfume, dye, brightener / SRP1 / Na carboxymethylcellulose/ photobleach / MgSO ₄ / PVPVI/ suds suppressor /high molecular PEG/clay.					

EXAMPLE 26

LIQUID LAUNDRY DETERGENTS

This Example provides various formulations for liquid laundry detergents. The following liquid laundry detergent formulations of the present invention are prepared:

Compound	Formulations					
	I	I	II	III	IV	V
LAS	11.5	11.5	9.0	-	4.0	-
C ₁₂ -C ₁₅ AE _{2.85} S	-	-	3.0	18.0	-	16.0
C ₁₄ -C ₁₅ E 2.5 S	11.5	11.5	3.0	-	16.0	-
C ₁₂ -C ₁₃ E ₉	-	-	3.0	2.0	2.0	1.0
C ₁₂ -C ₁₃ E ₇	3.2	3.2	-	-	-	-
CFAA	-	-	-	5.0	-	3.0
TPKFA	2.0	2.0	-	2.0	0.5	2.0

EXAMPLE 27

HIGH DENSITY DISHWASHING DETERGENTS

This Example provides various formulations for high density dishwashing detergents. The following compact high density dishwashing detergents of the present invention are prepared:

Compound	Formulations					
	I	II	III	IV	V	VI
STPP	-	45.0	45.0	-	-	40.0
3Na Citrate 2H ₂ O	17.0	-	-	50.0	40.2	-
Na Carbonate	17.5	14.0	20.0	-	8.0	33.6
Bicarbonate	-	-	-	26.0	-	-
Silicate	15.0	15.0	8.0	-	25.0	3.6
Metasilicate	2.5	4.5	4.5	-	-	-
PB1	-	-	4.5	-	-	-
PB4	-	-	-	5.0	-	-
Percarbonate	-	-	-	-	-	4.8
BB1	-	0.1	0.1	-	0.5	-
BB2	0.2	0.05		0.1	-	0.6
Nonionic	2.0	1.5	1.5	3.0	1.9	5.9
HEDP	1.0	-	-	-	-	-
DETPMP	0.6	-	-	-	-	-
PAAC	0.03	0.05	0.02	-	-	-
Paraffin	0.5	0.4	0.4	0.6	-	-
nprE	0.072	0.053	-	0.026	-	0.01
PMN	-	-	0.053	-	0.059	-
Protease B	-	-	-	-	-	0.01
Amylase	0.012	-	0.012	-	0.021	0.006
Lipase	-	0.001	-	0.005	-	-
Pectin Lyase	0.001	0.001	0.001	-	-	-
Aldose Oxidase	0.05	0.05	0.03	0.01	0.02	0.01
BTA	0.3	0.2	0.2	0.3	0.3	0.3
Polycarboxylate	6.0	-	-	-	4.0	0.9

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Perfume	0.2	0.1	0.1	0.2	0.2	0.2
Balance to 100% Moisture and/or Minors*						
*Brightener / dye / SRP1 / Na carboxymethylcellulose/ photobleach / MgSO ₄ / PVPVI/ suds suppressor /high molecular PEG/clay.						

The pH of Examples 27(I) through (VI) is from about 9.6 to about 11.3.

EXAMPLE 28

TABLET DETERGENT COMPOSITIONS

- 5 This Example provides various tablet detergent formulations. The following tablet detergent compositions of the present invention are prepared by compression of a granular dishwashing detergent composition at a pressure of 13KN/cm² using a standard 12 head rotary press:

Compound	Formulations							
	I	II	III	IV	V	VI	VII	VIII
STPP	-	48.8	44.7	38.2	-	42.4	46.1	46.0
3Na Citrate 2H ₂ O	20.0	-	-	-	35.9	-	-	-
Na Carbonate	20.0	5.0	14.0	15.4	8.0	23.0	20.0	-
Silicate	15.0	14.8	15.0	12.6	23.4	2.9	4.3	4.2
Lipase	0.001	-	0.01	-	0.02	-	-	-
Protease B	0.01	-	-	-	-	-	-	-
Protease C	-	-	-	-	-	0.01	-	-
nprE	0.01	0.08	-	0.04	-	0.023	-	0.05
PMN	-	-	0.05	-	0.052	-	0.023	-
Amylase	0.012	0.012	0.012	-	0.015	-	0.017	0.002
Pectin Lyase	0.005	-	-	0.002	-	-	-	-
Aldose Oxidase	-	0.03	-	0.02	0.02	-	0.03	-
PB1	-	-	3.8	-	7.8	-	-	4.5
Percarbonate	6.0	-	-	6.0	-	5.0	-	-
BB1	0.2	-	0.5	-	0.3	0.2	-	-
BB2	-	0.2	-	0.5	-	-	0.1	0.2
Nonionic	1.5	2.0	2.0	2.2	1.0	4.2	4.0	6.5
PAAC	0.01	0.01	0.02	-	-	-	-	-
DETBCHD	-	-	-	0.02	0.02	-	-	-
TAED	-	-	-	-	-	2.1	-	1.6

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HEDP	1.0	-	-	0.9	-	0.4	0.2	-
DETPMP	0.7	-	-	-	-	-	-	-
Paraffin	0.4	0.5	0.5	0.5	-	-	0.5	-
BTA	0.2	0.3	0.3	0.3	0.3	0.3	0.3	-
Polycarboxylate	4.0	-	-	-	4.9	0.6	0.8	-
PEG 400-30,000	-	-	-	-	-	2.0	-	2.0
Glycerol	-	-	-	-	-	0.4	-	0.5
Perfume	-	-	-	0.05	0.2	0.2	0.2	0.2
Balance to 100% Moisture and/or Minors*								
*Brightener / SRP / Na carboxymethylcellulose/ photobleach / MgSO ₄ / PVPVI/ suds suppressor /high molecular PEG/clay.								

The pH of Examples 28(I) through 28(VII) is from about 10 to about 11.5; pH of 15(VIII) is from 8-10. The tablet weight of Examples 28(I) through 28(VIII) is from about 20 grams to about 30 grams.

EXAMPLE 29

LIQUID HARD SURFACE CLEANING DETERGENTS

- 5 This Example provides various formulations for liquid hard surface cleaning detergents. The following liquid hard surface cleaning detergent compositions of the present invention are prepared:

Compound	Formulations						
	I	II	III	IV	V	VI	VII
C ₉ -C ₁₁ E ₅	2.4	1.9	2.5	2.5	2.5	2.4	2.5
C ₁₂ -C ₁₄ E ₅	3.6	2.9	2.5	2.5	2.5	3.6	2.5
C ₇ -C ₉ E ₆	-	-	-	-	8.0	-	-
C ₁₂ -C ₁₄ E ₂₁	1.0	0.8	4.0	2.0	2.0	1.0	2.0
LAS	-	-	-	0.8	0.8	-	0.8
Sodium culmene sulfonate	1.5	2.6	-	1.5	1.5	1.5	1.5
Isachem ® AS	0.6	0.6	-	-	-	0.6	-
Na ₂ O ₃	0.6	0.13	0.6	0.1	0.2	0.6	0.2
3Na Citrate 2H ₂ O	0.5	0.56	0.5	0.6	0.75	0.5	0.75
NaOH	0.3	0.33	0.3	0.3	0.5	0.3	0.5
Fatty Acid	0.6	0.13	0.6	0.1	0.4	0.6	0.4
2-butyl octanol	0.3	0.3		0.3	0.3	0.3	0.3
PEG DME-2000®	0.4		0.3	0.35	0.5	-	-

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PVP	0.3	0.4	0.6	0.3	0.5	-	-
MME PEG (2000) ®	-	-	-	-	-	0.5	0.5
Jeffamine® ED-2001	-	0.4	-	-	0.5	-	-
PAAC	-	-	-	0.03	0.03	0.03	-
DETBCHD	0.03	0.05	0.05	-	-	-	-
nprE	0.07	-	0.08	0.03	-	0.01	0.04
PMN	-	0.05	-	-	0.06	-	-
Protease B	-	-	-	-	-	0.01	-
Amylase	0.12	0.01	0.01	-	0.02	-	0.01
Lipase	-	0.001	-	0.005	-	0.005	-
Pectin Lyase	0.001	-	0.001	-	-	-	0.002
ZnCl ₂	0.02	0.01	0.03	0.05	0.1	0.05	0.02
Calcium Formate	0.03	0.03	0.01	-	-	-	-
PB1	-	4.6	-	3.8	-	-	-
Aldose Oxidase	0.05	-	0.03	-	0.02	0.02	0.05
Balance to 100% perfume / dye and/or water							

The pH of Examples 29(I) through (VII) is from about 7.4 to about 9.5.

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	tctaaccgga ccggttgcaaa caaaaacaaa gccgagctga aagcagcagc cacaaaagac 540		
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	tcttctgaaa gcggcaataa tgtgctgcgc gatctttcta aacctaccgg aacacaaatt 780		
	attacgtacg atctgcaaaa ccgcgagtat aacctgccgg gcacactcgt atccagcacc 840		
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Leu Lys Glu Asn Leu Thr Asn Phe Val Pro Lys His Ser Leu Val Gln
      35      40      45
Ser Glu Leu Pro Ser Val Ser Asp Lys Ala Ile Lys Gln Tyr Leu Lys
50      55      60
Gln Asn Gly Lys Val Phe Lys Gly Asn Pro Ser Glu Arg Leu Lys Leu
65      70      75      80
Ile Asp Gln Thr Thr Asp Asp Leu Gly Tyr Lys His Phe Arg Tyr Val
      85      90      95
Pro Val Val Asn Gly Val Pro Val Lys Asp Ser Gln Val Ile Ile His
100      105      110
Val Asp Lys Ser Asn Asn Val Tyr Ala Ile Asn Gly Glu Leu Asn Asn
115      120      125
Asp Val Ser Ala Lys Thr Ala Asn Ser Lys Lys Leu Ser Ala Asn Gln
130      135      140
Ala Leu Asp His Ala Tyr Lys Ala Ile Gly Lys Ser Pro Glu Ala Val
145      150      155      160
Ser Asn Gly Thr Val Ala Asn Lys Asn Lys Ala Glu Leu Lys Ala Ala
165      170      175
Ala Thr Lys Asp Gly Lys Tyr Arg Leu Ala Tyr Asp Val Thr Ile Arg
180      185      190
Tyr Ile Glu Pro Glu Pro Ala Asn Trp Glu Val Thr Val Asp Ala Glu
195      200      205
Thr Gly Lys Ile Leu Lys Lys Gln Asn Lys Val Glu His Ala Ala Thr
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Thr Gly Thr Gly Thr Thr Leu Lys Gly Lys Thr Val Ser Leu Asn Ile
225      230      235      240
Ser Ser Glu Ser Gly Lys Tyr Val Leu Arg Asp Leu Ser Lys Pro Thr
245      250      255
Gly Thr Gln Ile Ile Thr Tyr Asp Leu Gln Asn Arg Glu Tyr Asn Leu
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Pro Gly Thr Leu Val Ser Ser Thr Thr Asn Gln Phe Thr Thr Ser Ser
275      280      285
Gln Arg Ala Ala Val Asp Ala His Tyr Asn Leu Gly Lys Val Tyr Asp
290      295      300
Tyr Phe Tyr Gln Lys Phe Asn Arg Asn Ser Tyr Asp Asn Lys Gly Gly
305      310      315      320
Lys Ile Val Ser Ser Val His Tyr Gly Ser Arg Tyr Asn Asn Ala Ala
325      330      335
Trp Ile Gly Asp Gln Met Ile Tyr Gly Asp Gly Asp Gly Ser Phe Phe
340      345      350
Ser Pro Leu Ser Gly Ser Met Asp Val Thr Ala His Glu Met Thr His
355      360      365
Gly Val Thr Gln Glu Thr Ala Asn Leu Asn Tyr Glu Asn Gln Pro Gly
370      375      380
Ala Leu Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr Phe Asn Asp Thr
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Glu Asp Trp Asp Ile Gly Glu Asp Ile Thr Val Ser Gln Pro Ala Leu
405      410      415
Arg Ser Leu Ser Asn Pro Thr Lys Tyr Gly Gln Pro Asp Asn Phe Lys
420      425      430
Asn Tyr Lys Asn Leu Pro Asn Thr Asp Ala Gly Asp Tyr Gly Gly Val
435      440      445
His Thr Asn Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn Thr Ile Thr
450      455      460
Lys Ile Gly Val Asn Lys Ala Glu Gln Ile Tyr Arg Ala Leu Thr
465      470      475      480
Val Tyr Leu Thr Pro Ser Ser Thr Phe Lys Asp Ala Lys Ala Ala Leu
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Lys	Gln	Tyr	Leu	Lys	Gln	Asn	Gly	Lys	Val	Phe	Lys	Gly	Asn	Pro	Ser
		35					40					45			
Glu	Arg	Leu	Lys	Leu	Ile	Asp	Gln	Thr	Thr	Asp	Asp	Leu	Gly	Tyr	Lys
	50					55				60					
His	Phe	Arg	Tyr	Val	Pro	Val	Val	Asn	Gly	Val	Pro	Val	Lys	Asp	Ser
65				70						75				80	
Gln	Val	Ile	Ile	His	Val	Asp	Lys	Ser	Asn	Asn	Val	Tyr	Ala	Ile	Asn
				85					90				95		
Gly	Glu	Leu	Asn	Asn	Asp	Val	Ser	Ala	Lys	Thr	Ala	Asn	Ser	Lys	Lys
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Leu	Ser	Ala	Asn	Gln	Ala	Leu	Asp	His	Ala	Tyr	Lys	Ala	Ile	Gly	Lys
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Ser	Pro	Glu	Ala	Val	Ser	Asn	Gly	Thr	Val	Ala	Asn	Lys	Asn	Lys	Ala
	130					135					140				
Glu	Leu	Lys	Ala	Ala	Ala	Thr	Lys	Asp	Gly	Lys	Tyr	Arg	Leu	Ala	Tyr
145				150						155				160	
Asp	Val	Thr	Ile	Arg	Tyr	Ile	Glu	Pro	Glu	Pro	Ala	Asn	Trp	Glu	Val
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 35 40 45
 Tyr Asn Leu Pro Gly Thr Leu Val Ser Ser Thr Thr Asn Gln Phe Thr
 50 55 60
 Thr Ser Ser Gln Arg Ala Ala Val Asp Ala His Tyr Asn Leu Gly Lys
 65 70 75 80
 Val Tyr Asp Tyr Phe Tyr Gln Lys Phe Asn Arg Asn Ser Tyr Asp Asn
 85 90 95
 Lys Gly Gly Lys Ile Val Ser Ser Val His Tyr Gly Ser Arg Tyr Asn
 100 105 110
 Asn Ala Ala Trp Ile Gly Asp Gln Met Ile Tyr Gly Asp Gly Asp Gly
 115 120 125
 Ser Phe Phe Ser Pro Leu Ser Gly Ser Met Asp Val Thr Ala His Glu
 130 135 140
 Met Thr His Gly Val Thr Gln Glu Thr Ala Asn Leu Asn Tyr Glu Asn
 145 150 155 160
 Gln Pro Gly Ala Leu Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr Phe
 165 170 175
 Asn Asp Thr Glu Asp Trp Asp Ile Gly Glu Asp Ile Thr Val Ser Gln
 180 185 190
 Pro Ala Leu Arg Ser Leu Ser Asn Pro Thr Lys Tyr Gly Gln Pro Asp
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 Asn Phe Lys Asn Tyr Lys Asn Leu Pro Asn Thr Asp Ala Gly Asp Tyr
 210 215 220
 Gly Gly Val His Thr Asn Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn
 225 230 235 240
 Thr Ile Thr Lys Ile Gly Val Asn Lys Ala Glu Gln Ile Tyr Tyr Arg
 245 250 255
 Ala Leu Thr Val Tyr Leu Thr Pro Ser Ser Thr Phe Lys Asp Ala Lys
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- 141 -

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 <223> primer
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 <223> n is a, c, g, or t
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 15 <210> 65
 <211> 37
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 20 <223> primer
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 25 <400> 65
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 <210> 66
 <211> 37
 <212> DNA
 30 <213> Artificial Sequence
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 35 <222> (19)..(20)
 <223> n is a, c, g, or t

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<400> 66
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 <210> 67
 < 211> 37
 5 < 212> DNA
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 < 223> n is a, c, g, or t
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 15 <210> 68
 < 211> 37
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 20 < 223> primer
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 < 221> misc_feature
 < 222> (19)..(20)
 < 223> n is a, c, g, or t
 25 <400> 68
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 <210> 69
 < 211> 39
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 30 < 213> Artificial Sequence
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 < 223> primer
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 < 221> misc_feature
 35 < 222> (21)..(22)
 < 223> n is a, c, g, or t

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<400> 69
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 15 <210> 71
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 20 <223> primer
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 25 <400> 71
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 <210> 72
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 35 <222> (22)..(23)
 <223> n is a, c, g, or t

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<400> 72
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 <210> 73
 <211> 37
 5 <212> DNA
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 10 <221> misc_feature
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 15 <210> 74
 <211> 42
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 20 <223> primer
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 <222> (22)..(23)
 <223> n is a, c, g, or t
 25 <400> 74
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 <210> 75
 <211> 39
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 35 <222> (20)..(21)
 <223> n is a, c, g, or t

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<400> 75
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 <210> 76
 < 211> 38
 5 < 212> DNA
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 10 < 221> misc_feature
 < 222> (19)..(20)
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 15 <210> 77
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 20 < 223> primer
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 < 211> 40
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 < 223> n is a, c, g, or t

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<400> 78
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 < 211> 37
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 < 222> (19)..(20)
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 15 <210> 80
 < 211> 39
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 20 < 223> primer
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 < 221> misc_feature
 < 222> (21)..(22)
 < 223> n is a, c, g, or t
 25 <400> 80
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 <210> 81
 < 211> 44
 < 212> DNA
 30 < 213> Artificial Sequence
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 < 223> primer
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 < 221> misc_feature
 35 < 222> (19)..(20)
 < 223> n is a, c, g, or t

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<400> 81
 gtggagcatg ccgccacann sggaacaggt acgactotta aagg 44
 <210> 82
 < 211> 59
 5 < 212> DNA
 < 213> Artificial Sequence
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 < 223> primer
 <220>
 10 < 221> misc_feature
 < 222> (24)..(25)
 < 223> n is a, c, g, or t
 <400> 82
 ccggaacagg tacgactctt aaannsaaaa cgggtctcatt aatatattct tctgaaagc 59
 15 <210> 83
 < 211> 51
 < 212> DNA
 < 213> Artificial Sequence
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 20 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (29)..(30)
 < 223> n is a, c, g, or t
 25 <400> 83
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 <210> 84
 < 211> 49
 < 212> DNA
 30 < 213> Artificial Sequence
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 < 223> primer
 <220>
 < 221> misc_feature
 35 < 222> (23)..(24)
 < 223> n is a, c, g, or t

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<400> 84
 gtacgatctg caaaaccgcg agnnsaacct gccgggcaca ctctatcc 49
 <210> 85
 < 211> 51
 5 < 212> DNA
 < 213> Artificial Sequence
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 < 223> primer
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 10 < 221> misc_feature
 < 222> (26)..(27)
 < 223> n is a, c, g, or t
 <400> 85
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 15 <210> 86
 < 211> 48
 < 212> DNA
 < 213> Artificial Sequence
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 20 < 223> primer
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 < 221> misc_feature
 < 222> (24)..(25)
 < 223> n is a, c, g, or t
 25 <400> 86
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 < 211> 56
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 30 < 213> Artificial Sequence
 <220>
 < 223> primer
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 < 221> misc_feature
 35 < 222> (30)..(31)
 < 223> n is a, c, g, or t

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<400> 87
 gcagatacaa taacgcagcc tggatcggen nscaaatgat ttacggtgac ggcgac 56
 <210> 88
 < 211> 54
 5 < 212> DNA
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 < 222> (26)..(27)
 < 223> n is a, c, g, or t
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 15 <210> 89
 < 211> 41
 < 212> DNA
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 < 222> (19)..(20)
 < 223> n is a, c, g, or t
 25 <400> 89
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 <210> 90
 < 211> 48
 < 212> DNA
 30 < 213> Artificial Sequence
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 < 223> primer
 <220>
 < 221> misc_feature
 35 < 222> (25)..(26)
 < 223> n is a, c, g, or t

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<400> 90
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 < 211> 49
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 < 222> (28)..(29)
 < 223> n is a, c, g, or t
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 15 <210> 92
 < 211> 48
 < 212> DNA
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 25 <400> 92
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 <210> 93
 < 211> 48
 < 212> DNA
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 35 < 222> (27)..(28)
 < 223> n is a, c, g, or t

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<400> 93
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 <211> 53
 5 <212> DNA
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 <223> primer
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 10 <221> misc_feature
 <222> (24)..(25)
 <223> n is a, c, g, or t
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 15 <210> 95
 <211> 60
 <212> DNA
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 <220>
 20 <223> primer
 <220>
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 <222> (27)..(28)
 <223> n is a, c, g, or t
 25 <400> 95
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 <210> 96
 <211> 40
 <212> DNA
 30 <213> Artificial Sequence
 <220>
 <223> primer
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 <221> misc_feature
 35 <222> (20)..(21)
 <223> n is a, c, g, or t

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<400> 96
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 < 211> 49
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 < 222> (26)..(27)
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 15 <210> 98
 < 211> 56
 < 212> DNA
 < 213> Artificial Sequence
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 20 < 223> primer
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 < 221> misc_feature
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 < 223> n is a, c, g, or t
 25 <400> 98
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 <210> 99
 < 211> 53
 < 212> DNA
 30 < 213> Artificial Sequence
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 35 < 222> (26)..(27)
 < 223> n is a, c, g, or t

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<400> 99
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 <210> 100
 <211> 46
 5 <212> DNA
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 10 <221> misc_feature
 <222> (21)..(22)
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 15 <210> 101
 <211> 51
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 20 <223> primer
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 25 <400> 101
 ccgtcatcaa cttttaaga tgcaaaans gcttgatc aatctgegcg g 51
 <210> 102
 <211> 42
 <212> DNA
 30 <213> Artificial Sequence
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 <220>
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 35 <222> (20)..(21)
 <223> n is a, c, g, or t

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<400> 102
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 < 211> 39
 5 < 212> DNA
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 < 222> (18)..(19)
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 <400> 103
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 15 <210> 104
 < 211> 47
 < 212> DNA
 < 213> Artificial Sequence
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 20 < 223> primer
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 < 221> misc_feature
 < 222> (23)..(24)
 < 223> n is a, c, g, or t
 25 <400> 104
 cctttacggc tctcaagatg cttnsagcgt agaagctgcc tggaatg 47
 <210> 105
 < 211> 47
 < 212> DNA
 30 < 213> Artificial Sequence
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 35 < 222> (24)..(25)
 < 223> n is a, c, g, or t

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<400> 105
 ctcaagatgc tgcaagcgta gaannsgcct ggaatgcagt cggattg 47
 <210> 106
 < 211> 49
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 15 <210> 107
 < 211> 53
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 25 <400> 107
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 <210> 108
 < 211> 46
 < 212> DNA
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 < 223> n is a, c, g, or t

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<400> 108
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 <210> 109
 < 211> 43
 5 < 212> DNA
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 15 <210> 110
 < 211> 47
 < 212> DNA
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 < 223> n is a, c, g, or t
 25 <400> 110
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 <210> 111
 < 211> 44
 < 212> DNA
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 <220>
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 < 223> n is a, c, g, or t

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<400> 111
 cctttaagag tcgtacctgt tccsntgtg gcggcatgct ccac 44
 <210> 112
 <211> 59
 5 <212> DNA
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 <222> (35)..(36)
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 15 <210> 113
 <211> 51
 <212> DNA
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 25 <400> 113
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 <210> 114
 <211> 49
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 <221> misc_feature
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 <223> n is a, c, g, or t

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<400> 114
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 <210> 115
 < 211> 51
 5 < 212> DNA
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 < 222> (25).. (26)
 < 223> n is a, c, g, or t
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 15 <210> 116
 < 211> 48
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 < 223> n is a, c, g, or t
 25 <400> 116
 caacggcagc gcgctgagaa gasnntgtaa actggtttgt ggtgctgg 48
 <210> 117
 < 211> 56
 < 212> DNA
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 <220>
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 <220>
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 35 < 222> (26)..(27)
 < 223> n is a, c, g, or t

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<400> 117
 gtcgccgtca ccgtaaatca ttgsnngcc gatccaggct gcgttattgt atctgc 56
 <210> 118
 < 211> 54
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 < 222> (28)..(29)
 < 223> n is a, c, g, or t
 <400> 118
 gaaccggaaa gaggtgagaa gaatgasnng tcgccgtcac cgtaaatcat ttgg 54
 15 <210> 119
 < 211> 41
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 < 223> n is a, c, g, or t
 25 <400> 119
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 <210> 120
 < 211> 48
 < 212> DNA
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 <220>
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 35 < 222> (23)..(24)
 < 223> n is a, c, g, or t

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<400> 120
 gtagttcagg ttggctgttt csntgtaac gccatgtgtc atttcattg 48
 <210> 121
 <211> 49
 5 <212> DNA
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 10 <221> misc_feature
 <222> (21)..(22)
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 15 <210> 122
 <211> 48
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 25 <400> 122
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 <210> 123
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 <221> misc_feature
 35 <222> (21)..(22)
 <223> n is a, c, g, or t

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<400> 123
 caccgatatc ccagtcctcs nnatcgttga agtaccgaa tacatcag 48
 <210> 124
 < 211> 53
 5 < 212> DNA
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 10 < 221> misc_feature
 < 222> (29)..(30)
 < 223> n is a, c, g, or t
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 15 <210> 125
 < 211> 60
 < 212> DNA
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 <220>
 20 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (33)..(34)
 < 223> n is a, c, g, or t
 25 <400> 125
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 < 211> 40
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 <220>
 < 221> misc_feature
 35 < 222> (20)..(21)
 < 223> n is a, c, g, or t

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<400> 126
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 <210> 127
 <211> 49
 5 <212> DNA
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 10 <221> misc_feature
 <222> (23)..(24)
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 15 <210> 128
 <211> 56
 <212> DNA
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 20 <223> primer
 <220>
 <221> misc_feature
 <222> (30)..(31)
 <223> n is a, c, g, or t
 25 <400> 128
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 <210> 129
 <211> 53
 <212> DNA
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 <223> primer
 <220>
 <221> misc_feature
 35 <222> (27)..(28)
 <223> n is a, c, g, or t

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<400> 129
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 <210> 130
 < 211> 46
 5 < 212> DNA
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 < 223> primer
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 10 < 221> misc_feature
 < 222> (25)..(26)
 < 223> n is a, c, g, or t
 <4'00> 130
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 15 <210> 131
 < 211> 51
 < 212> DNA
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 20 < 223> primer
 <220>
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 25 <400> 131
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 <210> 132
 < 211> 42
 < 212> DNA
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 <220>
 < 223> primer
 <220>
 < 221> misc_feature
 35 < 222> (22)..(23)
 < 223> n is a, c, g, or t

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<400> 132
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 <210> 133
 < 211> 39
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 <400> 133
 ctacgettgc agcatcttgs nngccgtaaa ggtccegcg 39
 15 <210> 134
 < 211> 47
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 < 222> (24)..(25)
 < 223> n is a, c, g, or t
 25 <400> 134
 cattccaggc agcttctacg ctsnnagcat cttgagagcc gtaaagg 47
 <210> 135
 < 211> 47
 < 212> DNA
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 < 223> n is a, c, g, or t

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<400> 135
 caatccgact gcattccagg csnnctctac gcttcagca tcttgag 47
 <210> 136
 <211> 49
 5 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> primer
 <220>
 10 <221> misc_feature
 <222> (27)..(28)
 <223> n is a, c, g, or t
 <400> 136
 etttcttgt ttacaatccg actgcsnncc aggcagcttc tacgcttgc 49
 15 <210> 137
 <211> 53
 <212> DNA
 <213> Artificial Sequence
 <220>
 20 <223> primer
 <220>
 <221> misc feature
 <222> (31)..(32)
 <223> n is a, c, g, or t
 25 <400> 137
 ggatttccgg tctctttct tgtttacaas nngactgcat tccaggcagc ttc 53
 <210> 138
 <211> 46
 <212> DNA
 30 <213> Artificial Sequence
 <220>
 <223> primer
 <220>
 <221> misc feature
 35 <222> (26)..(27)
 <223> n is a, c, g, or t

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<400> 138
 ctgagaagaa gttgtaaact ggtsnnggt gctggatacg agtgtg 46
 <210> 139
 < 211> 43
 5 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 10 < 221> misc_feature
 < 222> (23)..(24)
 < 223> n is a, c, g, or t
 <400> 139
 gatccagget gcgttattgt asnngctgcc gtaatgaacg gag 43
 15 <210> 140
 < 211> 47
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 20 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (24)..(25)
 < 223> n is a, c, g, or t
 25 <400> 140
 gtgtcatttc atgagcgggt acsnncattg aaccggaag aggtgag 47
 <210> 141
 < 211> 51
 < 212> DNA
 30 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 < 221> misc_feature
 35 < 222> (27)..(28)
 < 223> n is a, c, g, or t

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<400> 141
 gcgacgggtc attcttctca cctctnnsg gttcaatgga cgtaaccgct c 51
 <210> 142
 < 211> 54
 5 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 10 < 221> misc feature
 < 222> (30)..(31)
 < 223> n is a, c, g, or t
 <400> 142
 gcgacgggtc attcttctca cctcttccn nstcaatgga cgtaaccgct catg 54
 15 <210> 143
 < 211> 41
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 20 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (20)..(21)
 < 223> n is a, c, g, or t
 25 <400> 143
 cttctcacct ctttccggtn nsatggacgt aaccgctcat g 41
 <210> 144
 < 211> 43
 < 212> DNA
 30 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 < 221> misc_feature
 35 < 222> (22)..(23)
 < 223> n is a, c, g, or t

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<400> 144
 cctctttccg gttcaatgga cnnsaccgct catgaaatga cac 43
 <210> 145
 < 211> 49
 5 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 10 < 221> misc feature
 < 222> (20)..(21)
 < 223> n is a, c, g, or t
 <400> 145
 cagccagccg gctctccgcn nsftatccaa tccgacaaaa tacggacag 49
 15 <210> 146
 < 211> 49
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 20 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (23)..(24)
 < 223> n is a, c, g, or t
 25 <400> 146
 cagccagccg gctctccgca gcnnstccaa tccgacaaaa tacggacag 49
 <210> 147
 < 211> 51
 < 212> DNA
 30 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 < 221> misc_feature
 35 < 222> (26)..(27)
 < 223> n is a, c, g, or t

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<400> 147
 cagccagccg gctctccgca gcttannsaa tccgacaaaa tacggacagc c 51
 <210> 148
 < 211> 54
 5 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 10 < 221> misc_feature
 < 222> (31)..(32)
 < 223> n is a, c, g, or t
 <400> 148
 cagcctgata atttcaaaaa ttacaaaaac nnsccgaaca ctgatgccgg cgac 54
 15 <210> 149
 < 211> 54
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 20 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (34)..(35)
 < 223> n is a, c, g, or t
 25 <400> 149
 cagcctgata atttcaaaaa ttacaaaaac cttnnsaaca ctgatgccgg cgac 54
 <210> 150
 < 211> 57
 < 212> DNA
 30 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 < 221> misc_feature
 35 < 222> (37)..(38)
 < 223> n is a, c, g, or t

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<400> 150
 cagcctgata atttcaaaaa ttacaaaaac ctccggnnsa ctgatgccgg cgactac 57
 <210> 151
 < 211> 59
 5 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 10 < 221> misc_feature
 < 222> (40)..(41)
 < 223> n is a, c, g, or t
 <400> 151
 cagcctgata atttcaaaaa ttacaaaaac ctccgaacn nsgatgccgg cgactacgg 59
 15 <210> 152
 < 211> 64
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 20 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (43)..(44)
 < 223> n is a, c, g, or t
 25 <400> 152
 cagcctgata atttcaaaaa ttacaaaaac ctccgaaca cttnsgccgg cgactacggc 60
 ggcg 64
 <210> 153
 < 211> 66
 < 212> DNA
 30 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 < 221> misc_feature
 35 < 222> (46)..(47)

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< 223> n is a, c, g, or t
 <400> 153
 cagcctgata atttcaaaaa ttacaaaaac cttccgaaca ctgatnnsgg cgactacggc 60
 ggcgtg 66
 <210> 154
 5 < 211> 42
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 10 <220>
 < 221> misc_feature
 < 222> (20)..(21)
 < 223> n is a, c, g, or t
 <400> 154
 15 cctccgaac actgatgcn nsgactacgg cggcgtgcat ac 42
 <210> 155
 < 211> 43
 < 212> DNA
 < 213> Artificial Sequence
 20 <220>
 < 223> primer
 <220>
 < 221> misc feature
 < 222> (19)..(20)
 25 < 223> n is a, c, g, or t
 <400> 155
 cgggacctt acgctctnn sgatgctgca agcgtagaag ctg 43
 <210> 156
 < 211> 52
 30 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 35 < 221> misc_feature

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< 222> (21)..(22)
 < 223> n is a, c, g, or t
 <400> 156
 gcgtagaagc tgcctggaat nnsctcgat tgtaacaag aaaagagacc gg 52
 5 <210> 157
 < 211> 51
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 10 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (24)..(25)
 < 223> n is a, c, g, or t
 15 <400> 157
 gagcggttac gtccattgaa ccsnnaagag gtgagaagaa tgaaccgtcg c 51
 <210> 158
 < 211> 54
 < 212> DNA
 20 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 < 221> misc_feature
 25 < 222> (24)..(25)
 < 223> n is a, c, g, or t
 <400> 158
 catgagcggc tacgtccatt gasnnggaaa gaggtgagaa gaatgaaccg tcgc 54
 <210> 159
 30 < 211> 41
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 35 <220>
 < 221> misc_feature

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< 222> (21)..(22)
 < 223> n is a, c, g, or t
 <400> 159
 catgagcggg tacgtccats nnaccggaaa gaggtgagaa g 41
 5 <210> 160
 < 211> 43
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 10 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (21)..(22)
 < 223> n is a, c, g, or t
 15 <400> 160
 gtgtcattc atgagcggts nngtcattg aaccggaaag agg 43
 <210> 161
 < 211> 49
 < 212> DNA
 20 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 < 221> misc_feature
 25 < 222> (29)..(30)
 < 223> n is a, c, g, or t
 <400> 161
 ctgtccgtat ttgtcggat tggataasn gcggagagcc ggctggctg 49
 <210> 162
 30 < 211> 49
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 35 <220>
 < 221> misc_feature

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< 222> (26)..(27)
 < 223> n is a, c, g, or t
 <400> 162
 ctgtccgtat ttgtcggat tggasnngct gcggagagcc ggctggctg 49
 5 <210> 163
 < 211> 51
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 10 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (25)..(26)
 < 223> n is a, c, g, or t
 15 <400> 163
 ggctgtccgt atttgtcgg attsnntaag ctgcggagag ccggctggct g 51
 <210> 164
 < 211> 54
 < 212> DNA
 20 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 < 221> misc_feature
 25 < 222> (23)..(24)
 < 223> n is a, c, g, or t
 <400> 164
 gtcgccggca tcagtgttcg gsnngtttt gtaattttg aaattatcag gctg 54
 <210> 165
 30 < 211> 54
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 35 <220>
 < 221> misc_feature

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< 222> (20)..(21)
 < 223> n is a, c, g, or t
 <400> 165
 gtcgccggca tcagtgttsn naaggttttt gtaatttttg aaattatcag gctg 54
 5 <210> 166
 < 211> 57
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 10 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (20)..(21)
 < 223> n is a, c, g, or t
 15 <400> 166
 gtagtcgccg gcatcagtsn ncggaagggt ttgtaattt ttgaaattat caggctg 57
 <210> 167
 < 211> 59
 < 212> DNA
 20 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 < 221> misc_feature
 25 < 222> (19)..(20)
 < 223> n is a, c, g, or t
 <400> 167
 ccgtagtcgc cggcatcsnn gtcggaagg ttttgtaat tttgaaatt atcaggctg 59
 <210> 168
 30 < 211> 64
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 35 <220>
 < 221> misc_feature

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< 222> (21)..(22)
 < 223> n is a, c, g, or t
 <400> 168
 cgccgccgta gtcgccgcs nnagtgttcg gaagggtttt gtaatttttg aaattatcag 60
 gctg 64
 5 <210> 169
 < 211> 66
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 10 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (20)..(21)
 < 223> n is a, c, g, or t
 15 <400> 169
 caccgccg tagtcgccsn natcagtgtt cggaagggtt ttgtaatttt tgaaattatc 60
 aggctg 66
 <210> 170
 < 211> 42
 < 212> DNA
 20 < 213> Artificial Sequence
 <220>
 < 223> primer
 <220>
 < 221> misc_feature
 25 < 222> (22)..(23)
 < 223> n is a, c, g, or t
 <400> 170
 gtatgcacgc cgccgtagtc snnggcacga gtgttcggaa gg 42
 <210> 171
 30 < 211> 43
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 35 <220>

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< 221> misc feature
 < 222> (24)..(25)
 < 223> n is a, c, g, or t
 <400> 171
 5 cagcttctac gcttgcagca tcsnnagagc cgtaaaggtc ccg 43
 <210> 172
 < 211> 52
 < 212> DNA
 < 213> Artificial Sequence
 10 <220>
 < 223> primer
 <220>
 < 221> misc_feature
 < 222> (31)..(32)
 15 < 223> n is a, c, g, or t
 <400> 172
 ccggtctctt ttctgttta caatccgacs nnattccagg cagcttctac gc 52
 <210> 173
 < 211> 521
 20 < 212> PRT
 < 213> Bacillus amyloliquefaciens
 <400> 173
 Met Gly Leu Gly Lys Lys Leu Ser Val Ala Val Ala Ala Ser Phe Met
 1 5 10 15

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Ser Leu Thr Ile Ser Leu Pro Gly Val Gln Ala Ala Glu Asn Pro Gln
20                                     30
Leu Lys Glu Asn Leu Thr Asn Phe Val Pro Lys His Ser Leu Val Gln
35                                     45
Ser Glu Leu Pro Ser Val Ser Asp Lys Ala Ile Lys Gln Tyr Leu Lys
50                                     60
Gln Asn Gly Lys Val Phe Lys Gly Asn Pro Ser Glu Arg Leu Lys Leu
65                                     75
Ile Asp Gln Thr Thr Asp Asp Leu Gly Tyr Lys His Phe Arg Tyr Val
85                                     95
Pro Val Val Asn Gly Val Pro Val Lys Asp Ser Gln Val Ile Ile His
100                                    110
Val Asp Lys Ser Asn Asn Val Tyr Ala Ile Asn Gly Glu Leu Asn Asn
115                                    125
Asp Val Ser Ala Lys Thr Ala Asn Ser Lys Lys Leu Ser Ala Asn Gln
130                                    140
Ala Leu Asp His Ala Tyr Lys Ala Ile Gly Lys Ser Pro Glu Ala Val
145                                    160
Ser Asn Gly Thr Val Ala Asn Lys Asn Lys Ala Glu Leu Lys Ala Ala
165                                    175
Ala Thr Lys Asp Gly Lys Tyr Arg Leu Ala Tyr Asp Val Thr Ile Arg
180                                    190
Tyr Ile Glu Pro Glu Pro Ala Asn Trp Glu Val Thr Val Asp Ala Glu
195                                    205
Thr Gly Lys Ile Leu Lys Lys Gln Asn Lys Val Glu His Ala Ala Thr
210                                    220
Thr Gly Thr Gly Thr Thr Leu Lys Gly Lys Thr Val Ser Leu Asn Ile
225                                    235
Ser Ser Glu Ser Gly Lys Tyr Val Leu Arg Asp Leu Ser Lys Pro Thr
245                                    255
Gly Thr Gln Ile Ile Thr Tyr Asp Leu Gln Asn Arg Glu Tyr Asn Leu
260                                    270
Pro Gly Thr Leu Val Ser Ser Thr Thr Asn Gln Phe Thr Thr Ser Ser
275                                    285
Gln Arg Ala Ala Val Asp Ala His Tyr Asn Leu Gly Lys Val Tyr Asp
290                                    300
Tyr Phe Tyr Gln Lys Phe Asn Arg Asn Ser Tyr Asp Asn Lys Gly Gly
305                                    315
Lys Ile Val Ser Ser Val His Tyr Gly Ser Arg Tyr Asn Asn Ala Ala
325                                    335
Trp Ile Gly Asp Gln Met Ile Tyr Gly Asp Gly Asp Gly Ser Phe Phe
340                                    350
Ser Pro Leu Ser Gly Ser Met Asp Val Thr Ala His Glu Met Thr His
355                                    365
Gly Val Thr Gln Glu Thr Ala Asn Leu Asn Tyr Glu Asn Gln Pro Gly
370                                    380
Ala Leu Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr Phe Asn Asp Thr
385                                    395
Glu Asp Trp Asp Ile Gly Glu Asp Ile Thr Val Ser Gln Pro Ala Leu
405                                    415
Arg Ser Leu Ser Asn Pro Thr Lys Tyr Gly Gln Pro Asp Asn Phe Lys
420                                    430
Asn Tyr Lys Asn Leu Pro Asn Thr Asp Ala Gly Asp Tyr Gly Gly Val
435                                    445
His Thr Asn Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn Thr Ile Thr
450                                    460
Lys Ile Gly Val Asn Lys Ala Glu Gln Ile Tyr Tyr Arg Ala Leu Thr
465                                    475
Val Tyr Leu Thr Pro Ser Ser Thr Phe Lys Asp Ala Lys Ala Ala Leu
485                                    495
Ile Gln Ser Ala Arg Asp Leu Tyr Gly Ser Gln Asp Ala Ala Ser Val
500                                    510
Glu Ala Ala Trp Asn Ala Val Gly Leu
515                                    520

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<210> 174

<211> 521

5

<212> PRT

<213> Bacillus subtilis

<400> 174

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Met Gly Leu Gly Lys Lys Leu Ser Val Arg Val Ala Ala Ser Phe Met
1      5      10      15
Ser Leu Ser Ile Ser Leu Pro Gly Val Gln Ala Ala Glu Gly His Gln
20      25      30
Leu Lys Glu Asn Gln Thr Asn Phe Leu Ser Lys Lys Pro Ile Ala Gln
35      40      45
Ser Glu Leu Ser Ala Pro Asn Asp Lys Ala Val Lys Gln Phe Leu Lys
50      55      60
Lys Asn Ser Asn Ile Phe Lys Gly Asp Pro Ser Lys Ser Val Lys Leu
65      70      75      80
Val Glu Ser Thr Thr Asp Ala Leu Gly Tyr Lys His Phe Arg Tyr Ala
85      90      95
Pro Val Val Asn Gly Val Pro Ile Lys Asp Ser Gln Val Ile Val His
100     105     110
Val Asp Lys Ser Asp Asn Val Tyr Ala Val Asn Gly Glu Leu His Asn
115     120     125
Gln Ser Ala Ala Lys Thr Asp Asn Ser Gln Lys Val Ser Ser Glu Lys
130     135     140
Ala Leu Ala Leu Ala Phe Lys Ala Ile Gly Lys Ser Pro Asp Ala Val
145     150     155     160
Ser Asn Gly Ala Ala Lys Asn Ser Asn Lys Ala Glu Leu Lys Ala Ile
165     170     175
Glu Thr Lys Asp Gly Ser Tyr Arg Leu Ala Tyr Asp Val Thr Ile Arg
180     185     190
Tyr Val Glu Pro Glu Pro Ala Asn Trp Glu Val Leu Val Asp Ala Glu
195     200     205
Thr Gly Ser Ile Leu Lys Gln Gln Asn Lys Val Glu His Ala Ala Ala
210     215     220
Thr Gly Ser Gly Thr Thr Leu Lys Gly Ala Thr Val Pro Leu Asn Ile
225     230     235     240
Ser Tyr Glu Gly Gly Lys Tyr Val Leu Arg Asp Leu Ser Lys Pro Thr
245     250     255
Gly Thr Gln Ile Ile Thr Tyr Asp Leu Gln Asn Arg Gln Ser Arg Leu
260     265     270
Pro Gly Thr Leu Val Ser Ser Thr Thr Lys Thr Phe Thr Ser Ser Ser
275     280     285
Gln Arg Ala Ala Val Asp Ala His Tyr Asn Leu Gly Lys Val Tyr Asp
290     295     300
Tyr Phe Tyr Ser Asn Phe Lys Arg Asn Ser Tyr Asp Asn Lys Gly Ser
305     310     315     320
Lys Ile Val Ser Ser Val His Tyr Gly Thr Gln Tyr Asn Asn Ala Ala
325     330     335
Trp Thr Gly Asp Gln Met Ile Tyr Gly Asp Gly Asp Gly Ser Phe Phe
340     345     350
Ser Pro Leu Ser Gly Ser Leu Asp Val Thr Ala His Glu Met Thr His
355     360     365
Gly Val Thr Gln Glu Thr Ala Asn Leu Ile Tyr Glu Asn Gln Pro Gly
370     375     380
Ala Leu Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr Phe Asn Asp Thr
385     390     395     400
Glu Asp Trp Asp Ile Gly Glu Asp Ile Thr Val Ser Gln Pro Ala Leu
405     410     415
Arg Ser Leu Ser Asn Pro Thr Lys Tyr Asn Gln Pro Asp Asn Tyr Ala
420     425     430
Asn Tyr Arg Asn Leu Pro Asn Thr Asp Glu Gly Asp Tyr Gly Gly Val
435     440     445
His Thr Asn Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn Thr Ile Thr
450     455     460
Lys Leu Gly Val Ser Lys Ser Gln Gln Ile Tyr Tyr Arg Ala Leu Thr
465     470     475     480
Thr Tyr Leu Thr Pro Ser Ser Thr Phe Lys Asp Ala Lys Ala Ala Leu
485     490     495
Ile Gln Ser Ala Arg Asp Leu Tyr Gly Ser Thr Asp Ala Ala Lys Val
500     505     510
Glu Ala Ala Trp Asn Ala Val Gly Leu
515     520

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<210> 175

<211> 509

5

<212> PRT

<213> Staphylococcus aureus

<400> 175

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Met Arg Lys Phe Ser Arg Tyr Ala Phe Thr Ser Met Ala Thr Val Thr
1      5      10      15
Leu Leu Ser Ser Leu Thr Pro Ala Ala Leu Ala Ser Asp Thr Asn His
20      25      30
Lys Pro Ala Thr Ser Asp Ile Asn Phe Glu Ile Thr Gln Lys Ser Asp
35      40      45
Ala Val Lys Ala Leu Lys Glu Leu Pro Lys Ser Glu Asn Val Lys Asn
50      55      60
His Tyr Gln Asp Tyr Ser Val Thr Asp Val Lys Thr Asp Lys Lys Gly
65      70      75      80
Phe Thr His Tyr Thr Leu Gln Pro Ser Val Asp Gly Val His Ala Pro
85      90      95
Asp Lys Glu Val Lys Val His Ala Asp Lys Ser Gly Lys Val Val Leu
100     105     110
Ile Asn Gly Asp Thr Asp Ala Lys Lys Val Lys Pro Thr Asn Lys Val
115     120     125
Thr Leu Ser Lys Asp Glu Ala Ala Asp Lys Ala Phe Asn Ala Val Lys
130     135     140
Ile Asp Lys Asn Lys Ala Lys Asn Leu Gln Asp Asp Val Ile Lys Glu
145     150     155     160
Asn Lys Val Glu Ile Asp Gly Asp Ser Asn Lys Tyr Ile Tyr Asn Ile
165     170     175
Glu Leu Ile Thr Val Thr Pro Glu Ile Ser His Trp Lys Val Lys Ile
180     185     190
Asp Ala Asp Thr Gly Ala Val Val Glu Lys Thr Asn Leu Val Lys Glu
195     200     205
Ala Ala Ala Thr Gly Thr Gly Lys Gly Val Leu Gly Asp Thr Lys Asp
210     215     220
Ile Asn Ile Asn Ser Ile Asp Gly Gly Phe Ser Leu Glu Asp Leu Thr
225     230     235     240
His Gln Gly Lys Leu Ser Ala Tyr Asn Phe Asn Asp Gln Thr Gly Gln
245     250     255
Ala Thr Leu Ile Thr Asn Glu Asp Glu Asn Phe Val Lys Asp Asp Gln
260     265     270
Arg Ala Gly Val Asp Ala Asn Tyr Tyr Ala Lys Gln Thr Tyr Asp Tyr
275     280     285
Tyr Lys Asn Thr Phe Gly Arg Glu Ser Tyr Asp Asn His Gly Ser Pro
290     295     300
Ile Val Ser Leu Thr His Val Asn His Tyr Gly Gly Gln Asp Asn Arg
305     310     315     320
Asn Asn Ala Ala Trp Ile Gly Asp Lys Met Ile Tyr Gly Asp Gly Asp
325     330     335
Gly Arg Thr Phe Thr Asn Leu Ser Gly Ala Asn Asp Val Val Ala His
340     345     350
Glu Leu Thr His Gly Val Thr Gln Glu Thr Ala Asn Leu Glu Tyr Lys
355     360     365
Asp Gln Ser Gly Ala Leu Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr
370     375     380
Phe Val Asp Asp Glu Asp Phe Leu Met Gly Glu Asp Val Tyr Thr Pro
385     390     395     400
Gly Lys Glu Gly Asp Ala Leu Arg Ser Met Ser Asn Pro Glu Gln Phe
405     410     415
Gly Gln Pro Ser His Met Lys Asp Tyr Val Tyr Thr Glu Lys Asp Asn
420     425     430
Gly Gly Val His Thr Asn Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn
435     440     445
Val Ile Gln Ala Ile Gly Lys Ser Lys Ser Glu Gln Ile Tyr Tyr Arg
450     455     460
Ala Leu Thr Glu Tyr Leu Thr Ser Asn Ser Asn Phe Lys Asp Cys Lys
465     470     475     480
Asp Ala Leu Tyr Gln Ala Ala Lys Asp Leu Tyr Asp Glu Gln Thr Ala
485     490     495
Glu Gln Val Tyr Glu Ala Trp Asn Glu Val Gly Val Glu
500     505

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<210> 176

<211> 548

5

<212> PRT

<213> *Bacillus stearothermophilis*

<400> 176

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Met Asn Lys Arg Ala Met Leu Gly Ala Ile Gly Leu Ala Phe Gly Leu
 1 5 10 15
 Leu Ala Ala Pro Ile Gly Ala Ser Ala Lys Gly Glu Ser Ile Val Trp
 20 25 30
 Asn Glu Gln Trp Lys Thr Pro Ser Phe Val Ser Gly Ser Leu Leu Asn
 35 40 45
 Gly Gly Glu Gln Ala Leu Glu Glu Leu Val Tyr Gln Tyr Val Asp Arg
 50 55 60
 Glu Asn Gly Thr Phe Arg Leu Gly Gly Arg Ala Arg Asp Arg Leu Ala
 65 70 75 80
 Leu Ile Gly Lys Gln Thr Asp Glu Leu Gly His Thr Val Met Arg Phe
 85 90 95
 Glu Gln Arg His His Gly Ile Pro Val Tyr Gly Thr Met Leu Ala Ala
 100 105 110
 His Val Lys Asp Gly Glu Leu Ile Ala Leu Ser Gly Ser Leu Ile Pro
 115 120 125
 Asn Leu Asp Gly Gln Pro Arg Leu Lys Lys Ala Lys Thr Val Thr Val
 130 135 140
 Gln Gln Ala Glu Ala Ile Ala Glu Gln Asp Val Thr Glu Thr Val Thr
 145 150 155 160
 Lys Glu Arg Pro Thr Thr Glu Asn Gly Glu Arg Thr Arg Leu Val Ile
 165 170 175
 Tyr Pro Thr Asp Gly Thr Ala Arg Leu Ala Tyr Glu Val Asn Val Arg
 180 185 190
 Phe Leu Thr Pro Val Pro Gly Asn Trp Val Tyr Ile Ile Asp Ala Thr
 195 200 205
 Asp Gly Ala Ile Leu Asn Lys Phe Asn Gln Ile Asp Ser Arg Gln Pro
 210 215 220
 Gly Gly Gly Gln Pro Val Ala Gly Ala Ser Thr Val Gly Val Gly Arg
 225 230 235 240
 Gly Val Leu Gly Asp Gln Lys Tyr Ile Asn Thr Thr Tyr Ser Ser Tyr
 245 250 255
 Tyr Gly Tyr Tyr Tyr Leu Gln Asp Asn Thr Arg Gly Ser Gly Ile Phe
 260 265 270
 Thr Tyr Asp Gly Arg Asn Arg Thr Val Leu Pro Gly Ser Leu Trp Thr
 275 280 285
 Asp Gly Asp Asn Gln Phe Thr Ala Ser Tyr Asp Ala Ala Val Asp
 290 295 300
 Ala His Tyr Tyr Ala Gly Val Val Tyr Asp Tyr Tyr Lys Asn Val His
 305 310 315 320
 Gly Arg Leu Ser Tyr Asp Gly Ser Asn Ala Ala Ile Arg Ser Thr Val
 325 330 335
 His Tyr Gly Arg Gly Tyr Asn Asn Ala Phe Trp Asn Gly Ser Gln Met
 340 345 350
 Val Tyr Gly Asp Gly Asp Gly Gln Thr Phe Leu Pro Phe Ser Gly Gly
 355 360 365
 Ile Asp Val Val Gly His Glu Leu Thr His Ala Val Thr Asp Tyr Thr
 370 375 380
 Ala Gly Leu Val Tyr Gln Asn Glu Ser Gly Ala Ile Asn Glu Ala Met
 385 390 395 400
 Ser Asp Ile Phe Gly Thr Leu Val Glu Phe Tyr Ala Asn Arg Asn Pro
 405 410 415
 Asp Trp Glu Ile Gly Glu Asp Ile Tyr Thr Pro Gly Val Ala Gly Asp
 420 425 430
 Ala Leu Arg Ser Met Ser Asp Pro Ala Lys Tyr Gly Asp Pro Asp His
 435 440 445
 Tyr Ser Lys Arg Tyr Thr Gly Thr Gln Asp Asn Gly Gly Val His Thr
 450 455 460
 Asn Ser Gly Ile Ile Asn Lys Ala Ala Tyr Leu Leu Ser Gln Gly Gly
 465 470 475 480
 Val His Tyr Gly Val Ser Val Asn Gly Ile Gly Arg Asp Lys Met Gly
 485 490 495
 Lys Ile Phe Tyr Arg Ala Leu Val Tyr Tyr Leu Thr Pro Thr Ser Asn
 500 505 510
 Phe Ser Gln Leu Arg Ala Ala Cys Val Gln Ala Ala Asp Leu Tyr
 515 520 525
 Gly Ser Thr Ser Gln Glu Val Asn Ser Val Lys Gln Ala Phe Asn Ala
 530 535 540
 Val Gly Val Tyr
 545

<210> 177

<211> 544

<212> PRT

- 189 -

<213> Bacillus caldolyticus

<400> 177

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Met Asn Lys Arg Ala Met Leu Gly Ala Ile Gly Leu Ala Phe Gly Leu
1      5      10      15
Met Ala Trp Pro Phe Gly Ala Ser Ala Lys Gly Lys Ser Met Val Trp
20     25     30
Asn Glu Gln Trp Lys Thr Pro Ser Phe Val Ser Gly Ser Leu Leu Gly
35     40     45
Arg Cys Ser Gln Glu Leu Val Tyr Arg Tyr Leu Asp Gln Glu Lys Asn
50     55     60
Thr Phe Gln Leu Gly Gly Gln Ala Arg Glu Arg Leu Ser Leu Ile Gly
65     70     75     80
Asn Lys Leu Asp Glu Leu Gly His Thr Val Met Arg Phe Glu Gln Ala
85     90     95
Ile Ala Ala Ser Leu Cys Met Gly Ala Val Leu Val Ala His Val Asn
100    105    110
Asp Gly Glu Leu Ser Ser Leu Ser Gly Thr Leu Ile Pro Asn Leu Asp
115    120    125
Lys Arg Thr Leu Lys Thr Glu Ala Ala Ile Ser Ile Gln Gln Ala Glu
130    135    140
Met Ile Ala Lys Gln Asp Val Ala Asp Arg Val Thr Lys Glu Arg Pro
145    150    155    160
Ala Ala Glu Glu Gly Lys Pro Thr Arg Leu Val Ile Tyr Pro Asp Glu
165    170    175
Glu Thr Pro Arg Leu Ala Tyr Glu Val Asn Val Arg Phe Leu Thr Pro
180    185    190
Val Pro Gly Asn Trp Ile Tyr Met Ile Asp Ala Ala Asp Gly Lys Val
195    200    205
Leu Asn Lys Trp Asn Gln Met Asp Glu Ala Lys Pro Gly Gly Ala Gln
210    215    220
Pro Val Ala Gly Thr Ser Thr Val Gly Val Gly Arg Gly Val Leu Gly
225    230    235    240
Asp Gln Lys Tyr Ile Asn Thr Thr Tyr Ser Ser Tyr Tyr Gly Tyr Tyr
245    250    255
Tyr Leu Gln Asp Asn Thr Arg Gly Ser Gly Ile Phe Thr Tyr Asp Gly
260    265    270
Arg Asn Arg Thr Val Leu Pro Gly Ser Leu Trp Ala Asp Gly Asp Asn
275    280    285    290
Gln Phe Phe Ala Ser Tyr Asp Ala Ala Val Asp Ala His Tyr Tyr
295    300
Ala Gly Val Val Tyr Asp Tyr Tyr Lys Asn Val His Gly Arg Leu Ser
305    310    315    320
Tyr Asp Gly Ser Asn Ala Ala Ile Arg Ser Thr Val His Tyr Gly Arg
325    330    335
Gly Tyr Asn Asn Ala Phe Trp Asn Gly Ser Gln Met Val Tyr Gly Asp
340    345    350
Gly Asp Gly Gln Thr Phe Leu Pro Phe Ser Gly Gly Ile Asp Val Val
355    360    365
Gly His Glu Leu Thr His Ala Val Thr Asp Tyr Thr Ala Gly Leu Val
370    375    380
Tyr Gln Asn Glu Ser Gly Ala Ile Asn Glu Ala Met Ser Asp Ile Phe
385    390    395    400
Gly Thr Leu Val Glu Phe Tyr Ala Asn Arg Asn Pro Asp Trp Glu Ile
405    410    415
Gly Glu Asp Ile Tyr Thr Pro Gly Val Ala Gly Asp Ala Leu Arg Ser
420    425    430
Met Ser Asp Pro Ala Lys Tyr Gly Asp Pro Asp His Tyr Ser Lys Arg
435    440    445
Tyr Thr Gly Thr Gln Asp Asn Gly Gly Val His Thr Asn Ser Gly Ile
450    455    460
Ile Asn Lys Ala Ala Tyr Leu Leu Ser Gln Gly Gly Val His Tyr Gly
465    470    475    480
Val Ser Val Thr Gly Ile Gly Arg Asp Lys Met Gly Lys Ile Phe Tyr
485    490    495
Arg Ala Leu Val Tyr Tyr Leu Thr Pro Thr Ser Asn Phe Ser Gln Leu
500    505    510
Arg Ala Ala Cys Val Gln Ala Ala Asp Leu Tyr Gly Ser Thr Ser
515    520    525
Gln Glu Val Asn Ser Val Lys Gln Ala Phe Asn Ala Val Gly Val Tyr
530    535    540

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5

<210> 178

<211> 566

- 190 -

< 212> PRT

< 213> *Bacillus thuringiensis*

<400> 178

```

Met Lys Lys Lys Ser Leu Ala Leu Val Leu Ala Thr Gly Met Ala Val
 1      5      10      15
Thr Thr Phe Gly Gly Thr Gly Ser Ala Phe Ala Asp Ser Lys Asn Val
 20     25     30
Leu Ser Thr Lys Lys Tyr Asn Glu Thr Val Gln Ser Pro Glu Phe Val
 35     40     45
Ser Gly Asp Leu Thr Glu Ala Thr Gly Lys Lys Ala Glu Ser Val Val
 50     55     60
Phe Asp Tyr Leu Asn Ala Ala Lys Gly Asp Tyr Lys Leu Gly Glu Lys
 65     70     75     80
Ser Ala Gln Asp Cys Phe Lys Val Lys Gln Ala Lys Lys Asp Ala Val
 85     90     95
Thr Asp Ser Thr Val Leu Arg Leu Gln Gln Val Tyr Glu Gly Val Pro
100    105    110
Val Trp Gly Ser Thr Gln Val Ala His Val Ser Lys Asp Gly Ser Leu
115    120    125
Lys Val Leu Ser Gly Thr Val Ala Pro Asp Leu Asp Lys Lys Glu Lys
130    135    140
Leu Lys Asn Lys Asn Lys Ile Glu Gly Ala Lys Ala Ile Glu Ile Ala
145    150    155    160
Gln Lys Asp Leu Gly Ile Thr Pro Lys Tyr Glu Val Glu Pro Lys Ala
165    170    175
Asp Leu Tyr Val Tyr Gln Asn Gly Glu Glu Thr Thr Tyr Ala Tyr Val
180    185    190
Val Asn Leu Asn Phe Leu Asp Pro Ser Pro Gly Asn Tyr Tyr Tyr Phe
195    200    205
Ile Glu Ala Asp Ser Gly Lys Val Leu Asn Lys Tyr Asn Lys Leu Asp
210    215    220
His Val Ala Asn Glu Asp Lys Ser Pro Val Lys Gln Glu Ala Pro Lys
225    230    235    240
Gln Glu Val Lys Pro Ala Val Lys Pro Val Thr Gly Thr Asn Ala Val
245    250    255

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- 191 -

Gly Thr Gly Lys Gly Val Leu Gly Asp Thr Lys Ser Leu Asn Thr Thr
 260 265 270
 Leu Ser Ser Ser Ser Tyr Tyr Leu Gln Asp Asn Thr Arg Gly Ala Thr
 275 280 285
 Ile Phe Thr Tyr Asp Ala Lys Asn Arg Thr Thr Leu Pro Gly Thr Leu
 290 295 300
 Trp Val Asp Ala Asp Asn Val Phe Asn Ala Ala Tyr Asp Ala Ala Ala
 305 310 315 320
 Val Asp Ala His Tyr Tyr Ala Gly Lys Thr Tyr Asp Tyr Tyr Lys Ala
 325 330 335
 Thr Phe Asn Arg Asn Ser Ile Asn Asp Ala Gly Ala Pro Leu Lys Ser
 340 345 350
 Thr Val His Tyr Gly Ser Lys Tyr Asn Asn Ala Phe Trp Asn Gly Ser
 355 360 365
 Gln Met Val Tyr Gly Asp Gly Asp Gly Val Thr Phe Thr Ser Leu Ser
 370 375 380
 Gly Gly Ile Asp Val Ile Gly His Glu Leu Thr His Ala Val Thr Glu
 385 390 395 400
 Tyr Ser Ser Asp Leu Ile Tyr Gln Asn Glu Ser Gly Ala Leu Asn Glu
 405 410 415
 Ala Ile Ser Asp Val Phe Gly Thr Leu Val Glu Phe Tyr Asp Asn Arg
 420 425 430
 Asn Pro Asp Trp Glu Ile Gly Glu Asp Ile Tyr Thr Pro Gly Lys Ala
 435 440 445
 Gly Asp Ala Leu Arg Ser Met Ser Asp Pro Thr Lys Tyr Gly Asp Pro
 450 455 460
 Asp His Tyr Ser Lys Arg Tyr Thr Gly Thr Ser Asp Asn Gly Gly Val
 465 470 475 480
 His Thr Asn Ser Gly Ile Ile Asn Lys Gln Ala Tyr Leu Leu Ala Asn
 485 490 495
 Gly Gly Thr His Tyr Gly Val Thr Val Thr Gly Ile Gly Lys Asp Lys
 500 505 510
 Leu Gly Ala Ile Tyr Tyr Arg Ala Asn Thr Gln Tyr Phe Thr Gln Ser
 515 520 525
 Thr Thr Phe Ser Gln Ala Arg Ala Gly Ala Val Gln Ala Ala Ala Asp
 530 535 540
 Leu Tyr Gly Ala Asn Ser Ala Glu Val Asn Ala Val Lys Gln Ser Phe
 545 550 555 560
 Ser Ala Val Gly Ile Asn
 565

<210> 179

<211> 566

<212> PRT

5

<213> *Bacillus cereus*

<400> 179

Met Lys Lys Lys Ser Leu Ala Leu Val Leu Ala Thr Gly Met Ala Val
 1 5 10 15
 Thr Thr Phe Gly Gly Thr Gly Ser Ala Phe Ala Asp Ser Lys Asn Val
 20 25 30
 Leu Ser Thr Lys Lys Tyr Asn Glu Thr Val Gln Ser Pro Glu Phe Ile
 35 40 45
 Ser Gly Asp Leu Thr Glu Ala Thr Gly Lys Lys Ala Glu Ser Val Val
 50 55 60
 Phe Asp Tyr Leu Asn Ala Ala Lys Gly Asp Tyr Lys Leu Gly Glu Lys
 65 70 75 80

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Ser Ala Gln Asp Ser Phe Lys Val Lys Gln Val Lys Lys Asp Ala Val
85
Thr Asp Ser Thr Val Val Arg Met Gln Gln Val Tyr Glu Gly Val Pro
100
Val Trp Gly Ser Thr Gln Val Ala His Val Ser Lys Asp Gly Ser Leu
115
Lys Val Leu Ser Gly Thr Val Ala Pro Asp Leu Asp Lys Lys Glu Lys
130
Leu Lys Asn Lys Asn Lys Ile Glu Gly Ala Lys Ala Ile Glu Ile Ala
145
Gln Gln Asp Leu Gly Val Thr Pro Lys Tyr Glu Val Glu Pro Lys Ala
165
Asp Leu Tyr Val Tyr Gln Asn Gly Glu Glu Thr Thr Tyr Ala Tyr Val
180
Val Asn Leu Asn Phe Leu Asp Pro Ser Pro Gly Asn Tyr Tyr Tyr Phe
195
Ile Glu Ala Asp Ser Gly Lys Val Leu Asn Lys Phe Asn Thr Ile Asp
210
His Val Thr Asn Asp Asp Lys Ser Pro Val Lys Gln Glu Ala Pro Lys
225
Gln Asp Ala Lys Ala Val Val Lys Pro Val Thr Gly Thr Asn Lys Val
245
Gly Thr Gly Lys Gly Val Leu Gly Asp Thr Lys Ser Leu Asn Thr Thr
260
Leu Ser Gly Ser Ser Tyr Tyr Leu Gln Asp Asn Thr Arg Gly Ala Thr
275
Ile Phe Thr Tyr Asp Ala Lys Asn Arg Ser Thr Leu Pro Gly Thr Leu
290
Trp Ala Asp Ala Asp Asn Val Phe Asn Ala Ala Tyr Asp Ala Ala Ala
305
Val Asp Ala His Tyr Tyr Ala Gly Lys Thr Tyr Asp Tyr Tyr Lys Ala
325
Thr Phe Asn Arg Asn Ser Ile Asn Asp Ala Gly Ala Pro Leu Lys Ser
340
Thr Val His Tyr Gly Ser Asn Tyr Asn Asn Ala Phe Trp Asn Gly Ser
355
Gln Met Val Tyr Gly Asp Gly Asp Gly Val Thr Phe Thr Ser Leu Ser
370
Gly Gly Ile Asp Val Ile Gly His Glu Leu Thr His Ala Val Thr Glu
385
Asn Ser Ser Asn Leu Ile Tyr Gln Asn Glu Ser Gly Ala Leu Asn Glu
405
Ala Ile Ser Asp Ile Phe Gly Thr Leu Val Glu Phe Tyr Asp Asn Arg
420
Asn Pro Asp Trp Glu Ile Gly Glu Asp Ile Tyr Thr Pro Gly Lys Ala
435
Gly Asp Ala Leu Arg Ser Met Ser Asp Pro Thr Lys Tyr Gly Asp Pro
450
Asp His Tyr Ser Lys Arg Tyr Thr Gly Ser Ser Asp Asn Gly Gly Val
465
His Thr Asn Ser Gly Ile Ile Asn Lys Gln Ala Tyr Leu Leu Ala Asn
485
Gly Gly Thr His Tyr Gly Val Thr Val Thr Gly Ile Gly Lys Asp Lys
500
Leu Gly Ala Ile Tyr Tyr Arg Ala Asn Thr Gln Tyr Phe Thr Gln Ser
515
Thr Thr Phe Ser Gln Ala Arg Ala Gly Ala Val Gln Ala Ala Ala Asp
530
Leu Tyr Gly Ala Asn Ser Ala Glu Val Ala Ala Val Lys Gln Ser Phe
545
Ser Ala Val Gly Val Asn
565

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<210> 180

<211> 538

<212> PRT

<213> Bacillus subtilis

<400> 180

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Met Arg Asn Leu Thr Lys Thr Ser Leu Leu Leu Ala Gly Leu Cys Thr
1      5      10      15
Ala Ala Gln Met Val Phe Val Thr His Ala Ser Ala Glu Glu Ser Ile
20      25      30
Glu Tyr Asp His Thr Tyr Gln Thr Pro Ser Tyr Ile Ile Glu Lys Ser
35      40      45
Pro Gln Lys Pro Val Gln Asn Thr Thr Gln Lys Glu Ser Leu Phe Ser
50      55      60
Tyr Leu Asp Lys His Gln Thr Gln Phe Lys Leu Lys Gly Asn Ala Asn
65      70      75      80
Ser His Phe Arg Val Ser Lys Thr Ile Lys Asp Pro Lys Thr Lys Gln
85      90      95
Thr Phe Phe Lys Leu Thr Glu Val Tyr Lys Gly Ile Pro Ile Tyr Gly
100      105      110
Phe Glu Gln Ala Val Ala Met Lys Glu Asn Lys Gln Val Lys Ser Phe
115      120      125
Phe Gly Lys Val His Pro Gln Ile Lys Asp Val Ser Val Thr Pro Ser
130      135      140
Ile Ser Glu Lys Lys Ala Ile His Thr Ala Arg Arg Glu Leu Glu Ala
145      150      155      160
Ser Ile Gly Lys Ile Glu Tyr Leu Asp Gly Glu Pro Lys Gly Glu Leu
165      170      175
Tyr Ile Tyr Pro His Asp Gly Glu Tyr Asp Leu Ala Tyr Leu Val Arg
180      185      190
Leu Ser Thr Ser Glu Pro Glu Pro Gly Tyr Trp His Tyr Phe Ile Asp
195      200      205
Ala Lys Asn Gly Lys Val Ile Glu Ser Phe Asn Ala Ile His Glu Ala
210      215      220
Ala Gly Thr Gly Ile Gly Val Ser Gly Asp Glu Lys Ser Phe Asp Val
225      230      235      240
Thr Glu Gln Asn Gly Arg Phe Tyr Leu Ala Asp Glu Thr Arg Gly Lys
245      250      255
Gly Ile Asn Thr Phe Asp Ala Lys Asn Leu Asn Glu Thr Leu Phe Thr
260      265      270
Leu Leu Ser Gln Leu Ile Gly Tyr Thr Gly Lys Glu Ile Val Ser Gly
275      280      285
Thr Ser Val Phe Asn Glu Pro Ala Ala Val Asp Ala His Ala Asn Ala
290      295      300
Gln Ala Val Tyr Asp Tyr Tyr Ser Lys Thr Phe Gly Arg Asp Ser Phe
305      310      315      320
Asp Gln Asn Gly Ala Arg Ile Thr Ser Thr Val His Val Gly Lys Gln
325      330      335
Trp Asn Asn Ala Ala Trp Asn Gly Val Gln Met Val Tyr Gly Asp Gly
340      345      350
Asp Gly Ser Lys Phe Lys Pro Leu Ser Gly Ser Leu Asp Ile Val Ala
355      360      365
His Glu Ile Thr His Ala Val Thr Gln Tyr Ser Ala Gly Leu Leu Tyr
370      375      380
Gln Gly Glu Pro Gly Ala Leu Asn Glu Ser Ile Ser Asp Ile Met Gly
385      390      395      400
Ala Met Ala Asp Arg Asp Asp Trp Glu Ile Gly Glu Asp Val Tyr Thr
405      410      415
Pro Gly Ile Ala Gly Asp Ser Leu Arg Ser Leu Glu Asp Pro Ser Lys
420      425      430
Gln Gly Asn Pro Asp His Tyr Ser Asn Arg Tyr Thr Gly Thr Glu Asp
435      440      445
Tyr Gly Gly Val His Ile Asn Ser Ser Ile His Asn Lys Ala Ala Tyr
450      455      460
Leu Leu Ala Glu Gly Gly Val His His Gly Val Gln Val Glu Gly Ile
465      470      475      480
Gly Arg Glu Ala Ser Glu Gln Ile Tyr Tyr Arg Ala Leu Thr Tyr Tyr
485      490      495
Val Thr Ala Ser Thr Asp Phe Ser Met Met Lys Gln Ala Ala Ile Glu
500      505      510
Ala Ala Asn Asp Leu Tyr Gly Glu Gly Ser Lys Gln Ser Ala Ser Val
515      520      525
Glu Lys Ala Tyr Glu Ala Val Gly Ile Leu
530      535

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<210> 181

<211> 568

<212> PRT

<213> Bacillus cereus

- 194 -

<400> 181

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Met Lys Asn Lys Lys Asn Phe Val Lys Ile Gly Leu Thr Thr Gly Val
1      5      10      15
Met Leu Ser Val Ile Met Pro Tyr Gly Asp Ala Tyr Ala Ala Thr Glu
20      25      30
Asp Leu Lys Val Glu Thr Lys Glu Asp Thr Phe Arg Thr Gly Asn Leu
35      40      45
Thr Val Pro Ser Gln Lys Ser Ala Glu Asn Val Ala Lys Asp Ala Leu
50      55      60
Lys Gly Lys Thr Glu Gln Ala Leu Ser Ser Lys Gln Val Asn Thr Glu
65      70      75      80
Ser Lys Val Asn Tyr Asn Val Thr Gln Ser Arg Lys Ser Tyr Asp Gly
85      90      95
Thr Thr Leu Val Arg Leu Gln Gln Thr Tyr Glu Gly Arg Asp Val Tyr
100     105     110
Gly Tyr Gln Leu Thr Ala His Ile Asn Asp Asp Gly Val Leu Thr Ser
115     120     125
Val Ser Gly Asp Ser Ala Gln Asp Leu Gln Gln Gln Glu Asp Leu Lys
130     135     140
Gln Pro Ile Thr Leu Ser Glu Glu Asp Ala Lys Lys Gln Leu Phe Lys
145     150     155     160
Ile Tyr Gly Asp Asn Leu Thr Phe Val Glu Glu Pro Glu Ile Lys Gln
165     170     175
Val Val Tyr Val Asp Glu Asn Thr Asn Lys Ala Thr Ser Ala Tyr Gln
180     185     190
Ile Thr Phe Ser Ala Ser Thr Pro Glu Tyr Val Ser Gly Thr Val Leu
195     200     205
Ile Asp Ala Phe Val Gly Asp Leu Leu Lys Glu Leu Val Gln Lys Leu
210     215     220
Gly Ile Gln Val Asp Ser Ser Ile Val Gln Ser Ala Thr Ser Asn Lys
225     230     235     240
Ser Gln Asp Pro Ser Lys Leu Thr Gly Thr Gly Lys Asp Asp Leu Gly
245     250     255
Met Asn Arg Thr Phe Gly Ile Ser Gln Arg Ser Asp Gly Thr Tyr Thr
260     265     270
Leu Ala Asp Tyr Ser Arg Gly Lys Gly Ile Glu Thr Tyr Thr Ala Asn
275     280     285
Tyr Lys Asp Tyr Asn Asn Tyr Arg Arg Asn Ile Trp Gly Tyr Leu Asp
290     295     300
Asp Leu Val Thr Ser Asn Ser Thr Asn Phe Thr Asp Pro Lys Ala Val
305     310     315     320
Ser Ala His Tyr Leu Ala Thr Lys Val Tyr Asp Phe Tyr Gln Glu Lys
325     330     335
Tyr Ser Arg Asn Ser Phe Asp Asn Asn Gly Gln Lys Val Ile Ser Val
340     345     350
Val His Gly Trp Asn Thr Asn Gly Thr Asn Lys Gly Asn Pro Lys Gln
355     360     365
Trp Phe Asn Ala Phe Ser Asn Gly Ala Met Leu Val Tyr Gly Asp Pro
370     375     380
Ile Val Arg Ala Phe Asp Val Ala Gly His Glu Phe Thr His Ala Val
385     390     395     400
Thr Arg Asn Glu Ser Gly Leu Glu Tyr Ala Gly Glu Ala Gly Ala Ile
405     410     415
Asn Glu Ala Ile Ser Asp Ile Leu Gly Val Ala Val Glu Lys Tyr Ala
420     425     430
Asn Asn Gly Lys Phe Asn Trp Thr Met Gly Glu Gln Ser Gly Arg Ile
435     440     445
Phe Arg Asp Met Lys Asn Pro Ser Ser Ile Ser Ser Arg Tyr Pro Glu
450     455     460
Asp Tyr Arg His Tyr Asn Asn Leu Pro Ile Asp Ala Ala His Asp His
465     470     475     480
Gly Gly Val His Thr Asn Ser Ser Ile Ile Asn Lys Val Ala Tyr Leu
485     490     495
Ile Ala Ser Gly Gly Asn His Asn Gly Val Asn Val Gln Gly Ile Gly
500     505     510
Glu Asp Lys Met Phe Asp Ile Phe Tyr Tyr Ala Asn Thr Asp Glu Leu
515     520     525
Asn Met Thr Ser Asp Phe Lys Glu Leu Lys Glu Ala Cys Ile Arg Val
530     535     540
Ala Thr Asn Leu Tyr Gly Lys Asp Ser Ser Glu Val Gln Ala Val Gln
545     550     555     560
Gln Ala Phe Lys Ala Ala Tyr Ile
565

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<210> 182

- 195 -

<211> 316

<212> PRT

<213> *Bacillus thermoproteolyticus*

<400> 182

Ile Thr Gly Thr Ser Thr Val Gly Val Gly Arg Gly Val Leu Gly Asp
 1 5 10 15
 Gln Lys Asn Ile Asn Thr Thr Tyr Ser Thr Tyr Tyr Tyr Leu Gln Asp
 20 25 30
 Asn Thr Arg Gly Asn Gly Ile Phe Thr Tyr Asp Ala Lys Tyr Arg Thr
 35 40 45
 Thr Leu Pro Gly Ser Leu Trp Ala Asp Ala Asp Asn Gln Phe Phe Ala
 50 55 60
 Ser Tyr Asp Ala Pro Ala Val Asp Ala His Tyr Tyr Ala Gly Val Thr
 65 70 75 80
 Tyr Asp Tyr Tyr Lys Asn Val His Asn Arg Leu Ser Tyr Asp Gly Asn
 85 90 95
 Asn Ala Ala Ile Arg Ser Ser Val His Tyr Ser Gln Gly Tyr Asn Asn
 100 105 110
 Ala Phe Trp Asn Gly Ser Gln Met Val Tyr Gly Asp Gly Asp Gly Gln
 115 120 125
 Thr Phe Ile Pro Leu Ser Gly Gly Ile Asp Val Val Ala His Glu Leu
 130 135 140
 Thr His Ala Val Thr Asp Tyr Thr Ala Gly Leu Ile Tyr Gln Asn Glu
 145 150 155 160
 Ser Gly Ala Ile Asn Glu Ala Ile Ser Asp Ile Phe Gly Thr Leu Val
 165 170 175
 Lys Phe Tyr Ala Asn Lys Asn Pro Asp Trp Glu Ile Gly Glu Asp Val
 180 185 190
 Tyr Thr Pro Gly Ile Ser Gly Asp Ser Leu Arg Ser Met Ser Asp Pro
 195 200 205
 Ala Lys Tyr Gly Asp Pro Asp His Tyr Ser Lys Arg Tyr Thr Gly Thr
 210 215 220
 Gln Asp Asn Gly Gly Val His Ile Asn Ser Gly Ile Ile Asn Lys Ala
 225 230 235 240
 Ala Tyr Leu Ile Ser Gln Gly Gly Thr His Tyr Gly Val Ser Val Val
 245 250 255
 Gly Ile Gly Arg Asp Lys Leu Gly Lys Ile Phe Tyr Arg Ala Leu Thr
 260 265 270
 Gln Tyr Leu Thr Pro Thr Ser Asn Phe Ser Gln Leu Arg Ala Ala Ala
 275 280 285
 Val Gln Ser Ala Thr Asp Leu Tyr Gly Ser Thr Ser Gln Glu Val Ala
 290 295 300
 Ser Val Lys Gln Ala Phe Asp Ala Val Gly Val Lys
 305 310 315

<210> 183

<211> 316

<212> PRT

<213> *Bacillus stearothermophilis*

<400> 183

Ile Thr Gly Thr Ser Thr Val Gly Val Gly Arg Gly Val Leu Gly Asp
 1 5 10 15
 Gln Lys Asn Ile Asn Thr Thr Tyr Ser Thr Tyr Tyr Tyr Leu Gln Asp
 20 25 30
 Asn Thr Arg Gly Asn Gly Ile Phe Thr Tyr Asp Ala Lys Tyr Arg Thr
 35 40 45
 Thr Leu Pro Gly Ser Leu Trp Ala Asp Ala Asp Asn Gln Phe Phe Ala
 50 55 60
 Ser Tyr Asp Ala Pro Ala Val Asp Ala His Tyr Tyr Ala Gly Val Thr
 65 70 75 80
 Tyr Asp Tyr Tyr Lys Asn Val His Asn Arg Leu Ser Tyr Asp Gly Asn
 85 90 95
 Asn Ala Ala Ile Arg Ser Ser Val His Tyr Ser Gln Gly Tyr Asn Asn
 100 105 110
 Ala Phe Trp Asn Gly Ser Gln Met Val Tyr Gly Asp Gly Asp Gly Gln

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      115      120      125
Thr Phe Ile Pro Leu Ser Gly Gly Ile Asp Val Val Ala His Glu Leu
      130      135      140
Thr His Ala Val Thr Asp Tyr Thr Ala Gly Leu Ile Tyr Gln Asn Glu
145      150      155      160
Ser Gly Ala Ile Asn Glu Ala Ile Ser Asp Ile Phe Gly Thr Leu Val
      165      170      175
Glu Phe Tyr Ala Asn Lys Asn Pro Asp Trp Glu Ile Gly Glu Asp Val
      180      185      190
Tyr Thr Pro Gly Ile Ser Gly Asp Ser Leu Arg Ser Met Ser Asp Pro
      195      200      205
Ala Lys Tyr Gly Asp Pro Asp His Tyr Ser Lys Arg Tyr Thr Gly Thr
      210      215      220
Gln Asp Asn Gly Gly Val His Ile Asn Ser Gly Ile Ile Asn Lys Ala
225      230      235      240
Ala Tyr Leu Ile Ser Gln Gly Gly Thr His Tyr Gly Val Ser Val Val
      245      250      255
Gly Ile Gly Arg Asp Lys Leu Gly Lys Ile Phe Tyr Arg Ala Leu Thr
      260      265      270
Gln Tyr Leu Thr Pro Thr Ser Asn Phe Ser Gln Leu Arg Ala Ala Ala
      275      280      285
Val Gln Ser Ala Thr Asp Leu Tyr Gly Ser Thr Ser Gln Glu Val Ala
290      295      300
Ser Val Lys Gln Ala Phe Asp Ala Val Gly Val Lys
305      310      315

```

<210> 184

<211> 319

<212> PRT

5

<213> Bacillus caldoyticus

<400> 184

```

Val Ala Gly Thr Ser Thr Val Gly Val Gly Arg Gly Val Leu Gly Asp
1      5      10      15
Gln Lys Tyr Ile Asn Thr Thr Tyr Ser Ser Tyr Tyr Gly Tyr Tyr Tyr
      20      25      30
Leu Gln Asp Asn Thr Arg Gly Ser Gly Ile Phe Thr Tyr Asp Gly Arg
      35      40      45
Asn Arg Thr Val Leu Pro Gly Ser Leu Trp Ala Asp Gly Asp Asn Gln
      50      55      60
Phe Phe Ala Ser Tyr Asp Ala Ala Ala Val Asp Ala His Tyr Tyr Ala
65      70      75      80
Gly Val Val Tyr Asp Tyr Tyr Lys Asn Val His Gly Arg Leu Ser Tyr
      85      90      95
Asp Gly Ser Asn Ala Ala Ile Arg Ser Thr Val His Tyr Gly Arg Gly
      100      105      110
Tyr Asn Asn Ala Phe Trp Asn Gly Ser Gln Met Val Tyr Gly Asp Gly
      115      120      125
Asp Gly Gln Thr Phe Leu Pro Phe Ser Gly Gly Ile Asp Val Val Gly
      130      135      140
His Glu Leu Thr His Ala Val Thr Asp Tyr Thr Ala Gly Leu Val Tyr
145      150      155      160
Gln Asn Glu Ser Gly Ala Ile Asn Glu Ala Met Ser Asp Ile Phe Gly
      165      170      175
Thr Leu Val Glu Phe Tyr Ala Asn Arg Asn Pro Asp Trp Glu Ile Gly
      180      185      190
Glu Asp Ile Tyr Thr Pro Gly Val Ala Gly Asp Ala Leu Arg Ser Met
      195      200      205
Ser Asp Pro Ala Lys Tyr Gly Asp Pro Asp His Tyr Ser Lys Arg Tyr
      210      215      220
Thr Gly Thr Gln Asp Asn Gly Gly Val His Thr Asn Ser Gly Ile Ile
225      230      235      240
Asn Lys Ala Ala Tyr Leu Leu Ser Gln Gly Gly Val His Tyr Gly Val
      245      250      255
Ser Val Thr Gly Ile Gly Arg Asp Lys Met Gly Lys Ile Phe Tyr Arg
      260      265      270
Ala Leu Val Tyr Tyr Leu Thr Pro Thr Ser Asn Phe Ser Gln Leu Arg
      275      280      285
Ala Ala Cys Val Gln Ala Ala Ala Asp Leu Tyr Gly Ser Thr Ser Gln
290      295      300
Glu Val Asn Ser Val Lys Gln Ala Phe Asn Ala Val Gly Val Tyr
305      310      315

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- 197 -

<210> 185

<211> 317

<212> PRT

<213> *Bacillus cereus*

5

<400> 185

```

Val Thr Gly Thr Asn Lys Val Gly Thr Gly Lys Gly Val Leu Gly Asp
1      5      10      15
Thr Lys Ser Leu Asn Thr Thr Leu Ser Gly Ser Ser Tyr Tyr Leu Gln
20      25      30
Asp Asn Thr Arg Gly Ala Thr Ile Phe Thr Tyr Asp Ala Lys Asn Arg
35      40      45
Ser Thr Leu Pro Gly Thr Leu Trp Ala Asp Ala Asp Asn Val Phe Asn
50      55      60
Ala Ala Tyr Asp Ala Ala Ala Val Asp Ala His Tyr Tyr Ala Gly Lys
65      70      75      80
Thr Tyr Asp Tyr Tyr Lys Ala Thr Phe Asn Arg Asn Ser Ile Asn Asp
85      90      95
Ala Gly Ala Pro Leu Lys Ser Thr Val His Tyr Gly Ser Asn Tyr Asn
100     105     110
Asn Ala Phe Trp Asn Gly Ser Gln Met Val Tyr Gly Asp Gly Asp Gly
115     120     125
Val Thr Phe Thr Ser Leu Ser Gly Gly Ile Asp Val Ile Gly His Glu
130     135     140
Leu Thr His Ala Val Thr Glu Asn Ser Ser Asn Leu Ile Tyr Gln Asn
145     150     155     160
Glu Ser Gly Ala Leu Asn Glu Ala Ile Ser Asp Ile Phe Gly Thr Leu
165     170     175
Val Glu Phe Tyr Asp Asn Arg Asn Pro Asp Trp Glu Ile Gly Glu Asp
180     185     190
Ile Tyr Thr Pro Gly Lys Ala Gly Asp Ala Leu Arg Ser Met Ser Asp
195     200     205
Pro Thr Lys Tyr Gly Asp Pro Asp His Tyr Ser Lys Arg Tyr Thr Gly
210     215     220
Ser Ser Asp Asn Gly Gly Val His Thr Asn Ser Gly Ile Ile Asn Lys
225     230     235     240
Gln Ala Tyr Leu Leu Ala Asn Gly Gly Thr His Tyr Gly Val Thr Val
245     250     255
Thr Gly Ile Gly Lys Asp Lys Leu Gly Ala Ile Tyr Tyr Arg Ala Asn
260     265     270
Thr Gln Tyr Phe Thr Gln Ser Thr Thr Phe Ser Gln Ala Arg Ala Gly
275     280     285
Ala Val Gln Ala Ala Ala Asp Leu Tyr Gly Ala Asn Ser Ala Glu Val
290     295     300
Ala Ala Val Lys Gln Ser Phe Ser Ala Val Gly Val Asn
305     310     315

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<210> 186

<211> 304

<212> PRT

<213> *Bacillus brevis*

<400> 186

10

- 198 -

```

Val Thr Ala Thr Gly Lys Gly Val Leu Gly Asp Thr Lys Gln Phe Glu
1      5      10      15
Thr Thr Lys Gln Gly Ser Thr Tyr Met Leu Lys Asp Thr Thr Arg Gly
20      25      30
Lys Gly Ile Glu Thr Tyr Thr Ala Asn Asn Arg Thr Ser Leu Pro Gly
35      40      45
Thr Leu Met Thr Asp Ser Asp Asn Tyr Trp Thr Asp Gly Ala Ala Val
50      55      60
Asp Ala His Ala His Ala Gln Lys Thr Tyr Asp Tyr Phe Arg Asn Val
65      70      75      80
His Asn Arg Asn Ser Tyr Asp Gly Asn Gly Ala Val Ile Arg Ser Thr
85      90      95
Val His Tyr Ser Thr Arg Tyr Asn Asn Ala Phe Trp Asn Gly Ser Gln
100     105     110
Met Val Tyr Gly Asp Gly Asp Gly Thr Thr Phe Leu Pro Leu Ser Gly
115     120     125
Gly Leu Asp Val Val Ala His Glu Leu Thr His Ala Val Thr Glu Arg
130     135     140
Thr Ala Gly Leu Val Tyr Gln Asn Glu Ser Gly Ala Leu Asn Glu Ser
145     150     155     160
Met Ser Asp Ile Phe Gly Ala Met Val Asp Asn Asp Asp Trp Leu Met
165     170     175
Gly Glu Asp Ile Tyr Thr Pro Gly Arg Ser Gly Asp Ala Leu Arg Ser
180     185     190
Leu Gln Asp Pro Ala Ala Tyr Gly Asp Pro Asp His Tyr Ser Lys Arg
195     200     205
Tyr Thr Gly Ser Gln Asp Asn Gly Gly Val His Thr Asn Ser Gly Ile
210     215     220
Asn Asn Lys Ala Ala Tyr Leu Leu Ala Glu Gly Gly Thr His Tyr Gly
225     230     235     240
Val Arg Val Asn Gly Ile Gly Arg Thr Asp Thr Ala Lys Ile Tyr Tyr
245     250     255
His Ala Leu Thr His Tyr Leu Thr Pro Tyr Ser Asn Phe Ser Ala Met
260     265     270
Arg Arg Ala Ala Val Leu Ser Ala Thr Asp Leu Phe Gly Ala Asn Ser
275     280     285
Arg Gln Val Gln Ala Val Asn Ala Ala Tyr Asp Ala Val Gly Val Lys
290     295     300

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<210> 187

<211> 302

<212> PRT

5

<213> Bacillus polymyxa

<400> 187

- 199 -

Ala Thr Gly Lys Gly Val Leu Gly Asp Ser Lys Ser Phe Thr Thr Thr
1 5 10 15
Ala Ser Gly Ser Ser Tyr Gln Leu Lys Asp Thr Thr Arg Gly Asn Gly
20 25 30
Ile Val Thr Tyr Thr Ala Ser Asn Arg Gln Ser Ile Pro Gly Thr Leu
35 40 45
Leu Thr Asp Ala Asp Asn Val Trp Asn Asp Pro Ala Gly Val Asp Ala
50 55 60
His Ala Tyr Ala Ala Lys Thr Tyr Asp Tyr Tyr Lys Ser Lys Phe Gly
65 70 75 80
Arg Asn Ser Ile Asp Gly Arg Gly Leu Gln Leu Arg Ser Thr Val His
85 90 95
Tyr Gly Ser Arg Tyr Asn Asn Ala Phe Trp Asn Gly Ser Gln Met Thr
100 105 110
Tyr Gly Asp Gly Asp Gly Asp Gly Ser Thr Phe Ile Ala Phe Ser Gly
115 120 125
Asp Pro Asp Val Val Gly His Glu Leu Thr His Gly Val Thr Glu Tyr
130 135 140
Thr Ser Asn Leu Glu Tyr Tyr Gly Glu Ser Gly Ala Leu Asn Glu Ala
145 150 155 160
Phe Ser Asp Val Ile Gly Asn Asp Ile Gln Arg Lys Asn Trp Leu Val
165 170 175
Gly Asp Asp Ile Tyr Thr Pro Asn Ile Cys Gly Asp Ala Leu Arg Ser
180 185 190
Met Ser Asn Pro Thr Leu Tyr Asp Gln Pro His His Tyr Ser Asn Leu
195 200 205
Tyr Lys Gly Ser Ser Asp Asn Gly Gly Val His Thr Asn Ser Gly Ile
210 215 220
Ile Asn Lys Ala Tyr Tyr Leu Leu Ala Gln Gly Gly Thr Phe His Gly
225 230 235 240
Val Thr Val Asn Gly Ile Gly Arg Asp Ala Ala Val Gln Ile Tyr Tyr
245 250 255
Ser Ala Phe Thr Asn Tyr Leu Thr Ser Ser Ser Asp Phe Ser Asn Ala
260 265 270
Arg Ala Ala Val Ile Gln Ala Ala Lys Asp Leu Tyr Gly Ala Asn Ser
275 280 285
Ala Glu Ala Thr Ala Ala Ala Lys Ser Phe Asp Ala Val Gly
290 295 300

<210> 188

<211> 300

<212> PRT

5

<213> *Bacillus pumilus*

<400> 188

Ala Ala Ala Thr Gly Ser Gly Thr Thr Leu Lys Gly Ala Thr Val Pro
1 5 10 15
Leu Asn Ile Ser Tyr Glu Gly Gly Lys Tyr Val Leu Arg Asp Leu Ser
20 25 30
Lys Pro Thr Gly Thr Gln Ile Ile Thr Tyr Asp Leu Gln Asn Arg Gln
35 40 45
Ser Arg Leu Pro Gly Thr Leu Val Ser Ser Thr Thr Lys Thr Phe Thr
50 55 60
Ser Ser Ser Gln Arg Ala Val Asp Ala His Tyr Asn Leu Gly Lys
65 70 75 80
Val Tyr Asp Tyr Phe Tyr Ser Asn Phe Lys Arg Asn Ser Tyr Asp Asn
85 90 95

- 200 -

Lys Gly Ser Lys Ile Val Ser Ser Val His Tyr Gly Thr Gln Tyr Asn
 100 105 110
 Asn Ala Ala Trp Thr Gly Asp Gln Met Ile Tyr Gly Asp Gly Asp Gly
 115 120 125
 Ser Phe Phe Ser Pro Leu Ser Gly Ser Leu Asp Val Thr Ala His Glu
 130 135 140
 Met Thr His Gly Val Thr Gln Glu Thr Ala Asn Leu Ile Tyr Glu Asn
 145 150 155 160
 Gln Pro Gly Ala Leu Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr Phe
 165 170 175
 Asn Asp Thr Glu Asp Trp Asp Ile Gly Glu Asp Ile Thr Val Ser Gln
 180 185 190
 Pro Ala Leu Arg Ser Leu Ser Asn Pro Thr Lys Tyr Asn Gln Pro Asp
 195 200 205
 Asn Tyr Ala Asn Tyr Arg Asn Leu Pro Asn Thr Asp Glu Gly Asp Tyr
 210 215 220
 Gly Gly Val His Thr Asn Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn
 225 230 235 240
 Thr Ile Thr Lys Leu Gly Val Ser Lys Ser Gln Gln Ile Tyr Tyr Arg
 245 250 255
 Ala Leu Thr Thr Tyr Leu Thr Pro Ser Ser Thr Phe Lys Asp Ala Lys
 260 265 270
 Ala Ala Leu Ile Gln Ser Ala Arg Asp Leu Tyr Gly Ser Thr Asp Ala
 275 280 285
 Ala Lys Val Glu Ala Ala Trp Asn Ala Val Gly Leu
 290 295 300

<210> 189

<211> 300

<212> PRT

<213> Bacillus subtilis var.

5

<400> 189

Ala Ala Ala Thr Gly Ser Gly Thr Thr Leu Lys Gly Ala Thr Val Pro
 1 5 10 15
 Leu Asn Ile Ser Tyr Glu Gly Gly Lys Tyr Val Leu Arg Asp Leu Ser
 20 25 30
 Lys Pro Thr Gly Thr Gln Ile Ile Thr Tyr Asp Leu Gln Asn Arg Gln
 35 40 45
 Ser Arg Leu Pro Gly Thr Leu Val Ser Ser Thr Thr Lys Thr Phe Thr
 50 55 60
 Ser Ser Ser Gln Arg Ala Val Asp Ala His Tyr Asn Leu Gly Lys
 65 70 75 80
 Val Tyr Asp Tyr Phe Tyr Ser Asn Phe Lys Arg Asn Ser Tyr Asp Asn
 85 90 95
 Lys Gly Ser Lys Ile Val Ser Ser Val His Tyr Gly Thr Gln Tyr Asn
 100 105 110
 Asn Ala Ala Trp Thr Gly Asp Gln Met Ile Tyr Gly Asp Gly Asp Gly
 115 120 125
 Ser Phe Phe Ser Pro Leu Ser Gly Ser Leu Asp Val Thr Ala His Glu
 130 135 140
 Met Thr His Gly Val Thr Gln Glu Thr Ala Asn Leu Ile Tyr Glu Asn
 145 150 155 160
 Gln Pro Gly Ala Leu Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr Phe
 165 170 175
 Asn Asp Thr Glu Asp Trp Asp Ile Gly Glu Asp Ile Thr Val Ser Gln
 180 185 190
 Pro Ala Leu Arg Ser Leu Ser Asn Pro Thr Lys Tyr Asn Gln Pro Asp
 195 200 205
 Asn Tyr Ala Asn Tyr Arg Asn Leu Pro Asn Thr Asp Glu Gly Asp Tyr
 210 215 220
 Gly Gly Val His Thr Asn Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn
 225 230 235 240
 Thr Ile Thr Lys Leu Gly Val Ser Lys Ser Gln Gln Ile Tyr Tyr Arg
 245 250 255
 Ala Leu Thr Thr Tyr Leu Thr Pro Ser Ser Thr Phe Lys Asp Ala Lys
 260 265 270
 Ala Ala Leu Ile Gln Ser Ala Arg Asp Leu Tyr Gly Ser Thr Asp Ala
 275 280 285
 Ala Lys Val Glu Ala Ala Trp Asn Ala Val Gly Leu
 290 295 300

<210> 190

- 201 -

< 211> 230

< 212> PRT

< 213> *Bacillus subtilis*

<400> 190

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Ala Val Asp Ala His Tyr Asn Leu Gly Lys Val Tyr Asp Tyr Phe Tyr
1      5      10      15
Ser Asn Phe Lys Arg Asn Ser Tyr Asp Asn Lys Gly Ser Lys Ile Val
20      25      30
Ser Ser Val His Tyr Gly Thr Gln Tyr Asn Asn Ala Ala Trp Thr Gly
35      40      45
Asp Gln Met Ile Tyr Gly Asp Gly Asp Gly Ser Phe Phe Ser Pro Leu
50      55      60
Ser Gly Ser Leu Asp Val Thr Ala His Glu Met Thr His Gly Val Thr
65      70      75      80
Gln Glu Thr Ala Asn Leu Ile Tyr Glu Asn Gln Pro Gly Ala Leu Asn
85      90      95
Glu Ser Phe Ser Asp Val Phe Gly Tyr Phe Asn Asp Thr Glu Asp Trp
100     105     110
Asp Ile Gly Glu Asp Ile Thr Val Ser Gln Pro Ala Leu Arg Ser Leu
115     120     125
Ser Asn Pro Thr Lys Tyr Asn Gln Pro Asp Asn Tyr Ala Asn Tyr Arg
130     135     140
Asn Leu Pro Asn Thr Asp Glu Gly Asp Tyr Gly Gly Val His Thr Asn
145     150     155     160
Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn Thr Ile Thr Lys Leu Gly
165     170     175
Val Ser Lys Ser Gln Gln Ile Tyr Tyr Arg Ala Leu Thr Thr Tyr Leu
180     185     190
Thr Pro Ser Ser Thr Phe Lys Asp Ala Lys Ala Ala Leu Ile Gln Ser
195     200     205
Ala Arg Asp Leu Tyr Gly Ser Thr Asp Ala Ala Lys Val Glu Ala Ala
210     215     220
Trp Asn Ala Val Gly Leu
225     230

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5

<210> 191

< 211> 300

< 212> PRT

< 213> *Bacillus amyloliquefaciens*

<400> 191

10

- 202 -

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Ala Ala Thr Thr Gly Thr Gly Thr Thr Leu Lys Gly Lys Thr Val Ser
1      5      10      15
Leu Asn Ile Ser Ser Glu Ser Gly Lys Tyr Val Leu Arg Asp Leu Ser
20     25     30
Lys Pro Thr Gly Thr Gln Ile Ile Thr Tyr Asp Leu Gln Asn Arg Glu
35     40     45
Tyr Asn Leu Pro Gly Thr Leu Val Ser Ser Thr Thr Asn Gln Phe Thr
50     55     60
Thr Ser Ser Gln Arg Ala Ala Val Asp Ala His Tyr Asn Leu Gly Lys
65     70     75     80
Val Tyr Asp Tyr Phe Tyr Gln Lys Phe Asn Arg Asn Ser Tyr Asp Asn
85     90     95
Lys Gly Gly Lys Ile Val Ser Ser Val His Tyr Gly Ser Arg Tyr Asn
100    105    110
Asn Ala Ala Trp Ile Gly Asp Gln Met Ile Tyr Gly Asp Gly Asp Gly
115    120    125
Ser Phe Phe Ser Pro Leu Ser Gly Ser Met Asp Val Thr Ala His Glu
130    135    140
Met Thr His Gly Val Thr Gln Glu Thr Ala Asn Leu Asn Tyr Glu Asn
145    150    155    160
Gln Pro Gly Ala Leu Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr Phe
165    170    175
Asn Asp Thr Glu Asp Trp Asp Ile Gly Glu Asp Ile Thr Val Ser Gln
180    185    190
Pro Ala Leu Arg Ser Leu Ser Asn Pro Thr Lys Tyr Gly Gln Pro Asp
195    200    205
Asn Phe Lys Asn Tyr Lys Asn Leu Pro Asn Thr Asp Ala Gly Asp Tyr
210    215    220
Gly Gly Val His Thr Asn Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn
225    230    235    240
Thr Ile Thr Lys Ile Gly Val Asn Lys Ala Glu Gln Ile Tyr Tyr Arg
245    250    255
Ala Leu Thr Val Tyr Leu Thr Pro Ser Ser Thr Phe Lys Asp Ala Lys
260    265    270
Ala Ala Leu Ile Gln Ser Ala Arg Asp Leu Tyr Gly Ser Gln Asp Ala
275    280    285
Ala Ser Val Glu Ala Ala Trp Asn Ala Val Gly Leu
290    295    300

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<210> 192

<211> 300

<212> PRT

5

<213> Bacillus amyloliquefaciens

<400> 192

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Ala Ala Thr Thr Gly Thr Gly Thr Thr Leu Lys Gly Lys Thr Val Ser
1      5      10      15
Leu Asn Ile Ser Ser Glu Ser Gly Lys Tyr Val Leu Arg Asp Leu Ser
20     25     30
Lys Pro Thr Gly Thr Gln Ile Ile Thr Tyr Asp Leu Gln Asn Arg Glu
35     40     45
Tyr Asn Leu Pro Gly Thr Leu Val Ser Ser Thr Thr Asn Gln Phe Thr
50     55     60
Thr Ser Ser Gln Arg Ala Ala Val Asp Ala His Tyr Asn Leu Gly Lys
65     70     75     80
Val Tyr Asp Tyr Phe Tyr Gln Lys Phe Asn Arg Asn Ser Tyr Asp Asn

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- 203 -

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      85      90      95
Lys Gly Gly Lys Ile Val Ser Ser Val His Tyr Gly Ser Arg Tyr Asn
      100      105      110
Asn Ala Ala Trp Ile Gly Asp Gln Met Ile Tyr Gly Asp Gly Asp Gly
      115      120      125
Ser Phe Phe Ser Pro Leu Ser Gly Ser Met Asp Val Thr Ala His Glu
      130      135      140
Met Thr His Gly Val Thr Gln Glu Thr Ala Asn Leu Asn Tyr Glu Asn
      145      150      155
Gln Pro Gly Ala Leu Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr Phe
      165      170      175
Asn Asp Thr Glu Asp Trp Asp Ile Gly Glu Asp Ile Thr Val Ser Gln
      180      185      190
Pro Ala Leu Arg Ser Leu Ser Asn Pro Thr Lys Tyr Gly Gln Pro Asp
      195      200      205
Asn Phe Lys Asn Tyr Lys Asn Leu Pro Asn Thr Asp Ala Gly Asp Tyr
      210      215      220
Gly Gly Val His Thr Asn Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn
      225      230      235
Thr Ile Thr Lys Ile Gly Val Asn Lys Ala Glu Gln Ile Tyr Tyr Arg
      245      250      255
Ala Leu Thr Val Tyr Leu Thr Pro Ser Ser Thr Phe Lys Asp Ala Lys
      260      265      270
Ala Ala Leu Ile Gln Ser Ala Arg Asp Leu Tyr Gly Ser Gln Asp Ala
      275      280      285
Ala Ser Val Glu Ala Ala Trp Asn Ala Val Gly Leu
      290      295      300

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<210> 193

<211> 301

<212> PRT

5

<213> Staphylococcus aureus

<400> 193

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Ala Ala Ala Thr Gly Thr Gly Lys Gly Val Leu Gly Asp Thr Lys Asp
1 5 10 15
Ile Asn Ile Asn Ser Ile Asp Gly Gly Phe Ser Leu Glu Asp Leu Thr
20 25 30
His Gln Gly Lys Leu Ser Ala Tyr Asn Phe Asn Asp Gln Thr Gly Gln
35 40 45
Ala Thr Leu Ile Thr Asn Glu Asp Glu Asn Phe Val Lys Asp Asp Gln
50 55 60
Arg Ala Gly Val Asp Ala Asn Tyr Tyr Ala Lys Gln Thr Tyr Asp Tyr
65 70 75 80
Tyr Lys Asn Thr Phe Gly Arg Glu Ser Tyr Asp Asn His Gly Ser Pro
85 90 95
Ile Val Ser Leu Thr His Val Asn His Tyr Gly Gly Gln Asp Asn Arg
100 105 110
Asn Asn Ala Ala Trp Ile Gly Asp Lys Met Ile Tyr Gly Asp Gly Asp
115 120 125
Gly Arg Thr Phe Thr Asn Leu Ser Gly Ala Asn Asp Val Val Ala His
130 135 140
Glu Ile Thr His Gly Val Thr Gln Gln Thr Ala Asn Leu Glu Tyr Lys
145 150 155
Asp Gln Ser Gly Ala Leu Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr
165 170 175
Phe Val Asp Asp Glu Asp Phe Leu Met Gly Glu Asp Val Tyr Thr Pro
180 185 190
Gly Lys Glu Gly Asp Ala Leu Arg Ser Met Ser Asn Pro Glu Gln Phe
195 200 205
Gly Gln Pro Ser His Met Lys Asp Tyr Val Tyr Thr Glu Lys Asp Asn
210 215 220
Gly Gly Val His Thr Asn Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn
225 230 235
Val Ile Gln Ala Ile Gly Lys Ser Lys Ser Glu Gln Ile Tyr Tyr Arg
245 250 255
Ala Leu Thr Glu Tyr Leu Thr Ser Asn Ser Asn Phe Lys Asp Leu Lys
260 265 270
Asp Ala Leu Tyr Gln Ala Ala Lys Asp Leu Tyr Glu Gln Gln Thr Ala
275 280 285
Glu Gln Val Tyr Glu Ala Trp Asn Glu Val Gly Val Glu
290 295 300

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<210> 194

<211> 298

<212> PRT

<213> *Pseudomonas aeruginosa*

5

<400> 194

Ala Glu Ala Gly Gly Pro Gly Gly Asn Gln Lys Ile Gly Lys Tyr Thr
 1 5 10 15
 Tyr Gly Ser Asp Tyr Gly Pro Leu Ile Val Asn Asp Arg Cys Glu Met
 20 25 30
 Asp Asp Gly Asn Val Ile Thr Val Asp Met Asn Ser Ser Thr Asp Asp
 35 40 45
 Ser Lys Thr Thr Pro Phe Arg Phe Ala Cys Pro Thr Asn Thr Tyr Lys
 50 55 60
 Gln Val Asn Gly Ala Tyr Ser Pro Leu Asn Asp Ala His Phe Phe Gly
 65 70 75 80
 Gly Val Val Phe Lys Leu Tyr Arg Asp Trp Phe Gly Thr Ser Pro Leu
 85 90 95
 Thr His Lys Leu Tyr Met Lys Val His Tyr Gly Arg Ser Val Glu Asn
 100 105 110
 Ala Tyr Trp Asp Gly Thr Ala Met Leu Phe Gly Asp Gly Ala Thr Met
 115 120 125
 Phe Tyr Pro Leu Val Ser Leu Asp Val Ala Ala His Glu Val Ser His
 130 135 140
 Gly Phe Thr Glu Gln Asn Ser Gly Leu Ile Tyr Arg Gly Gln Ser Gly
 145 150 155 160
 Gly Met Asn Glu Ala Phe Ser Asp Met Ala Gly Glu Ala Ala Glu Phe
 165 170 175
 Tyr Met Arg Gly Lys Asn Asp Phe Leu Ile Gly Tyr Asp Ile Lys Lys
 180 185 190
 Gly Ser Gly Ala Leu Arg Tyr Met Asp Gln Pro Ser Arg Asp Gly Arg
 195 200 205
 Ser Ile Asp Asn Ala Ser Gln Tyr Tyr Asn Gly Ile Asp Val His His
 210 215 220
 Ser Ser Gly Val Tyr Asn Arg Ala Phe Tyr Leu Leu Ala Asn Ser Pro
 225 230 235 240
 Gly Trp Asp Thr Arg Lys Ala Phe Glu Val Phe Val Asp Ala Asn Arg
 245 250 255
 Tyr Tyr Trp Thr Ala Thr Ser Asn Tyr Asn Ser Gly Ala Cys Gly Val
 260 265 270
 Ile Arg Ser Ala Gln Asn Arg Asn Tyr Ser Ala Ala Asp Val Thr Arg
 275 280 285
 Ala Phe Ser Thr Val Gly Val Thr Cys Pro
 290 295

<210> 195

<211> 317

<212> PRT

<213> *Bacillus cereus*

10

<400> 195

- 205 -

Val Thr Gly Thr Asn Lys Val Gly Thr Gly Lys Gly Val Leu Gly Asp
 1 5 10 15
 Thr Lys Ser Leu Asn Thr Thr Leu Ser Gly Ser Ser Tyr Tyr Leu Gln
 20 25 30
 Asp Asn Thr Arg Gly Ala Thr Ile Phe Thr Tyr Asp Ala Lys Asn Arg
 35 40 45
 Ser Thr Leu Pro Gly Thr Leu Trp Ala Asp Ala Asp Asn Val Phe Asn
 50 55 60
 Ala Ala Tyr Asp Ala Ala Val Asp Ala His Tyr Tyr Ala Gly Lys
 65 70 75 80
 Thr Tyr Asp Tyr Tyr Lys Ala Thr Phe Asn Arg Asn Ser Ile Asn Asp
 85 90 95
 Ala Gly Ala Pro Leu Lys Ser Thr Val His Tyr Gly Ser Asn Tyr Asn
 100 105 110
 Asn Ala Phe Trp Asn Gly Ser Gln Met Val Tyr Gly Asp Gly Asp Gly
 115 120 125
 Val Thr Phe Thr Ser Leu Ser Gly Gly Ile Asp Val Ile Gly His Glu
 130 135 140
 Leu Thr His Ala Val Thr Glu Asn Ser Ser Asn Leu Ile Tyr Gln Asn
 145 150 155 160
 Glu Ser Gly Ala Leu Asn Glu Ala Ile Ser Asp Ile Phe Gly Thr Leu
 165 170 175
 Val Glu Phe Tyr Asp Asn Arg Asn Pro Asp Trp Glu Ile Gly Glu Asp
 180 185 190
 Ile Tyr Thr Pro Gly Lys Ala Gly Asp Ala Leu Arg Ser Met Ser Asp
 195 200 205
 Pro Thr Lys Tyr Gly Asp Pro Asp His Tyr Ser Lys Arg Tyr Thr Gly
 210 215 220
 Ser Ser Asp Asn Gly Gly Val His Thr Asn Ser Gly Ile Ile Asn Lys
 225 230 235 240
 Gln Ala Tyr Leu Leu Ala Asn Gly Gly Thr His Tyr Gly Val Thr Val
 245 250 255
 Thr Gly Ile Gly Lys Asp Lys Leu Gly Ala Ile Tyr Tyr Arg Ala Asn
 260 265 270
 Thr Gln Tyr Phe Thr Gln Ser Thr Thr Phe Ser Gln Ala Arg Ala Gly
 275 280 285
 Ala Val Gln Ala Ala Ala Asp Leu Tyr Gly Ala Asn Ser Ala Glu Val
 290 295 300
 Ala Ala Val Lys Gln Ser Phe Ser Ala Val Gly Val Asn
 305 310 315

<210> 196

<211> 30

<212> DNA

5 <213> Artificial Sequence

<220>

<223> primer

<400> 196

cgtcttcaac aattgtccat tttctctgc 30

10 <210> 197

<211> 53

<212> DNA

<213> Artificial Sequence

<220>

15 <223> primer

<400> 197

cagacaattt cttacctaaa cccactcttt accctctcct tttaaaaaaa ttc 53

<210> 198

- 206 -

< 211> 53
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 5 < 223> primer
 <400> 198
 gaattttttt aaaaggagag ggtaaagagt gggtttaggt aagaaattgt ctg 53
 <210> 199
 < 211> 32
 10 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 <400> 199
 15 gcttatggat cccgtcggtt cagctgagag ag 32
 <210> 200
 < 211> 51
 < 212> DNA
 < 213> Artificial Sequence
 20 <220>
 < 223> primer
 <400> 200
 gatgtcttgg tcaagttgcg cactctttac cctctccttt taataaaatt c 51
 <210> 201
 25 < 211> 51
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 30 <400> 201
 gaattttttt aaaaggagag ggtaaagagt gcgcaacttg accaagacat c 51
 <210> 202
 < 211> 50
 < 212> DNA
 35 < 213> Artificial Sequence
 <220>

- 207 -

< 223> primer
 <400> 202
 ccaaggccgg tttttatgt aagcttatag aatgccgaca gcctcatacg 50
 <210> 203
 5 < 211> 50
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 10 <400> 203
 cgtatgaggc tgcggcatt ctataagctt acataaaaaa ccggccttgg 50
 <210> 204
 < 211> 24
 < 212> DNA
 15 < 213> Artificial Sequence
 <220>
 < 223> primer
 <400> 204
 aatggtgcat gcaaggagat ggcg 24
 20 <210> 205
 < 211> 31
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 25 < 223> primer
 <400> 205
 cgtcttcaag aattctctca tttctctg c 31
 <210> 206
 < 211> 54
 30 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 <400> 206
 35 gcaccaaca ttgcacgttt attcaactctt taccctctcc ttttaaaaaa attc 54
 <210> 207

- 208 -

< 211> 54
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 5 < 223> primer
 <400> 207
 gaattttttt aaaaggagag ggtaaagagt gaataaacgt gcaatgttgg gtgc 54
 <210> 208
 < 211> 32
 10 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 <400> 208
 15 gcttataagc ttaatatact ccaaccgcgt tg 32
 <210> 209
 < 211> 50
 < 212> DNA
 < 213> Artificial Sequence
 20 <220>
 < 223> primer
 <400> 209
 ccagcatagc gcgtttgttc actctttacc ctctcctttt aaaaaaatc 50
 <210> 210
 25 < 211> 50
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 30 <400> 210
 gaattttttt aaaaggagag ggtaaagagt gaacaaacgc gctatgctgg 50
 <210> 211
 < 211> 38
 < 212> DNA
 35 < 213> Artificial Sequence
 <220>

- 209 -

< 223> primer
 <400> 211
 gcttataagc ttaatagaca cccacggcat taaacgcc 38
 <210> 212
 5 < 211> 58
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 < 223> primer
 10 <400> 212
 caggacaaga gctaaggact ttttttcac tctttaccct ctcttttaa aaaaattc 58
 <210> 213
 < 211> 58
 < 212> DNA
 15 < 213> Artificial Sequence
 <220>
 < 223> primer
 <400> 213
 gaattttttt aaaaggagag ggtaaagagt gaaaaaaaaag tccttagctc ttgtcctg 58
 20 <210> 214
 < 211> 30
 < 212> DNA
 < 213> Artificial Sequence
 <220>
 25 < 223> primer
 <400> 214
 gcttataagc ttaattaatg ccgacggcac 30
 <210> 215
 < 211> 10
 30 < 212> PRT
 < 213> Bacillus amyloliquefaciens
 <400> 215
 Ala Ala Thr Thr Gly Thr Gly Thr Thr Leu
 1 5 10
 <210> 216
 35 < 211> 10
 < 212> PRT

- 210 -

< 213> Bacillus amyloliquefaciens
 <400> 216
 Asp Ala Gly Asp Tyr Gly Gly Val His Thr
 1 5 10
 <210> 217
 5 < 211> 10
 < 212> PRT
 < 213> Bacillus amyloliquefaciens
 <400> 217
 Ala Gly Asp Tyr Gly Gly Val His Thr Asn
 10 1 5 10
 <210> 218
 < 211> 9
 < 212> PRT
 < 213> Bacillus amyloliquefaciens
 15 <400> 218
 Gly Asp Tyr Gly Gly Val His Thr Asn
 1 5
 <210> 219
 < 211> 10
 < 212> PRT
 20 < 213> Bacillus amyloliquefaciens
 <400> 219
 Ala Ala Thr Thr Gly Thr Gly Thr Thr Leu
 1 5 10
 <210> 220
 < 211> 10
 25 < 212> PRT
 < 213> Bacillus amyloliquefaciens
 <400> 220
 Ala Ala Thr Thr Gly Thr Gly Thr Thr Leu
 1 5 10
 <210> 221
 30 < 211> 10
 < 212> PRT
 < 213> Bacillus amyloliquefaciens
 <400> 221
 Leu Ser Asn Pro Thr Lys Tyr Gly Gln Pro
 1 5 10
 35 <210> 222
 < 211> 219

- 211 -

< 212> PRT

< 213> Bacillus amyloliquefaciens

<400> 222

Ala Ala Thr Thr Gly Thr Gly Thr Thr Leu Lys Gly Lys Thr Val Ser
 1 5 10 15
 Leu Asn Ile Ser Ser Glu Ser Gly Lys Tyr Val Leu Arg Asp Leu Ser
 20 25 30
 Lys Pro Thr Gly Thr Gln Ile Ile Thr Tyr Asp Leu Gln Asn Arg Glu
 35 40 45
 Tyr Asn Leu Pro Gly Thr Leu Val Ser Ser Thr Thr Asn Gln Phe Thr
 50 55 60
 Thr Ser Ser Gln Arg Ala Ala Val Asp Ala His Tyr Asn Leu Gly Lys
 65 70 75 80
 Val Tyr Asp Tyr Phe Tyr Gln Lys Phe Asn Arg Asn Ser Tyr Asp Asn
 85 90 95
 Lys Gly Gly Lys Ile Val Ser Ser Val His Tyr Gly Ser Arg Tyr Asn
 100 105 110
 Asn Ala Ala Trp Ile Gly Asp Gln Met Ile Tyr Gly Asp Gly Asp Gly
 115 120 125
 Ser Phe Phe Ser Pro Leu Ser Gly Ser Met Asp Val Thr Ala His Glu
 130 135 140
 Met Thr His Gly Val Thr Gln Glu Thr Ala Asn Leu Asn Tyr Glu Asn
 145 150 155 160
 Gln Pro Gly Ala Leu Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr Phe
 165 170 175
 Asn Asp Thr Glu Asp Trp Asp Ile Gly Glu Asp Ile Thr Val Ser Gln
 180 185 190
 Pro Ala Leu Arg Ser Leu Ser Asn Pro Thr Lys Tyr Gly Gln Pro Asp
 195 200 205
 Asn Phe Lys Asn Tyr Lys Asn Leu Pro Asn Thr
 210 215

5

<210> 223

< 211> 81

< 212> PRT

< 213> Bacillus amyloliquefaciens

<400> 223

Asp Ala Gly Asp Tyr Gly Gly Val His Thr Asn Ser Gly Ile Pro Asn
 1 5 10 15
 Lys Ala Ala Tyr Asn Thr Ile Thr Lys Ile Gly Val Asn Lys Ala Glu
 20 25 30
 Gln Ile Tyr Tyr Arg Ala Leu Thr Val Tyr Leu Thr Pro Ser Ser Thr
 35 40 45
 Phe Lys Asp Ala Lys Ala Ala Leu Ile Gln Ser Ala Arg Asp Leu Tyr
 50 55 60
 Gly Ser Gln Asp Ala Ala Ser Val Glu Ala Ala Trp Asn Ala Val Gly
 65 70 75 80
 Leu

<210> 224

< 211> 197

< 212> PRT

< 213> Bacillus amyloliquefaciens

<400> 224

15

- 212 -

Ala Ala Thr Thr Gly Thr Gly Thr Thr Leu Lys Gly Lys Thr Val Ser
 1 5 10 15
 Leu Asn Ile Ser Ser Glu Ser Gly Lys Tyr Val Leu Arg Asp Leu Ser
 20 25 30
 Lys Pro Thr Gly Thr Gln Ile Ile Thr Tyr Asp Leu Gln Asn Arg Glu
 35 40 45
 Tyr Asn Leu Pro Gly Thr Leu Val Ser Ser Thr Thr Asn Gln Phe Thr
 50 55 60
 Thr Ser Ser Gln Arg Ala Ala Val Asp Ala His Tyr Asn Leu Gly Lys
 65 70 75 80
 Val Tyr Asp Tyr Phe Tyr Gln Lys Phe Asn Arg Asn Ser Tyr Asp Asn
 85 90 95
 Lys Gly Gly Lys Ile Val Ser Ser Val His Tyr Gly Ser Arg Tyr Asn
 100 105 110
 Asn Ala Ala Trp Ile Gly Asp Gln Met Ile Tyr Gly Asp Gly Asp Gly
 115 120 125
 Ser Phe Phe Ser Pro Leu Ser Gly Ser Met Asp Val Thr Ala His Glu
 130 135 140
 Met Thr His Gly Val Thr Gln Glu Thr Ala Asn Leu Asn Tyr Glu Asn
 145 150 155 160
 Gln Pro Gly Ala Leu Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr Phe
 165 170 175
 Asn Asp Thr Glu Asp Trp Asp Ile Gly Glu Asp Ile Thr Val Ser Gln
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 Pro Ala Leu Arg Ser
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<211> 140

5

<212> PRT

<213> *Bacillus amyloliquefaciens*

<400> 225

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 20 25 30
 Lys Pro Thr Gly Thr Gln Ile Ile Thr Tyr Asp Leu Gln Asn Arg Glu
 35 40 45
 Tyr Asn Leu Pro Gly Thr Leu Val Ser Ser Thr Thr Asn Gln Phe Thr
 50 55 60
 Thr Ser Ser Gln Arg Ala Ala Val Asp Ala His Tyr Asn Leu Gly Lys
 65 70 75 80
 Val Tyr Asp Tyr Phe Tyr Gln Lys Phe Asn Arg Asn Ser Tyr Asp Asn
 85 90 95
 Lys Gly Gly Lys Ile Val Ser Ser Val His Tyr Gly Ser Arg Tyr Asn
 100 105 110
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 Ser Phe Phe Ser Pro Leu Ser Gly Ser Met Asp Val
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<210> 226

10

<211> 103

<212> PRT

<213> *Bacillus amyloliquefaciens*

<400> 226

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Lys Asn Leu Pro Asn Thr Asp Ala Gly Asp Tyr Gly Gly Val His Thr
20          25          30
Asn Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn Thr Ile Thr Lys Ile
35          40          45
Gly Val Asn Lys Ala Glu Gln Ile Tyr Tyr Arg Ala Leu Thr Val Tyr
50          55          60
Leu Thr Pro Ser Ser Thr Phe Lys Asp Ala Lys Ala Ala Leu Ile Gln
65          70          75          80
Ser Ala Arg Asp Leu Tyr Gly Ser Gln Asp Ala Ala Ser Val Glu Ala
85          90          95
Ala Trp Asn Ala Val Gly Leu

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100

Anvendelse og fremstilling af en opbevaringsstabil neutral metalloprotease

PATENTKRAV

1. Isoleret neutral metalloproteasevariant med mindst 70% aminosyreidentitet med den neutrale metalloprotease omfattende SEQ ID NO: 3 eller SEQ ID NO: 18, og med en aminosyresekvens, der mindst
- 5 omfatter én mutation svarende til en mutation i en neutral metalloprotease, der omfatter aminosyresekvensen ifølge SEQ ID NO:18, hvor mutationerne er udvalgt fra

T004C, T004E, T004H, T004I, T004K, T004L, T004M, T004N, T004P, T004R, T004S, T004V, T004W, T004Y, G012D, G012E, G012I, G012K, G012L, G012M, G012Q, G012T, G012V, G012W, K013C, K013D, K013E, K013F, K013G, K013H, K013I, K013L, K013M, K013N, K013Q, K013S, K013T, K013V, K013Y, T014F, T014G, T014H, T014I, T014K, T014L, T014M, T014P, T014Q, T014R, T014S, T014V, T014W, T014Y, S023A, S023D, S023F, S023I, S023K, S023L, S023M, S023N, S023P, S023Q, S023R, S023T, S023V, S023W, S023Y, G024A, G024D, G024F, G024G, G024H, G024I, G024K, G024L, G024M, G024N, G024P, G024R, G024S, G024T, G024V, G024W, G024Y, K033H, Q045C, Q045D, Q045E, Q045F, Q045H, Q045I, Q045K, Q045L, Q045M, Q045N, Q045P, Q045R, Q045T, Q045W, N046A, N046C, N046E, N046F, N046G, N046H, N046I, N046K, N046L, N046M, N046P, N046Q, N046R, N046S, N046T, N046V, N046W, N046Y, R047E, R047K, R047L, R047M, R047Q, R047S, R047T, Y049A, Y049C, Y049D, Y049E, Y049F, Y049H, Y049I, Y049K, Y049L, Y049N, Y049R, Y049T, Y049V, Y049W, N050D, N050F, N050G, N050H, N050I, N050K, N050L, N050M, N050P, N050Q, N050W, N050Y, T054C, T054D, T054E, T054F, T054G, T054H, T054I, T054K, T054L, T054M, T054N, T054P, T054Q, T054R, T054S, T054V, T054W, T054Y, S058D, S058H, S058I, S058L, S058N, S058P, S058Q, T059A, T059C, T059E, T059G, T059H, T059I, T059K, T059L, T059M, T059N, T059P, T059Q, T059R, T059S, T059V, T059W, T060D, T060F, T060I, T060K, T060L, T060N, T060Q, T060R, T060V, T060W, T060Y, T065C, T065E, T065F, T065H, T065I, T065K, T065L, T065M, T065P, T065Q, T065R, T065V, T065Y, S066C, S066D, S066E, S066F, S066H, S066I, S066K, S066L, S066N, S066P, S066Q, S066R, S066T, S066V, S066W, S066Y, Q087A, Q087D, Q087E, Q087H, Q087I, Q087K, Q087L, Q087M, Q087N, Q087R, Q087T, Q087V, Q087W, N090C, N090D, N090E, N090F, N090G, N090H, N090I, N090R, N090T, N096G, N096H, N096K, N096R, K097H, K097Q, K097W, K100A, K100D, K100E, K100F, K100H, K100N, K100P, K100Q, K100R, K100S, K100V, K100Y, R110A, R110C, R110E, R110H, R110K, R110L, R110M, R110N, R110S, R110Y, D119E, D119H, D119I, D119L, D119Q, D119R, D119S, D119T, D119V, D119W, G128C, G128F, G128H, G128K, G128L, G128M, G128N, G128Q, G128R, G128W, G128Y, S129A, S129C, S129D, S129F, S129G, S129H, S129I, S129K, S129L, S129M, S129Q, S129R, S129T, S129V, S129W, S129Y, F130I, F130K, F130L, F130M, F130Q, F130R, F130T, F130V, F130Y, S135P, G136I, G136L, G136P, G136V, G136W, G136Y, S137A, M138I, M138K, M138Q, M138V, D139A, D139C, D139E, D139G, D139H, D139I, D139K, D139L, D139M, D139P, D139R, D139S, D139V, D139W, D139Y, V140C, Q151I, E152A, E152C, E152D, E152F, E152G, E152H, E152L, E152M, E152N, E152R, E152S, E152W, N155D, N155K, N155Q, N155R, D178A, D178C, D178G, D178H, D178K, D178L, D178M, D178N, D178P, D178Q, D178R, D178S, D178T, D178V, D178W, D178Y, T179A, T179E, T179H, T179I, T179K, T179L, T179M, T179N, T179P, T179Q, T179R, T179S, T179V, T179W, T179Y, E186A, E186C, E186D, E186G, E186H, E186K, E186L, E186M, E186N, E186P, E186Q, E186R, E186S, E186T, E186V, E186W, E186Y, V190H, V190I, V190K, V190L, V190Q, V190R, S191F, S191G, S191H, S191I, S191K, S191L, S191N, S191Q, S191R, S191W, L198M, L198V, S199C, S199D, S199E, S199F, S199I, S199K, S199L, S199N, S199Q, S199R, S199V, Y204H, Y204T, G205F, G205H, G205I, G205M, G205R, G205S, G205Y, K211C, K211D, K211G, K211M, K211N, K211Q, K211R, K211S, K211T, K211V, K214A, K214C, K214E, K214I, K214L, K214M, K214N, K214Q, K214S, K214V, L216A,

L216C, L216F, L216H, L216Q, L216R, L216S, L216Y, N218K, N218P, T219D, D220A, D220E, D220H, D220K, D220N, D220P, A221D, A221F, A221I, A221K, A221L, A221M, A221N, A221S, A221V, A221Y, G222C, G222H, G222N, G222R, Y224F, Y224H, Y224N, Y224R, T243C, T243G, T243H, T243I, T243K, T243L, T243Q, T243R, T243W, T243Y, K244A, K244C, K244D, K244E, K244F, K244G, K244L, K244M, K244N, K244Q, K244S, K244T, K244V, K244W, K244Y, V260A, V260D, V260E, V260G, V260H, V260I, V260K, V260L, V260M, V260P, V260Q, V260R, V260S, V260T, V260W, V260Y, Y261C, Y261F, Y261H, Y261L, T263E, T263F, T263H, T263I, T263L, T263M, T263Q, T263V, T263W, T263Y, S265A, S265C, S265D, S265E, S265K, S265N, S265P, S265Q, S265R, S265T, S265V, S265W, K269E, K269F, K269G, K269H, K269I, K269L, K269M, K269N, K269P, K269Q, K269S, K269T, K269V, K269W, K269Y, A273C, A273D, A273H, A273I, A273K, A273L, A273N, A273Q, A273R, A273Y, R280A, R280C, R280D, R280E, R280F, R280G, R280H, R280K, R280L, R280M, R280S, R280T, R280V, R280W, R280Y, L282F, L282G, L282H, L282I, L282K, L282M, L282N, L282Q, L282R, L282V, L282Y, S285A, S285C, S285D, S285E, S285K, S285P, S285Q, S285R, S285W, Q286A, Q286D, Q286E, Q286K, Q286P, Q286R, A289C, A289D, A289E, A289K, A289L, A289R, A293C, A293R, N296C, N296D, N296E, N296K, N296R, N296V, A297C, A297K, A297N, A297Q, A297R, og G299N;

hvor aminosyresekvensidentiteten med SEQ ID NO: 3 eller SEQ ID NO: 18 er bestemt ved anvendelse af BLAST med standardparametre;

hvor den neutrale metalloproteasevariant har forbedret termostabilitet sammenlignet med vildtype

- 5 B. *amyloliquefaciens* neutral metalloprotease med sekvensen SEQ ID NO:18;

forbedret performance under lavere eller højere pH-forhold sammenlignet med vildtype B. *amyloliquefaciens* neutral metalloprotease med sekvensen SEQ ID NO: 18; eller

forbedret autolytisk stabilitet sammenlignet med vildtype B. *amyloliquefaciens* neutral metalloprotease med sekvensen SEQ ID NO: 18.

- 10 2. Isoleret neutral metalloproteasevariant ifølge krav 1, hvor proteasen omfatter flere mutationer udvalgt fra

S023W/G024M,
T004V/S023W/G024W, S023W/G024Y/A288V, T004L/S023W/G024Y,
N046Q/N050F/T054L, N050Y/T059R/S129Q, S023W/G024W, A273H/S285P/E292G,
S023Y/G024Y, S023Y/G024W, T004S/S023Y/G024W, N046Q/T054K, S023W/G024Y,
T004V/S023W, T059K/S066N, N046Q/N050W/T054H/T153A, T004V/S023W/G024Y,
L282M/Q286P/A289R, N046Q/R047K/N050Y/T054K, L044Q/T263W/S285R,
T004L/S023W/G024W, R047K/N050F/T054K, A273H/S285R, N050Y/T059K/S066Q,
T054K/Q192K, N046Q/N050W, L282M/Q286K, T059K/S066Q, T004S/S023W,
L282M/Q286R/A289R/K011N, L282M/A289R, N046Q/N050W/T054H, T059K/S129Q,
T004S/S023N/G024Y/F210L, T004V/S023W/G024M/A289V,
L282M/Q286K/A289R/S132T, N050W/T054H, L282M/Q286R, L282F/Q286K/A289R,
T059R/S066Q, R047K/N050W/T054H,
S265P/L282M/Q286K/A289R, L282M/Q286R/T229S, L282F/Q286K, T263W/S285R,
S265P/L282M/Q286K, T263H/A273H/S285R, T059R/S129V,
S032T/T263H/A273H/S285R,
T059R/S066Q/S129Q, T004S/G024W, T004V/S023W/G024M, T059K/S066Q/S129Q,
L282M/Q286K/A289R/I253V, T004V/S023Y/G024W, T059R/S066N/S129Q,
N050F/T054L,

T004S/S023N/G024W, T059R/S066N, T059R/S066N/S129V, Q286R/A289R,
 N046Q/R047K/N050F/T054K, S265P/L282M/Q286P/A289R, S265P/L282M/Q286R/A289R,
 Q062K/S066Q/S129I, S023N/G024W, N046Q/R047K/N050W/T054H, R047K/T054K,
 T004L/G024W, T014M/T059R/S129V, T059R/S066Q/N092S/S129I,
 R047K/N050W/T054K, T004V/G024W, N047K/N050F/T054K,
 S265P/L282F/Q286K/N061Y, L282F/Q286K/E159V, T004V/S023Y/G024M,
 S265P/L282F/A289R/T063S, T059K/F063L/S066N/S129V, T004L/S023W, N050F/T054H,
 T059R/S066Q/S129V, V190I/D220E/S265W/L282F, T004S/S023Y/G024M,
 T004L/S023N/G024Y, T059K/S066N/S129I, T059R/S066N/S129I,
 L282M/Q286R/A289R/P162S, N046Q/N050F/T179N, T059K/Y082C/S129V,
 T059K/S129I,
 N050Y/T054K, T059K/S066Q/V102A/S129Q, T059R/S066Q/S129I,
 T059W/S066N/S129V/S290R, T059R/S129I, T059K/S066Q/S129I, T059K/S066Q/S129V,
 S265P/L282M/Q286R/A289R/T202S/K203N, T004V/S023N/G024W, S265P/Q286K,
 S265P/L282F/A289R, D220P/S265W, L055F/T059W/S129V, T059R/S129Q/S191R,
 N050W/T054K, T004S/S023W/G024M, R047K/N050F/T054H, T059K/S066N/K088E,
 T059K/S066Q/S129I/V291L, L282M/Q286R/A289R, T059R/S066N/F085S/S129I,
 L282F/Q286P/A289R, L282F/Q286R/A289R, G099D/S265P/L282F/Q286K/A289R,
 N046Q/N050F, N050Y/T059W/S066N/S129V, T009I/D220P/S265N,
 V190F/D220P/S265W, N157Y/T263W/A273H/S285R, T263W/A273H/S285R,
 T263W/S285W, T004V/S023Y, N046Q/R047K/N050W, N050W/T054L,
 N200Y/S265P/L282F/Q286P/A289R, T059R/S066Q/P264Q, T004V/G024Y,
 T004L/G024Y, N050Y/S191I, N050Y/T054L, T004L/S023W/G024Y/N155K,
 F169I/L282F/Q286R/A289R, L282M/Q286K/A289R, F130L/M138L/E152W/D183N,
 N046Q/R047K/N050Y/T054H, T004V/G024M, N050Y/T059W/S066Q/S129V,
 S023N/G024Y, T054H/P162Q, T004S/S023W/G024Y, N050Y/T054H,
 L282F/Q286R/A289R/F169I, R047K/N050W, V190F/D220P, L282M/F173Y,
 T004L/S023Y, N050W/A288D, V190I/D220P/S265Q, S265P/L282F/Q286P/A289R,
 S265P/L282F/Q286R/A289R, N046Q/N050Y/T054K, T059W/S066Q,
 T263W/A273H/S285W, T263W/A273H/S285P, S023Y/G024M, T004L/S023N/G024W,
 T004V/S023N/G024Y, T059W/S066N/S129Q, T004S/S023Y, T004S/S023N/G024M,
 T059W/S066N/A070T, T059W/S066Q/S129Q, T263W/A273H, A273H/S285P,
 N046Q/R047K/N050Y/T054L, N046Q/R047K/N050Y, R047K/N050Y, T263H/S285W,
 R047K/N050F, N046Q/R047K/N050F/T054H, S023N/G024M, T004S/G024Y,
 R047K/N050Y/T054H, T059W/S066N/S129I, R047K/T054L, T004S/S023W/G024W,
 M138L/E152F/T146S, D220P/S265N, T004S/G024M, T004V/S023N,
 N046Q/N050F/T054K, N046Q/N050Y/T054H, Q062H/S066Q/S129Q, T059W/S129Q,

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T059W/S129V, N050F/T054K, R047K/N050F/T054L, V190I/D220P/S265W,
 N112I/T263H/A273H/S285R, T059W/S066N/S129V, T059W/S066Q/S129I, T059W/S129I,
 T263W/S285P, V190I/D220P, A289V/T263H/A273H, T263H/A273H/S285P,
 N90S/A273H/S285P, R047K/N050Y/T054L,
 T004S/S023N, T059R/S129Q, N046Q/R047K/T054H, T059W/S066Q/S129V,
 E152W/T179P, N050Y/S066Q/S129V, T202S/T263W/A273H, T263W/A273H/S285P,
 M138L/E152W/T179P,
 N046Q/R047K, N046Q/T054H/F176L, T004L/G024M, T004S/L282M, T263H/A273H,
 T263H/A273H/S285W, T004L/S023Y/G024M, L282F/Q286P, T004V/S023Y/G024Y,
 V190F/S265W, M138L/E152F, V190F/D220E/S265W, N046Q/N050F/T054H,
 N157Y/S285W,
 T004F/S023Y/G024M, T004V/S023N/G024M, L198I/D220E/S265Q,
 N046Q/N050Y/T054K/A154T, S016L/D220E/S265W, D220E/S265W,
 D220E/A237S/S265W, S066Q/S129Q, V190F/D220E/S265Q/T267I, L282M/F173Y/T219S,
 E152F/T179P, V190I/S265W, M138L/S066Q, M138L/E152W,
 T059W/S066Q/A070T/S129I, V190F/D220E/S265N, V190F/S265N, N046Q/N050Y, og
 M138L/E152F/T179P.

3. Isoleret neutral metalloproteasevariant ifølge krav 1, hvor proteasen omfatter flere mutationer udvalgt fra

V190I/D220P, V190I/D220P/S265Q,
 V190L/D220E, V190I/D220E/S265Q, V190I/D220E/S265W/L282F, V190L/D220E/S265Q,
 V190I/D220E/S265W, V190L/D220E/S265N, T059R/S066Q/S129I, V190I/D220E/S265N,
 V190L/D220E/S265W, V190I/D220E, T059W/S066N/S129V, T059K/S066Q/S129V,
 T059K/Y082C/S129V, T059R/S066N/S129I, S066Q/S129V, T059R/S066Q/S129V,
 T059R/S129I, N050Y/T059W/S066N/S129V, D220P/S265N, S066Q/S129I,
 T059W/S066Q/S129V, T059K/S066Q/S129I, T059R/S129V, N050Y/S066Q/S129V,
 T059W/S066Q/S129I, N050Y/T059W/S066Q/S129V, T059K/S129I, D220P/S265W,
 F130L/M138L/T179P, S066N/S129I, T059R/S066N/S129V, F130I/M138L/T179P,
 T059R/S066Q/N092S/S129I, S066N/S129V, D220E/S265Q, F130L/M138L/E152W/T179P,
 T059W/S129V, S265P/L282M/Q286R/A289R, S265P/L282F/Q286R/A289R,
 T059W/S066N/S129I, V190I/D220P/S265W, F130L/E152W/T179P,
 F130L/M138L/E152F/T179P, Q062K/S066Q/S129I, T059K/S066N/S129I, E152H/T179P,
 S265P/L282M/Q286K/A289R, F130L/M138L/E152H/T179P, T263W/A273H/S285R,
 D220E/S265N, F130I/M138L/E152H/T179P, F130V/M138L/E152W/T179P,
 F130I/M138L/E152W/T179P, T059W/S129I, D220E/S265W, F130V/M138L/T179P,
 F130L/E152V/T179P, T059R/S129Q, T263W/S285P, F130I/M138L/E152F/T179P,
 E152W/T179P, V190L/S265Q, F130L/E152F/T179P, L282M/Q286R/A289R/P162S,

D220P/S265Q, M138L/E152F/T179P, F130I/E152H/T179P, M138L/E152W/T179P,
 F130L/T179P, F130L/M138L/E152W/T179P/Q286H, F130L/M138L/E152H,
 T263W/A273H/S285W, S265P/Q286K, T059W/S066Q/S129Q, T263W/S285R,
 T059W/S066N/S129Q, T263W/S285W, T059R/S066N/S129Q,
 S265P/L282M/Q286R/A289R/T202S/K203N, T059W/S129Q, Q062H/S066Q/S129Q,
 L282M/Q286R/A289R, V190L/D220E/S265N/V291I, V190L/S265N,
 F130L/M138L/E152W, N050Y/T059R/S129Q, F130I/T179P, T059K/S066Q/S129Q,
 T059K/S129Q, S265P/L282M/Q286P/A289R, S265P/L282F/Q286P/A289R,
 T263W/A273H/S285P, S265P/L282M/Q286K, S016L/D220E/S265W, S066Q/S129Q,
 S265P/L282M/Q286P, L282F/Q286R/A289R, F130V/E152W/T179P,
 L044Q/T263W/S285R L055F/T059W/S129V, V190L/S265W, Q286R/A289R,
 G99D/S265P/L282F/Q286K/A289R, F130L/M138L/E152F, T059R/S066Q/S129Q,
 F130L/E152H, S066N/S129Q, T004S/S023N/G024M/K269N, S265P/L282M,
 E152F/T179P, T059W/S066N/S129V/S290R, L282F/Q286K/A289R, F130L/M138L,
 F130I/M138L/E152W, S265P/L282F, F130I/M138L/E152H, F130V/M138L/E152H,
 V190I/S265Q, M138L/E152M, S265P/L282F/Q286P, M138L/E152H,
 T059K/S066N/K088E, V190I/S265W, F130L/E152W, L282M/Q286K/A289R,
 L282M/Q286K/A289R/I253V, T263W/A273H, V190I/S265N, M138L/E152W,
 A273H/S285R, F130I/M138L, F130L/E152F, F130V/M138L/E152W,
 T059K/S066Q/V102A/S129Q, F130V/E152H/T179P, F130I/M138L/E152F,
 F130V/M138L/E152F, M138L/E152F, L282M/Q286R, F130I/E152H,
 S265P/L282F/A289R/T065S, T263H/A273H/S285R, F130V/M138L,
 T014M/T059R/S129V, L282M/Q286R/A289R/K11N, A273H/S285P,
 L282M/Q286K/A289R/S132T, T263H/A273H/S285W, F130V/E152W,
 S265P/L282F/Q286K/N061Y, F130I/E152W, L198U/D220E/S265Q, V190I/S265L,
 T263H/S285W, S265P/L282F/A289R, M138L/S066Q, F130I/E152F, N90S/A273H/S285P,
 S032I/T263H/A273H/S285R, L282F/Q286P/A289R, N157Y/T263W/A273H/S285R,
 V105A/S129V, T263H/A273H/S285P, S129Q/L282H, T059W/S066Q, F130V/E152H,
 S023W/G024Y, T004V/S023N, T059R/S066Q, N050W/T054L, L282M/Q286P/A289R,
 A115V/V190L/S265W, L282M/Q286K, T059R/S066N, L282F/Q286P,
 T004V/S023W/G024M, S265P/L282F/Q286R/L78H, L282F/Q286K,
 T004V/S023W/G024Y, S023W/G024M, T059R/R256S, F130V/E152F, T004V/G024W,
 N050W/T054K, S023Y/G024M, T004V/S023Y, T004V/S023Y/G024M, N050Y/T054H,
 S023W/G024W, T004V/S023Y/G024Y, T004V/S023N/G024W,
 F130L/M138L/E152F/T179P/V291I, N050Y/T059K/S066Q, T004V/S023Y/G024W,
 T059K/S066N, T004V/S023N/G024Y, S023Y/G024W, N050F/T054L, R047K/T054K,
 S023N/G024W, L282M/A289R, S023Y/G024Y, T004V/G024M, R047K/N050F/T054K,

 N050F/T054K, T059K/S066Q, S023N/G024M, S023N/G024Y, T004L/S023N,
 R047K/N050W/T054H, T004L/S023W/G024Y, T004S/S023W,
 N046Q/N050W/T054H/A142T, T004L/S023Y, T004V/S023W, N050W/T054H,
 T004S/S023N, T004S/L282M, T004L/S023W, N050F/T054H, N050Y/T054L, og
 R047K/N050W/T054K.

4. Isoleret neutral metalloproteasevariant ifølge krav 1, hvor proteasen omfatter flere mutationer udvalgt fra

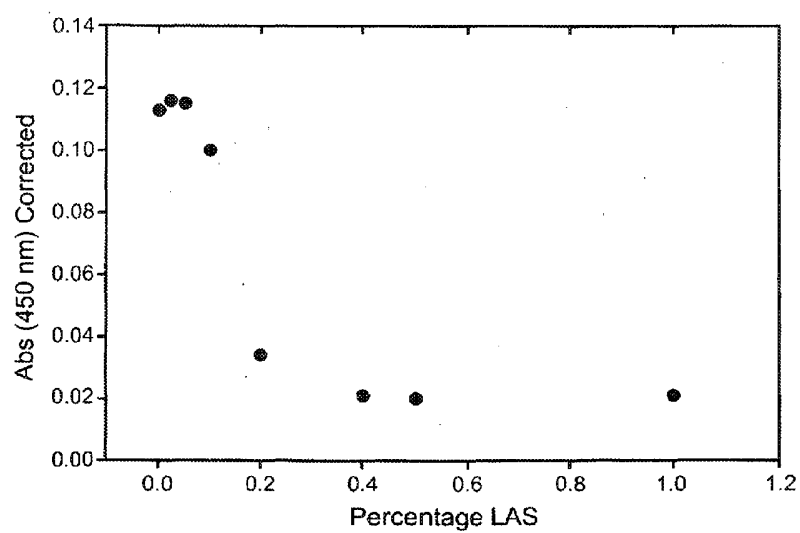
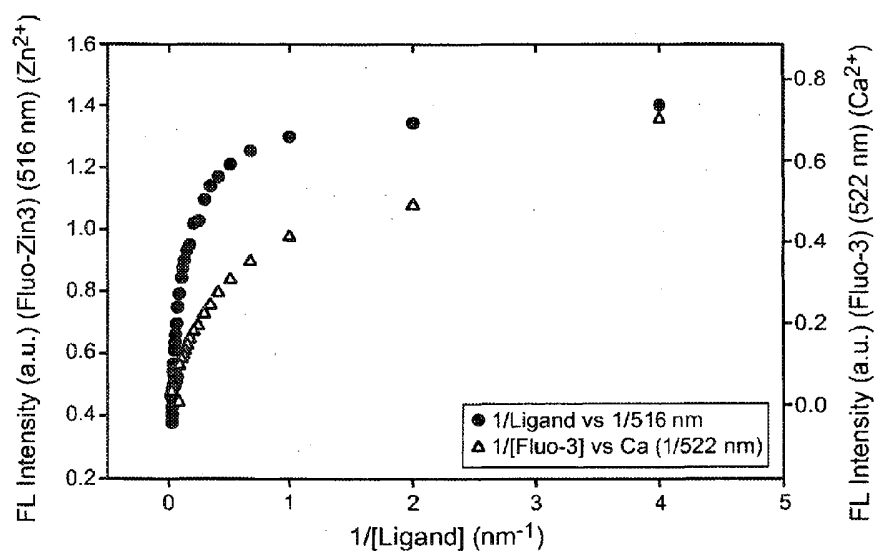
S066Q/S129V, S066Q/S129I,
 N050Y/S066Q/S129V, S066N/S129I, T059K/S066Q/S129V, S066N/S129V,
 F130L/E152W/T179P, S265P/L282M/Q286R/A289R, F130L/E152V/T179P,
 T059K/S066Q/S129I, T263W/S285P, T059K/S066N/S129I, T263W/A273H/S285P,
 S265P/L282F/Q286R/A289R, F130V/E152W/T179P, T263W/A273H/S285R,
 V190I/D220P/S265W, F130L/E152H, S066N/S129Q, S265P/L282M/Q286K/A289R,
 V190I/D220E, T059R/S066N/S129I, V190I/D220E/S265W, T059K/S129I,
 T059R/S066Q/S129I, F130I/M138L/E152H/T179P, F130I/T179P, T263W/A273H/S285W,
 S016I/D220E/S265W, S066Q/S129Q, V190I/D220E/S265Q, T059R/S066Q/S129V,
 D220E/S265N, V190L/D220E, D220E/S265W, V190I/D220P, V190L/D220E/S265N,
 L044Q/T263W/S285R, S265P/L282M/Q286P/A289R, F130L/M138L/E152H/T179P,
 T263W/S285R, L282M/Q286R/A289R, T263W/S285W, F130I/E152H/T179P,
 V190I/D220E/S265N, V190L/D220E/S265W, V190I/D220P/S265Q, T059R/S066N/S129V,
 V190L/D220E/S265Q, E152H/T179P, F130L/M138L/E152F/T179P, Q062H/S066Q/S129Q,
 T059R/S129V, V190I/D220E/S265W/L282F, V190I/S265Q, F130L/E152F/T179P,
 D220E/S265Q, E152W/T179P, T059K/S066Q/S129Q, F130L/M138L/T179P,
 F130I/M138L/E152F/T179P, F130L/M138L/E152W/T179P, N050Y/T059W/S066Q/S129V,
 S265P/L282M/Q286K, T059R/S129I, F130V/E152H/T179P, D220P/S265N,
 S265P/L282M/Q286P, F130I/E152H, T059R/S066Q/N092S/S129I, F130L/T179P,
 G99D/S265P/L282F/Q286K/A289R, T263W/A273H, V190I/S265N, D220P/S265W,
 F130L/E152W, F130L/M138L/E152H, S265P/L282M, V190I/S265Q, F130L/E152F,
 T059K/S129Q, Q286R/A289R, M138L/E152W/T179P, F130I/M138L/E152H,
 D220P/S265Q, V190L/S265N, F130I/M138L/E152W, S265P/Q286K, V190L/S265Q,
 V190I/S265W, F130L/M138L/E152F, F130V/E152H, E152F/T179P,
 N050Y/T059W/S066N/S129V, T059R/S066N/S129Q, F130I/E152W, F130V/E152W,
 T059R/S066Q/S129Q, T263H/A273H/S285P, N90S/A273H/S285P,
 V190L/D220E/S265N/V291I, T059R/S129Q, A273H/S285P, F130I/M138L/E152W/T179P,
 F130V/M138L/E152F, N050Y/T059R/S129Q, T059W/S066Q/S129I, F130V/M138L/T179P,
 F130V/M138L/E152W/T179P, V190L/S265W, F130V/M138L/E152W,
 T059W/S066Q/S129V, V190I/S265Q, F130V/M138L/E152H, F130I/E152F,

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N157Y/T263W/A273H/S285R, T263H/S285W, M138L/E152F/T179P, A115V/V190L/S265W, M138L/E152M, T263H/A273H/S285W, F130L/M138L/E152W, T059K/S066N/K088E, F130I/M138L/E152F, F130I/M138L/T179P, T004V/S023N, T059K/S066Q/V102A/S129Q, F130L/M138L, N047K/N050F/T054K, T263H/A273H/S285R, F130L/M138L/E152W/T179P/Q286H, M138L/E152H, M138L/S066Q, L282M/Q286R/A289R/P162S, L282F/Q286R/A289R, Q062K/S066Q/S129I, A273H/S285R, S265P/L282F/Q286P, S265P/L282F/Q286P/A289R, S265P/L282M/Q286R/A289R/T202S/K203N, T059W/S066N/S129I, V190I/S265L, T059W/S066N/S129V, F130I/M138L, L282M/Q286K/A289R/I253V, R047K/N050F/T054K, M138L/E152F, N050W/T054K, L198I/D220E/S265Q, L282F/Q286K/A289R, N050F/T054K, L282M/Q286R, M138L/E152W, S265P/L282F, F130V/E152F, T059W/S066N/S129Q, F130V/M138L, T263H/A273H, L282M/Q286K/A289R, N046Q/N050W/T054H/A142T, T059W/S066Q/S129Q, S265P/L282F/A289R/T065S, N050F/T054H, S129Q/L282H, L282M/Q286K/A289R/S132T, L282M/Q286R/A289R/K11N, T059K/S066N, R047K/N050W/T054K, T059K/S066Q, T004V/S023Y, T059W/S066N/S129V/S290R, N050Y/T059K/S066Q, og R047K/N050Y.

5. Polynukleotid, der koder for den neutrale metalloprotease ifølge et hvilket som helst af kravene 1 til 4.
6. Ekspressionsvektor omfattende nukleotidsekvensen ifølge krav 5.
- 5 7. Værtscelle omfattende ekspressionsvektoren ifølge krav 6.
8. Fremgangsmåde til fremstilling af et enzym med neutral metalloproteaseaktivitet, omfattende dyrkning af værtscellen ifølge krav 7 på betingelser, der er egnede til fremstillingen af den neutrale metalloproteasevariant.
9. Fremgangsmåde ifølge krav 8, der endvidere omfatter trinnet med høstning af den fremstillede
- 10 neutrale metalloproteasevariant.
10. Fremgangsmåde ifølge krav 7 eller krav 8, hvor værtscellen er en *Bacillus*-art.
11. Rengørings sammensætning omfattende den neutrale metalloproteasevariant ifølge et hvilket som helst af kravene 1 til 4.
12. Fremgangsmåde til rengøring, omfattende trinnet med at bringe en overflade og/eller en artikel
- 15 omfattende et stof i kontakt med rengørings sammensætningen ifølge krav 11.
13. Fremgangsmåde ifølge krav 12, hvor overfladen og/eller en artikel omfattende et stof omfatter en græsplet og trinnet med etablering af kontakt omfatter at bringe græspletten i kontakt med rengørings sammensætningen.
14. Dyrefodersammensætning, tekstilbehandlingssammensætning eller læderbehandlings-
- 20 sammensætning, der omfatter den isolerede neutrale metalloproteasevariant ifølge et hvilket som helst af kravene 1 til 4.

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**FIG. 1****FIG. 2**

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B.amylo_nprE -----MGLGKKLSVAVAAAFMSLTISLPGVQAAENPQIKENLTNFPVKHSLVQSEL
B.sub_nprE -----MGLGKKLSVRVAASFMSLSISLPGVQAAEGHQIKENQTNFLSKKPIAQSEL
S.aur_aur -----MRKFSRYAFTSMATVTLLSSLTPAALASDTNKKPATSDINFEI
B.ste_nprT -----MNKRAMLGAIGLAFGLLAAPIGASAKGESIVWNEQWKTSPFVSGSLNGGEQAL
B.cal_npr -----MNKRAMLGAIGLAFGLMAWPPGASAKGKSMVWNEQWKTSPFVSGSLNG---RCS
B.thu_nprB MKKKSLALVLATGMVTTFGGTGSAFADSKNVLSTKKYNETVQSPFVSGDLTEATGKKA
B.cer_nprC MKKKSLALVLATGMVTTFGGTGSAFADSKNVLSTKKYNETVQSPFVSGDLTEATGKKA
B.sub_nprB --MRNLTKTSLLLAGLCTAAQMVFTVTHASAEESIEYDHTYQTPSYIIEKSPQKPVQNTTQ
B.cerE33L_npr --MKNKKNFVKIGLTTGVMLSVIMPYGDAYAATEDLKVETKEDTFRGTGNLTVPFSQKSAEN

B.amylo_nprE PSVSDKAIKQYLKONGKVFKNPSERLKLIQTTDDLGKHFYVPVWNGVPVKDSQVII
B.sub_nprE SAPNDKAVKQFLKKNNSNIFKGDPSKSVKLVESTTDALGYKHFRYAFVWNGVFIKDSQVIV
S.aur_aur TQKSD-AVKALKELPKSENVKNHYQDYSVTDVKTDDKGFTHYTLQPSVDGVHAPDKEVKV
B.ste_nprT EELVYQYVDRENGTFRLGGRARDRLALI--GKQTDDELGHTVMRFEQRHHGIPVYGTMLAA
B.cal_npr QELVYRYLDQEKNTFQLGGQARERLSLI--GNKLEDELGHTVMRFEQAIAASLCMGAVLVA
B.thu_nprB ESVVFDYLNAAKGDYKLGKSAQDCFRVKQAKKDAVTDSTVLRQQVYEGVFWGSTQVA
B.cer_nprC ESVVFDYLNAAKGDYKLGKSAQDSFVKQVKKDAVTDSTVVRMQQVYEGVFWGSTQVA
B.sub_nprB KESLFSYLDKHQTQFKLKGNSHFRVSK-TIKDPKTKQTFFKLETVYKGIPIYGFQAV
B.cerE33L_npr VAKDALKGKTEQALSSKQVNTSEKVNYNVTQSRKSYDGTTLVRLQQTYEGRDVYGYQLTA

B.amylo_nprE HVDKSNVYAIINGELNNDVSAKTANSKKLSANQALDHAYKAIGKSPEAVSNGTVANKKA
B.sub_nprE HVDKSDNVYAVNGELHNQSAAKTDNSQKVSSEKALALAFKAIGKSPDAVSNGAAKNSNKA
S.aur_aur HADKSGKVVLING----DTDAKKVKPTNKVTLSDKDEAADKAFNAVKIDKNKAKNLQDDVI
B.ste_nprT HV-KDGLIELIALSGSLIPNLDGQPRLLKAKTVTVQQAETAEQDVETVTKERPTTNGER
B.cal_npr HV-NDGELSSLSGTLIPNLD-KRILKTEAATISIQQAEMIAKQDVADRVTKERPAEEGKP
B.thu_nprB HVSKDGSIKVLSGTVAPDIDKKEKLNKNKIEGAKAIEIAQKDLGIT----PKYEVEPK
B.cer_nprC HVSKDGSILKVLSGTVAPDIDKKEKLNKNKIEGAKAIEIAQQDLGVT----PKYEVEPK
B.sub_nprB AMKENQVKSFFGKVHPQIKDVSVTPSISEKKAHTARRELEASIGKI----EYLDGEPK
B.cerE33L_npr HYNDDGVLTSVSGSDAQLQQLQEDLKQPIITLSEEDAKKQLEFKIYGDNLIT---FVEEPEIK
.. : . * : :

B.amylo_nprE ELKAAATKDGKRYLAYDVTIRYIEPEPANWEVTVDAETGKILKKQNKVE-----
B.sub_nprE ELKAIETKDGSYRLAYDVTIRYVEPEPANWEVLVDAETGKILKKQNKVE-----
S.aur_aur KENKVEIDGDSNKYIYNIELITVTPEISHKVKIDADTGAVVEKTNLVK-----
B.ste_nprT TRLVITYPTDGTARLAYEVNVRFLTVPVPGNWWYIIDATDGAILNKFNQID-----
B.cal_npr TRLVITYPDEETPRLAYEVNVRFLTVPVPGNWIYMIDAADGKVLNKNQMD-----
B.thu_nprB ADLYVYQNGEETTYAYVNLNLFDPSPGNYYYFIEADSGKVLNKNKLDHVANEDKSPVK
B.cer_nprC ADLYVYQNGEETTYAYVNLNLFDPSPGNYYYFIEADSGKVLNKNKNTIDHVTNDDKSPVK
B.sub_nprB GELYIYPHDGEYDLAYLVRLLSTSEPEPGYWHYFIDAKNGKVIESFNAIH-----
B.cerE33L_npr QVVYVDENTNKATSAYQITFSASTPEYVSGTVLIDAFVGDLLKELVQKLGIQVDSI---
* : . * : : * * : :

B.amylo_nprE -----HAATTGTGTTLKGTVSLNISSESGKYVLRDLSKPTGTQIITYD
B.sub_nprE -----HAAATGSGTTLKCATVPLNISYEGGKYVLRDLSKPTGTQIITYD
S.aur_aur -----EAAATGTGKGLVLDTKDININSIDGGESLEDLTHQKLSAYNFN
B.ste_nprT -----SRQPGGGQPVAGASTVGVGRGVLGDQKYINTTYSYYGYLQDNTRGSGIFTYD
B.cal_npr -----EAKPGGAQPVAGSTVGVGRGVLGDQKYINTTYSYYGYLQDNTRGSGIFTYD
B.thu_nprB QEAPKQEVKPAVKPVTGTNAVGTGKGLVLDTKSLNTTLSS--SSYYLQDNTRGATIFTYD
B.cer_nprC QEAPKQDAKAVVKPVTGTNKVGTGKGLVLDTKSLNTTLSC--SSYYLQDNTRGATIFTYD
B.sub_nprB -----EAGTGIGVSGDEKSFVTEQN--GREYLADETRGKGINTFD
B.cerE33L_npr -----VQSATSNKSQDPSKLTGKDDLGMRNRTFGISQRS-DGYTTLADYSRGKGIETYT
* * * * : :

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FIG. 3A

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B.amylo_nprE LQNRXY-----NLPGTLVSSTTNQETTSQRAAVDAHYNLGKVYDYFYQKFNRRNSYDN
B.sub_nprE LQNRQS-----RLPGTLVSSTTKTFTSSSQRAAVDAHYNLGKVYDYFYSNFKRNSYDN
S.aur_aur DQTGQ-----ATLITNEDENFVKDDQAGVDANYAKQTYDYKNTFGRESYDN
B.ste_nprT GRNR-----TVLPGLWTDGDNQFTASIDAAAVDAHYAGVVDYKKNVHGRLSYDG
B.cal_npr GRNR-----TVLPGLWADGDNQFFASYDAAAVDAHYAGVVDYKKNVHGRLSYDG
B.thu_nprB AKNR-----TTLPGTLWVDADNVFNAAAYDAAAVDAHYAGKTYDYKATFNRRNSIND
B.cer_nprC AKNR-----STLPGTLWADADNVFNAAAYDAAAVDAHYAGKTYDYKATFNRRNSIND
B.sub_nprB AKNLNETLFTLLSQLIGYTGKEIVSGTSVFNEPAAVDAHANAQAVYDYYSKTFGRDSFDQ
B.cerE33L_npr ANYKDYNNYR--RNIWGYLDDLVTNSNTNFTDPKAVSAHYLATKVYDFYQEKYSRNSFDN
      . . . . . : . * . : . * . : . * . : . * . :
B.amylo_nprE KGGKIYSSVHYG-----SRYNNAAWIGDQMIYGDGDSFFSPLSGSMDVTAHEMT
B.sub_nprE KGSKIYSSVHYG-----TQYNNAAWTGDMYIGDGDGDSFFSPLSGSLDVTAEHMT
S.aur_aur HGSPIVSLTHVNHYGQ-----DNRNNAAWIGDKMIYGDGDGRFTTNLSSGANDVVAHELT
B.ste_nprT SNAAIRSTVHYG-----RGYNNAFWNGSQMVYGDGDGQTFLPFSGGIDVVGHELT
B.cal_npr SNAAIRSTVHYG-----RGYNNAFWNGSQMVYGDGDGQTFLPFSGGIDVVGHELT
B.thu_nprB AGAPLKSTVHYG-----SKYNNAFWNGSQMVYGDGDGVTFTSLSGGIDVIGHELT
B.cer_nprC AGAPLKSTVHYG-----SNYNNAFWNGSQMVYGDGDGVTFTSLSGGIDVIGHELT
B.sub_nprB NGARITSTVHYG-----KQWNNAAWNGVQMVYGDGDGSKFKPLSGSLDIVAHEIT
B.cerE33L_npr NQQKVISVVGWNTNCTNKGKQWFAFNGAMLVYGD-----PIVRAFDVAGHEFT
      . : * . * : * . * : * . * : * . * : * . * : * . * :
B.amylo_nprE HGVQTETANLNYENQPGALNESFSDVFGYFND-----TEDWDIGEDITVSQPALRSLSNP
B.sub_nprE HGVQTETANLIYENQPGALNESFSDVFGYFND-----TEDWDIGEDITVSQPALRSLSNP
S.aur_aur HGVQTETANLEYKQSGALNESFSDVFGYFVD-----DEDFLMGEDVYTPGKEGDALRSM
B.ste_nprT HAVTDYTAGLVYQNESGALNEAMSDFPGTLVEFYANRNPDEWIGEDIYTPGVACDALRSM
B.cal_npr HAVTDYTAGLVYQNESGALNEAMSDFPGTLVEFYANRNPDEWIGEDIYTPGVAGDALRSM
B.thu_nprB HAVTEYSSDLIYQNESGALNEAISDFPGTLVEFYDNRNPDEWIGEDIYTPGKAGDALRSM
B.cer_nprC HAVTENSSNLIYQNESGALNEAISDFPGTLVEFYDNRNPDEWIGEDIYTPGKAGDALRSM
B.sub_nprB HAVTQYSAGLLYQCEPGALNESISDIMCAMAD-----RDDWEIGEDVYTPGIAGDSLRSL
B.cerE33L_npr HAVTRNESGLEIYAGEAGALNEAISDILGVAVEKYANNKFNWMTGEQSGRIFRDMKNPSS
      * . * . : * . * : * . * : * . * : * . * : * . * :
B.amylo_nprE TKYGPDPNFKNYKNLNTDAGDYGGVHTNSGIPNKAAYN-----TITKIGVNKAE
B.sub_nprE TKYNQPDNYANYRNLENTDEGDYGGVHTNSGIPNKAAYN-----TITKLGVSQSKSQ
S.aur_aur SNPEQFGQPSHMKDYVYTEK-DNGGVHTNSGIPNKAAYN-----VIQAIKSKSE
B.ste_nprT SDPAKYGDPDHYSKRYTGTO-DNGGVHTNSGIPNKAAYLLSQGGVHYGVSVNGIGRDKMG
B.cal_npr SDPAKYGDPDHYSKRYTGTO-DNGGVHTNSGIPNKAAYLLSQGGVHYGVSVNGIGRDKMG
B.thu_nprB SDFTKYGDPDHYSKRYTGTS-DNGGVHTNSGIPNKAAYLLANGGTHYGVTVTGIGKDKLG
B.cer_nprC SDFTKYGDPDHYSKRYTGSS-DNGGVHTNSGIPNKAAYLLANGGTHYGVTVTGIGKDKLG
B.sub_nprB EDPSKQGNPDHYSNRYTGTE-DYGGVHTNSGIPNKAAYLLAEGGVHHCQVQVEGIGREASE
B.cerE33L_npr ISSKYPEDYRHYNNLPIDAADHGGVHTNSGIPNKAAYLLAEGGVHHCQVQVEGIGREASE
      . : . : * * * * * * . * . * . : * . :
B.amylo_nprE QIYYRALTVYLTPTSSTFKDAKALIQSARDLYG--SQDAASVEAAWNAVGL- (SEQ ID NO:173)
B.sub_nprE QIYYRALTYLTPTSSTFKDAKALIQSARDLYG--STDAAKVEAAWNAVGL- (SEQ ID NO:174)
S.aur_aur QIYYRALTEYLTGNSNFKDCKDALYQAAKDLYD-EQTAEQVYFAWNEVGVE (SEQ ID NO:175)
B.ste_nprT KIFYRALVYVLTPTSNSQLRAACVQAAADLYGSTSQEVNSVKQAFNAVGVY (SEQ ID NO:176)
B.cal_npr KIFYRALVYVLTPTSNSQLRAACVQAAADLYGSTSQEVNSVKQAFNAVGVY (SEQ ID NO:177)
B.thu_nprB AIYYRANTQYFTQSTITFSQARAGAVQAAADLYGANSAEVNAVQSTSAVGIN (SEQ ID NO:178)
B.cer_nprC AIYYRANTQYFTQSTITFSQARAGAVQAAADLYGANSAEVNAVQSTSAVGIN (SEQ ID NO:179)
B.sub_nprB QIYYRALTYVYVASTIDFSMMKQAAIEAANDLYGEGSKQSASVEKAYEAVGIL (SEQ ID NO:180)
B.cerE33L_npr DIFYYANTDELNMTSDFKELKEACIRVATNLYGKDSSEVQAVQAFKAAYI- (SEQ ID NO:181)
      * . * . : * . * : * . * : * . * : * . * :

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FIG. 3B

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Thermolysin          ITGTSTVGVRGVLGDQKNINTTYSTYY---YLQDNTR--GNGIFTYDAK
B. stearrowthermophilus ITGTSTVGVRGVLGDQKNINTTYSTYY---YLQDNTR--GNGIFTYDAK
B. caldoyticus        VAGTSTVGVRGVLGDQKYINTTYSSYYGYYYIQDNTR--GSGIFTYDGR
B. cereus             VTGTNKGVTGKGVLDGDKSLNTTLGSSS---YLQDNTR--GATIFTYDAK
B. brevis             VTAT-----GKGVLDGDKQFETTKQGST---YMLKDTTR--GKGIETYTAN
B. polymyxa           -----ATGKGVLDGSKSFTTASGSS---YQLKDTTR--GNGIVTYTAS
B. pumilus            ---AAATGSGTTLKGATVPLNISYEGGK---YVLRDLSKPTGTQIITYDLQ
B. subtilis var.      ---AAATGSGTTLKGATVPLNISYEGGK---YVLRDLSKPTGTQIITYDLQ
B. subtilis           -----
B. amyloliquefaciens ---AATTGTGTLKGKTVSLNISSESGK---YVLRDLSKPTGTQIITYDLQ
                                                                94
Thermolysin          YRRT-LPGSLWADADNQFFASYDAPAVDAHYYAGVTYDYKYNVHNRLSYD
B. stearrowthermophilus YRRT-LPGSLWADADNQFFASYDAPAVDAHYYAGVTYDYKYNVHNRLSYD
B. caldoyticus        NRTV-LPGSLWADGDNQFFASYDAAAVDAHYYAGVVYDYKYNVHNRLSYD
B. cereus             NRST-LPGTLWADADNVENAAAYDAAAVDAHYYAGKTYDYKATFNKNSID
B. brevis             NRTS-LPGTLMTDSQNYWT---DGAAVDAHAAQKTYDYFRNVHNRNSYD
B. polymyxa           NRQS-IPGTLTADADNVWN---DPAGVDAHAAKTYDYKSKFGRNSID
B. pumilus            NRQSLPGTLVSSSTTKTFTSSSQRAAVDAHYNLGKVYDYFYSNFKRNSYD
B. subtilis var.      NRQSLPGTLVSSSTTKTFTSSSQRAAVDAHYNLGKVYDYFYSNFKRNSYD
B. subtilis           -----AVDAHYNLGKVYDYFYSNFKRNSYD
B. amyloliquefaciens NREYNLPGTLVSSSTTNQFTTSSQRAAVDAHYNLGKVYDYFYQKFNKNSYD
                                                                *****
                                                                142
Thermolysin          GNNAAIRSSVHYSGGYNNAFWNGSQMVYGDGDC--QTFLPLSGGIDVVAH
B. stearrowthermophilus GNNAAIRSSVHYSGGYNNAFWNGSQMVYGDGDC--QTFLPLSGGIDVVAH
B. caldoyticus        GSNAAIRSTVHYGRCYNNAFWNGSQMVYGDGDC--QTFLPLSGGIDVVGH
B. cereus             DAGAPLKSTVHYGSYNNAFWNGSQMVYGDGDC--VTFTSLSGGIDVIGH
B. brevis             GNCAGVIRSTVHYSTRYNNAFWNGSQMVYGDGDC--TTFPLPLSGGIDVVAH
B. polymyxa           GRGLQLRSTVHYGSRYNNAFWNGSQMTYGDGDCGSGTFIAPSGGDPVVGH
B. pumilus            NKGSKIVSSVHYGTQYNNAAWTGDMYGDGDC--SFFSPLSGSLDVTAH
B. subtilis var.      NKGSKIVSSVHYGTQYNNAAWTGDMYGDGDC--SFFSPLSGSLDVTAH
B. subtilis            NKGSKIVSSVHYGTQYNNAAWTGDMYGDGDC--SFFSPLSGSLDVTAH
B. amyloliquefaciens NKGSKIVSSVHYGSRYNNAAWIGDMYGDGDC--SFFSPLSGSMDVTAH
                                                                *:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*
                                                                192
Thermolysin          ELTHAVTDYTAGLIYQNESGAINAISDIFGTLVKFYANKNPDWEIGEDV
B. stearrowthermophilus ELTHAVTDYTAGLIYQNESGAINAISDIFGTLVEFYANKNPDWEIGEDV
B. caldoyticus        ELTHAVTDYTAGLVYQNESGAINAAMDIFGTLVEFYANKNPDWEIGEDI
B. cereus             ELTHAVTENSNNLIYQNESGALNEAISDIFGTLVEFYDNRPDWEIGEDI
B. brevis             ELTHAVTERTAGLVYQNESGALNESMSDIFGAMVD-----NDDWLMGEDI
B. polymyxa           ELTHGVTETYSNLEYGESGALNEAFSDVIGNDIQ-----RKNWLVGDDI
B. pumilus            EMTHGVTQETANLIYENQPGALNESFSDVFGYFND-----TEDWDIGEDI
B. subtilis var.      EMTHGVTQETANLIYENQPGALNESFSDVFGYFND-----TEDWDIGEDI
B. subtilis            EMTHGVTQETANLIYENQPGALNESFSDVFGYFND-----TEDWDIGEDI
B. amyloliquefaciens EMTHGVTQETANLNENQPGALNESFSDVFGYFND-----TEDWDIGEDI
                                                                *:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*
                                                                238
Thermolysin          YTPGISGDSLRMSDPAKYGDPDHYSKR---YTGTQDNNGGVHINSGIIN
B. stearrowthermophilus YTPGISGDSLRMSDPAKYGDPDHYSKR---YTGTQDNNGGVHINSGIIN
B. caldoyticus        YTPGVAGDALRMSDPAKYGDPDHYSKR---YTGTQDNNGGVHTNSGIIN
B. cereus             YTPGKAGDALRMSDPTKYGDPDHYSKR---YTGSNDNGGVHTNSGIIN
B. brevis             YTPGRSGDALRSLQDPAAYGDPDHYSKR---YTGSQDNNGGVHTNSGIIN
B. polymyxa           YTPNTCGDALRMSNPTLYDQPHYSNL---YKGSNDNGGVHTNSGIIN
B. pumilus            T---VSQPALRSLSNPTKYNQPDNYANYRNLPNTDEGDYGGVHTNSGIPN
B. subtilis var.      T---VSQPALRSLSNPTKYNQPDNYANYRNLPNTDEGDYGGVHTNSGIPN
B. subtilis            T---VSQPALRSLSNPTKYNQPDNYANYRNLPNTDEGDYGGVHTNSGIPN
B. amyloliquefaciens T---VSQPALRSLSNPTKYGQPDNFKYKNLPNTDAGDYGGVHTNSGIPN
                                                                *:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*

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FIG. 4A

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Thermolysin      KAAYLISQGGTHYGVSVVGIGRDKLGKIFYRALTQYLTPTSNTFSQLRAAA
B. stearothermophilus KAAYLISQGGTHYGVSVVGIGRDKLGKIFYRALTQYLTPTSNTFSQLRAAA
B. caldoyticus    KAAYLLSQGGVHYGVSVVTGIGRDKMGKIFYRALVYYLTPTSNTFSQLRAAC
B. cereus         KQAYLLANGGTHYGVTVTGIGKDKLGAIYYRANTQYFTQSTTFEQARAGA
B. brevis         KAAYLLAEGGTHYGVVRVNGIGRTDTAKIYYHALTHYLTFFYSNFSAMRRAA
B. polymyxa       KAYYLLAQGGTFHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARAAV
B. pumilus        KAAYN-----TITKLGVSQSQQIYYRALTTYLTTPSSTFKDAKAAL
B. subtilis var.  KAAYN-----TITKLGVSQSQQIYYRALTTYLTTPSSTFKDAKAAL
B. subtilis       KAAYN-----TITKLGVSQSQQIYYRALTTYLTTPSSTFKDAKAAL
B. amyloliquefaciens KAAYN-----TITKIGVNKAEQIYYRALTVYLTTPSSTFKDAKAAL
                  * * * : * * * * : * : .

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Thermolysin      VQSATDLYGSTSQEVASVKQAFDAVGVK (SEQ ID NO:182)
B. stearothermophilus VQSATDLYGSTSQEVASVKQAFDAVGVK (SEQ ID NO:183)
B. caldoyticus    VQAAADLYGSTSQEVNSVKQAFNAVGVY (SEQ ID NO:184)
B. cereus         VQAAADLYGANSAEVAAVKQSFSAVGVN (SEQ ID NO:185)
B. brevis         VLSATDLFGANSRQVQAVNAAYDAVGVK (SEQ ID NO:186)
B. polymyxa       IQAAKDLYGANSAAEATAAASFDVAG- (SEQ ID NO:187)
B. pumilus        IQSARDLYGST--DAKVEAAWNAVGL- (SEQ ID NO:188)
B. subtilis var.  IQSARDLYGST--DAKVEAAWNAVGL- (SEQ ID NO:189)
B. subtilis       IQSARDLYGST--DAKVEAAWNAVGL- (SEQ ID NO:190)
B. amyloliquefaciens IQSARDLYGSQ--DAASVEAAWNAVGL- (SEQ ID NO:191)
                  : * * *: : . : : * *

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FIG. 4B

```

NprE      ---AATTGCTGTTLK--GKTVSLNISSESCKYVLRDLKSKPTGTQITITYDLQNREYNLPGT-
1BQB.A    ---AAATGTGKGVL--GDTKDININSIDGGSLEOLT--HQGKLSAYNFNDQTGQ--AT-
1EZM      ----AEAGGPGGNOKIGKYTGSDYGLIVNDRCEMD---DGNVITVDMNSSTDDSKTTP
1NPC      VTGTNKVGTGKGVL--GDTKS--LNTTSLGSSYYLQDNTRGATIFTYDAKNRS-TLPGT-

NprE      LVSSTTNQFTTSS---QRAAVDAHYNLGKVYDYFYQKFNRSYDNKGGKIVSSVHYGS--
1BQB.A    LITNEDENFVKDE---QRAGVDANYAKQTYDYKNTFGRESYDNHGSPIVSLTHVNHYG
1EZM      FRFACPTNTYKQVNGAYSPLNDAH- FGGVVFKLYRDWFGTSPLT--HKLYMKVHYGR--
1NPC      LWADAENVFNAAY---DAAAVDAHYAGKTYDYKATFNRSINDAGAPLKSTVHYGS-

NprE      ---RYNNAAWIGDQMIYGDGSGSFFSPLSGSMOVTAHEMTHGVTQETANLNYENQPGALN
1BQB.A    GQDNRNNAAWIGDKMIYGDGDCRTFTNLSCANDVVAHEITHGVTQQTANLEYKQDSGALN
1EZM      ---SVENAYWDGTAMLFEGDGAT-MEYPLV-SLDVAAHEVSHGFTEQNSGLIYRGQSGGMN
1NPC      ---NYNNAFWNGSQMVYGDGSGVTFTSLSGGIDVIGHELTHAVTENSNNLIYQNESGALN

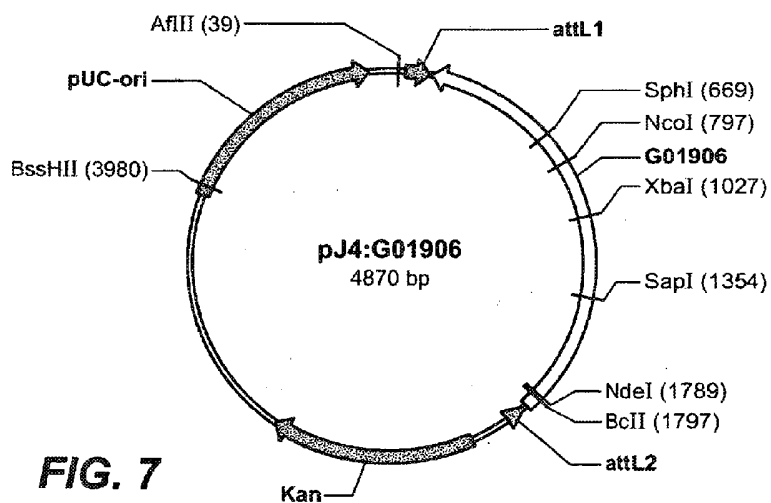
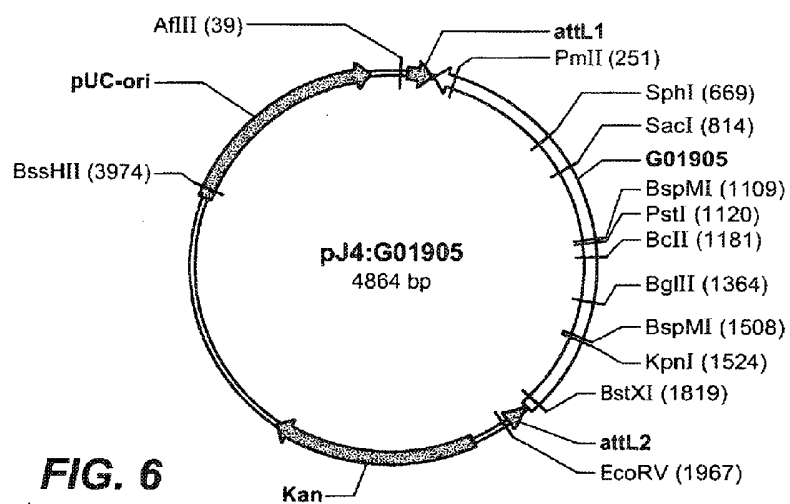
NprE      ESFSDVFGYFND-----TEDWDIGEDITVS---QPALRSLSNPTKYG-QPDNFKKNYNLP
1BQB.A    ESFSDVFGYFVD-----DEDFLMCEDVYTPCKEGDALRSMNSNPEQFG-QPSHMKDYVY--
1EZM      EAFSDMAGEAAEFYMRGKNDFLIGYDIKKG---SGALRYMDQPSRDGRSIDNASQYYNG-
1NPC      EATSDIFGTLVEFYDNRPDWEIGEDIYTPGKAGDALRSMDSPTKYG-DPDHYSKRYTGS

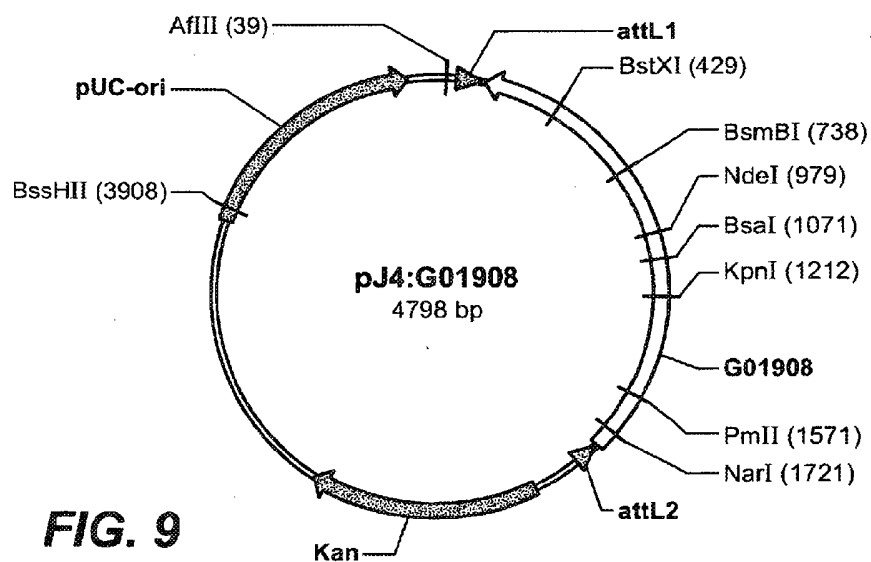
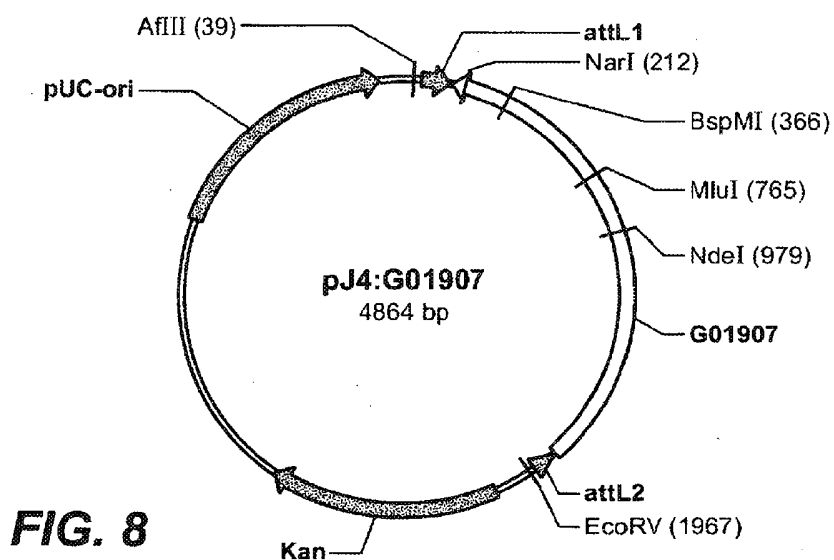
NprE      NTDAGDYGGVHTNSGIPNKAAYNTIT-----KIGVN--KAEQIYYRALTVYLTFS
1BQB.A    --TEKDNGGVHTNSGIPNKAAYNVIQ-----AIGKS--KSEQIYYRALTEYLTSN
1EZM      -----IDVHSSGVYNRAFYLLAN-----SPGWDRKAFEVFDANRYWTAT
1NPC      ----SDNGGVHTNSGILNKQAYLLANGGTHYGVTVTGIGKDKLGAIYYRANTQYFTQS

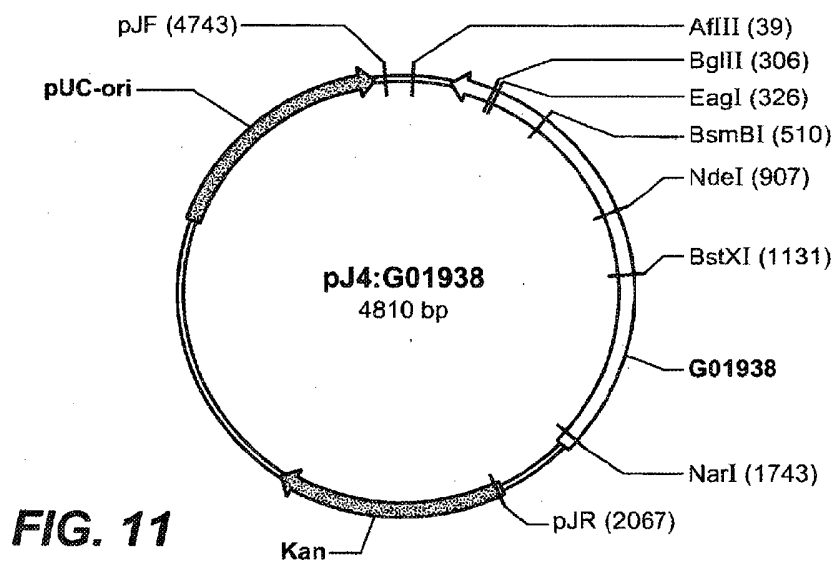
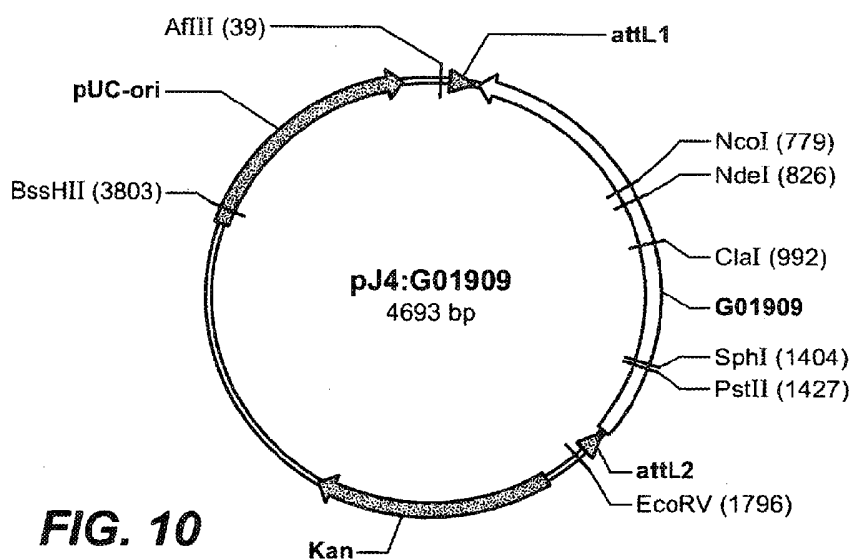
NprE      STFKDAKAALIQSARDLYG--SQDAASVEAAWNAVGL- (SEQ ID NO:192)
1BQB.A    SNFKDLKDZLYQAAKDLYE--QQTAEQVYEAWNEVGVE (SEQ ID NO:193)
1EZM      SNYNSGACGVIRSAQNRY--SAADVTRAFSTVGVTCP (SEQ ID NO:194)
1NPC      TTFSQARAGAVQAAADLYGANSAEVAAVKQSFSAVGVN (SEQ ID NO:195)

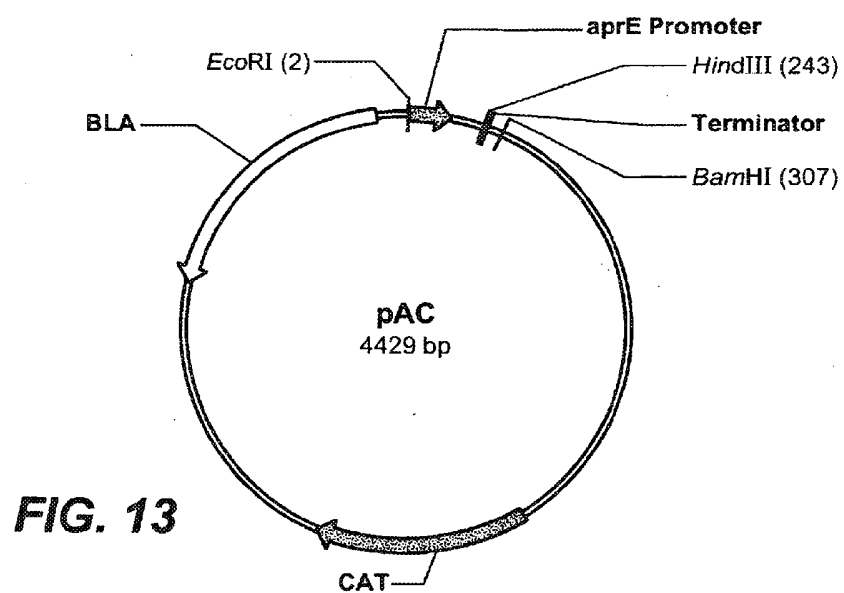
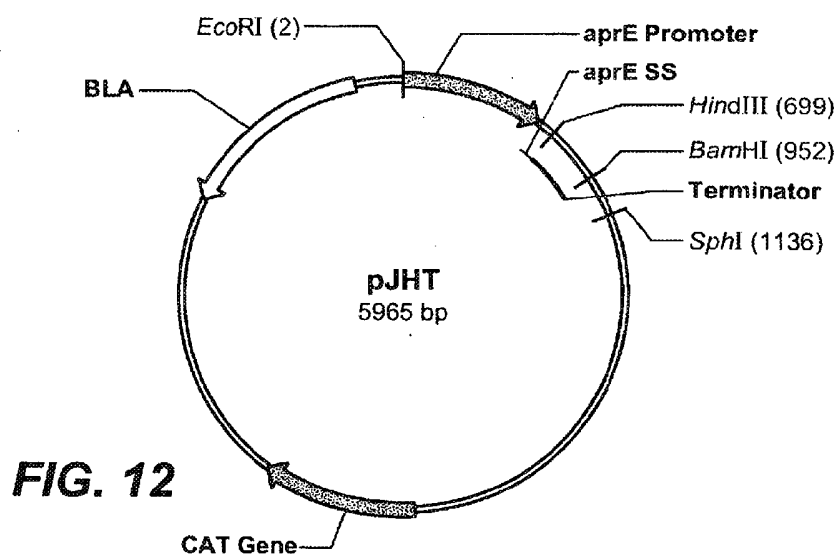
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FIG. 5









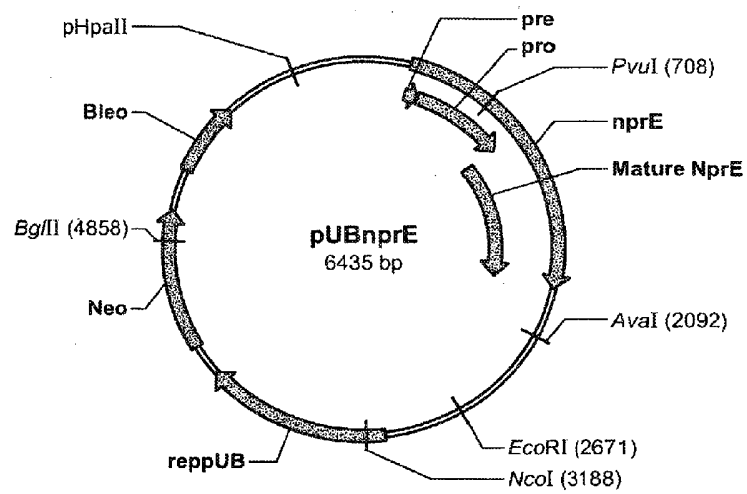


FIG. 14

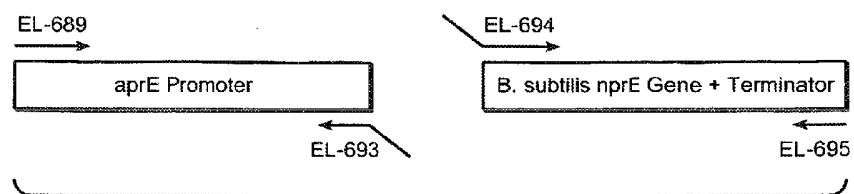


FIG. 15

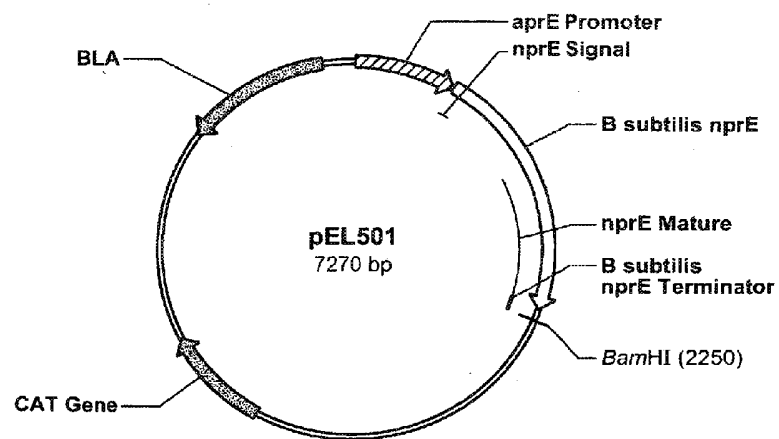


FIG. 16

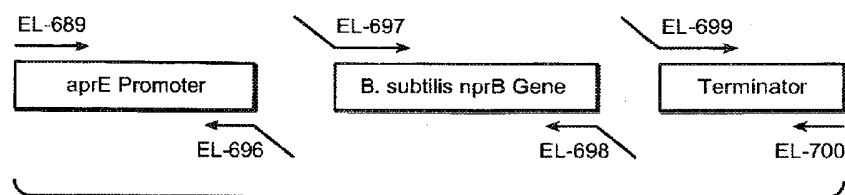
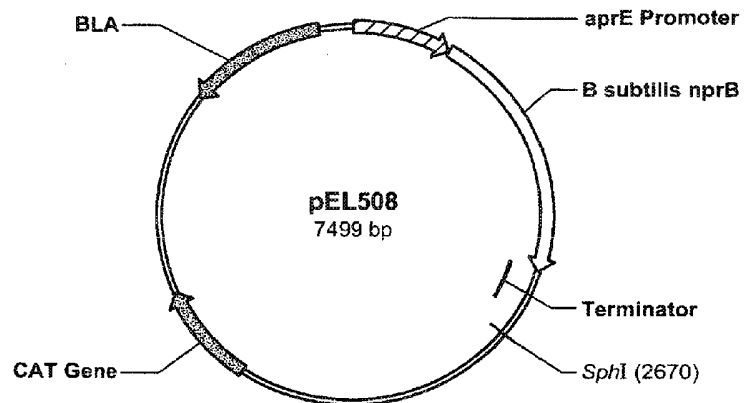
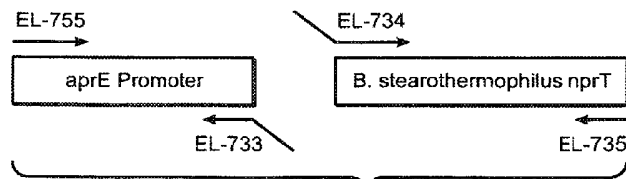
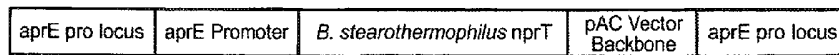
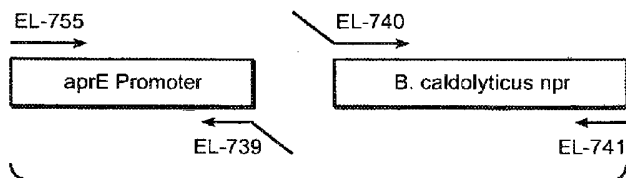
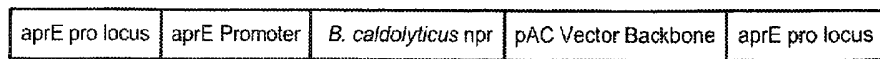
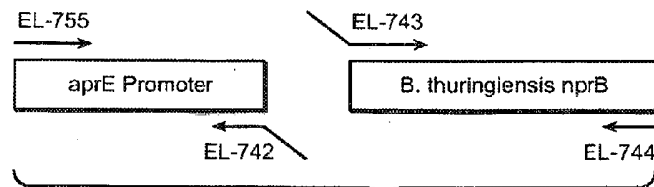
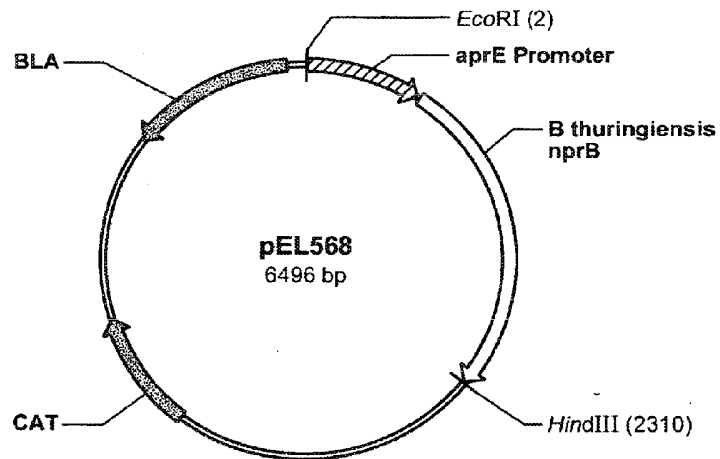


FIG. 17

**FIG. 18****FIG. 19****FIG. 20****FIG. 21**

**FIG. 22****FIG. 23****FIG. 24**

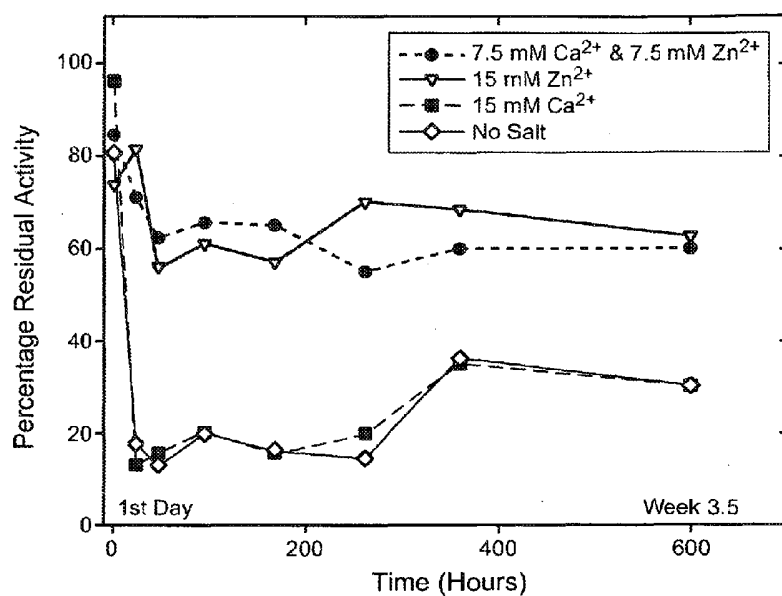


FIG. 25

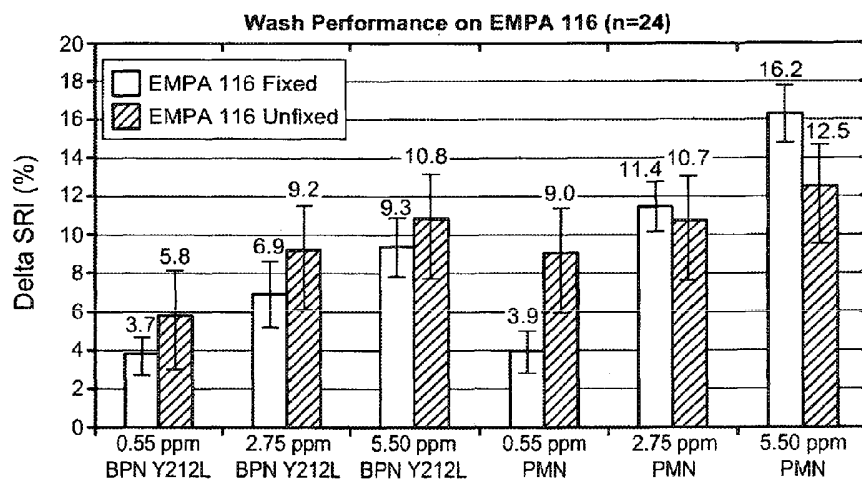
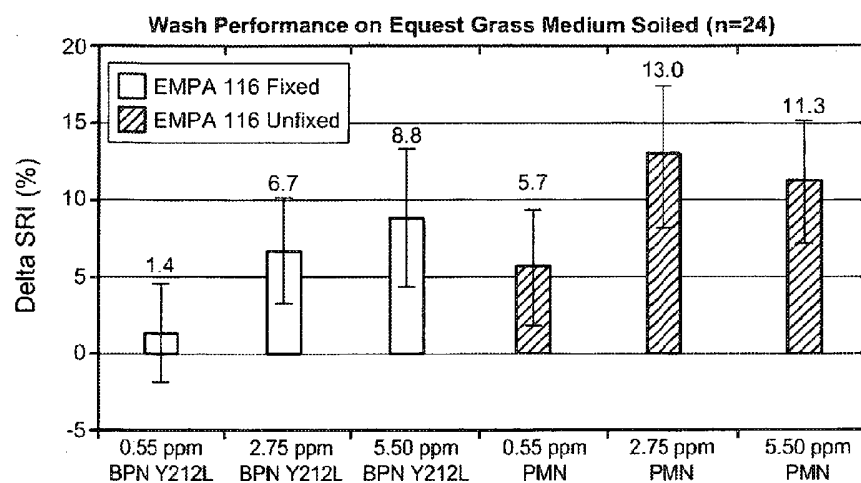
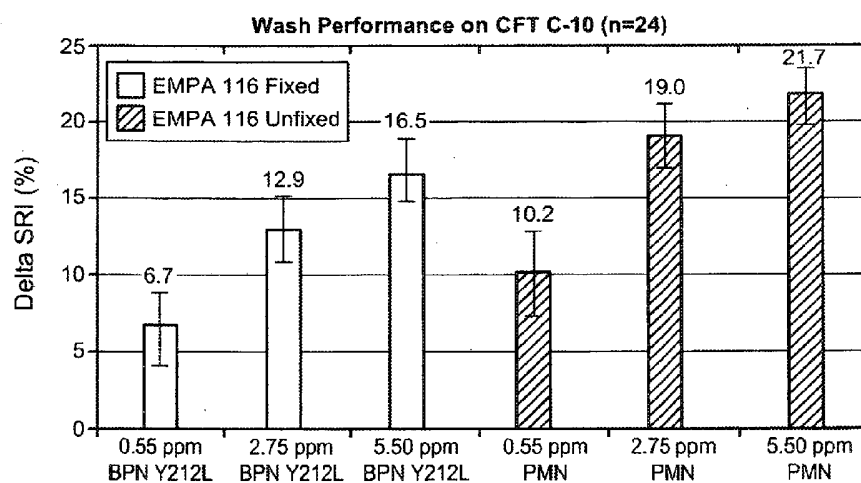
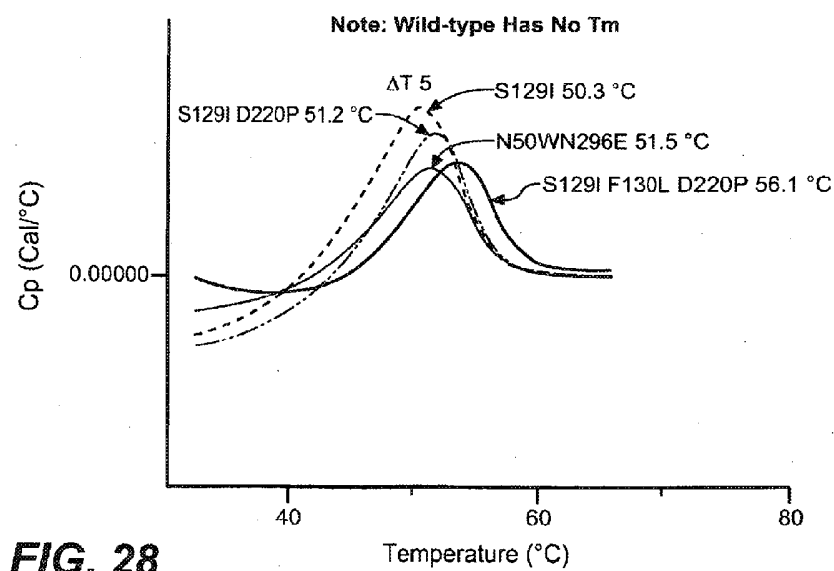
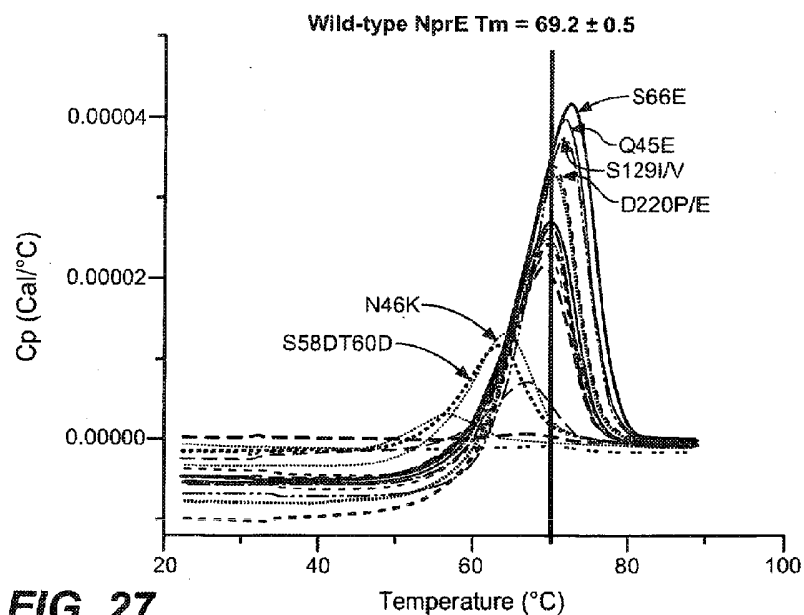


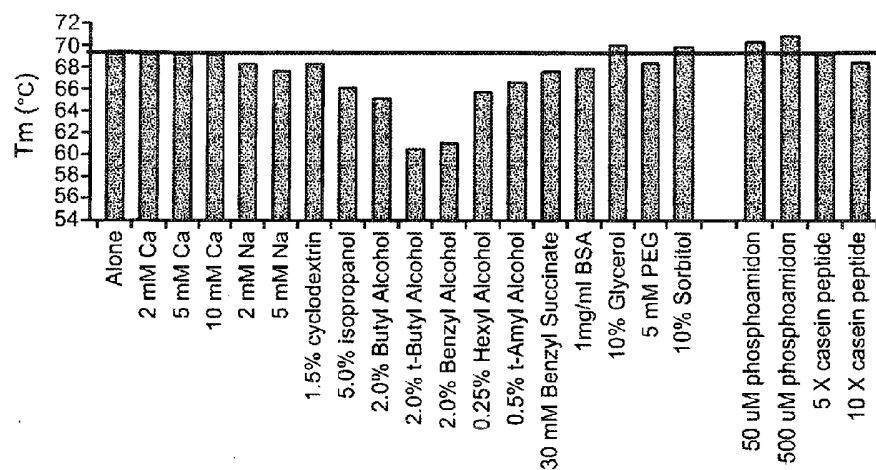
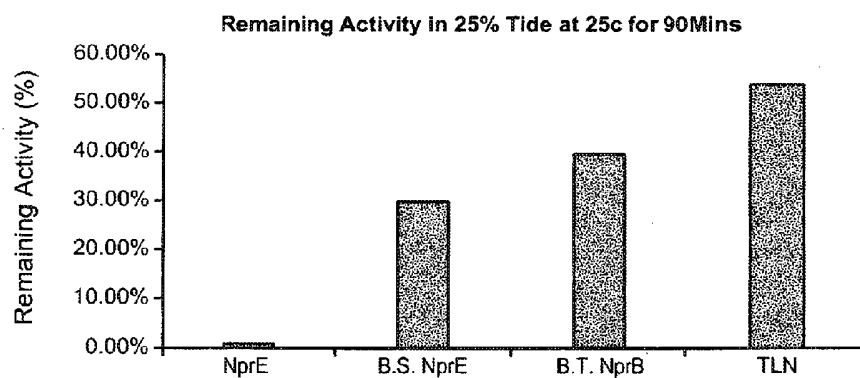
FIG. 26A

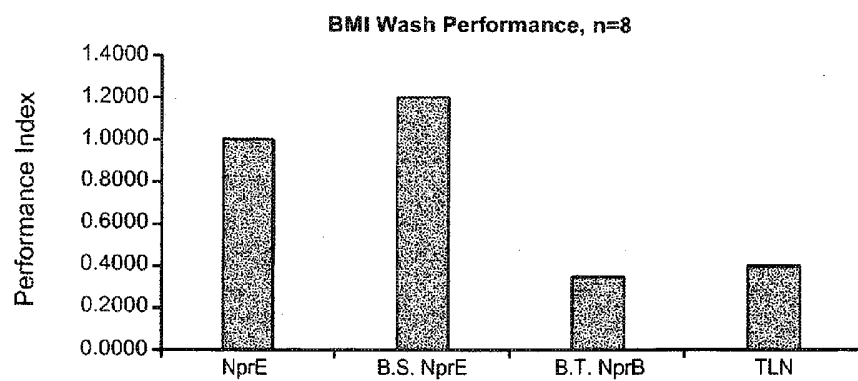
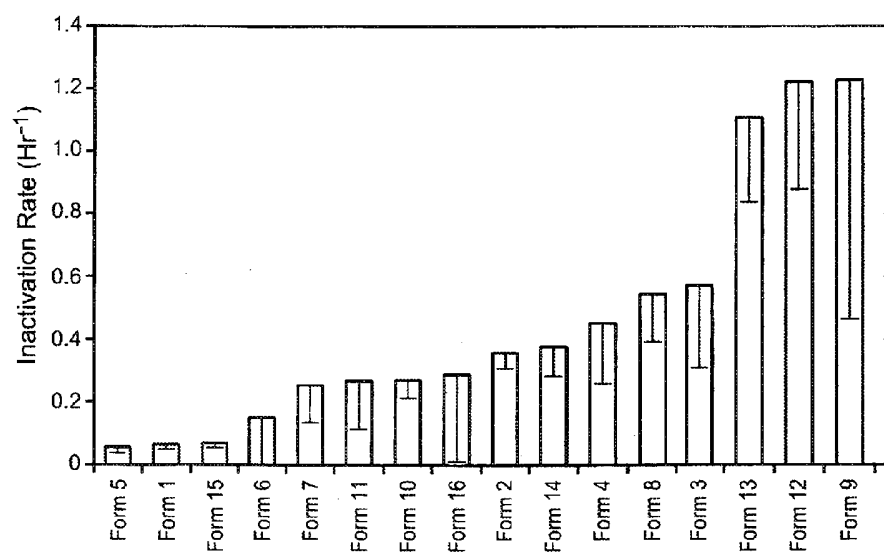
**FIG. 26B****FIG. 26C**

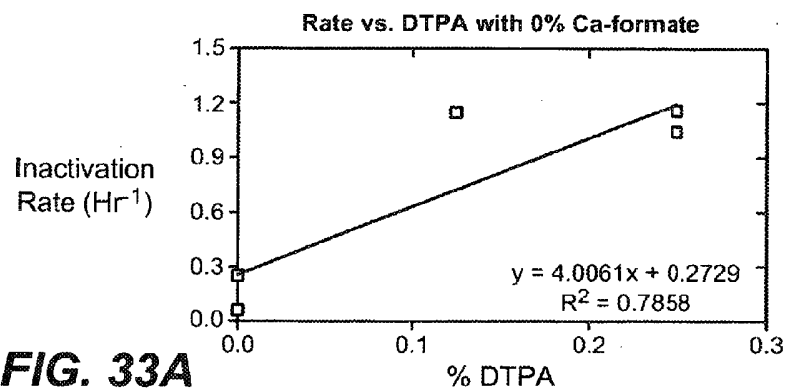
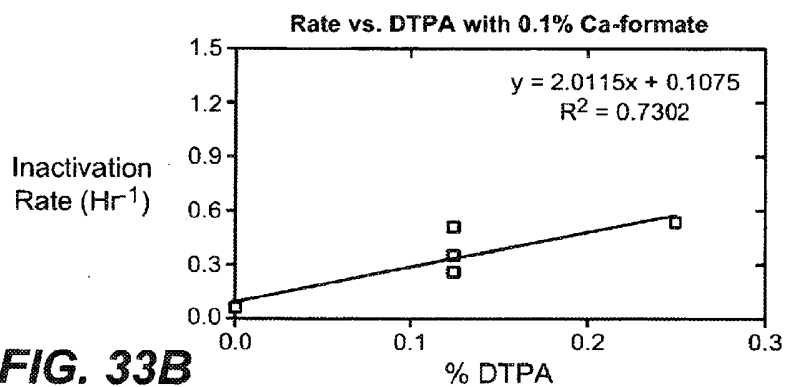
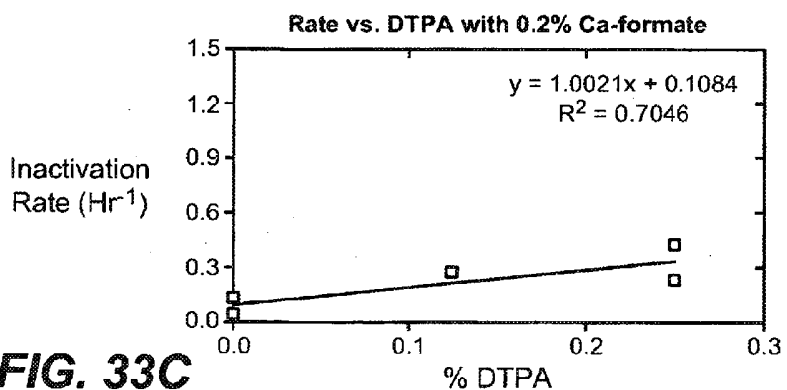
- 236 -



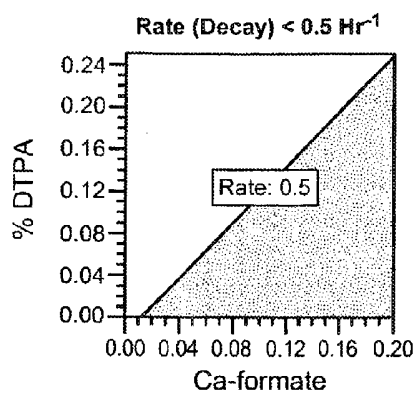
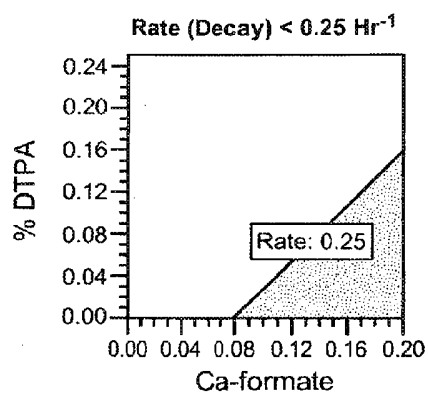
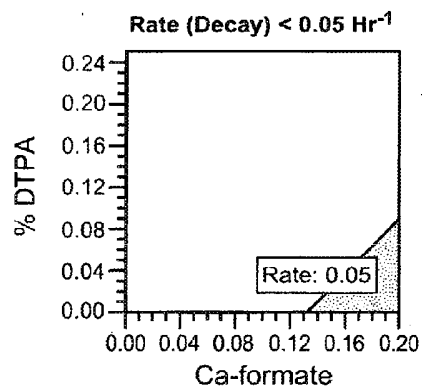
- 237 -

**FIG. 29****FIG. 30**

**FIG. 31****FIG. 32**

**FIG. 33A****FIG. 33B****FIG. 33C**

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**FIG. 34A****FIG. 34B****FIG. 34C**

Fragment 1:

AATTGTGTTLKGKTVSLNISSESGKYVLRDLSKPTGTQIITYDLQNREYNLPGLTVSSTTNQFTT
 SSQRAAVDAHYNLGKVYDYFYQKFNRNSYDNKGGKIVSSVHYGSRYNNAAWIGDQMIYGDGDGGSF
 FSPLSGSMDVTAHEMTHGVTQETANLNYENQPGALNESFSDFVFGYENDTEDWDIGEDITVSQPAL
 RLSNPTKYGQPDNFKNYKNLPNT (SEQ ID NO:222)

Fragment 2 (Frayed):

DAGDYGGVHTNSGIPNKAAYNTITKIGVNKAEQIYYRALTVYLTPTSSTFKDAKAALIQSARDLYG
 SQDAASVEAAWNAVGL (SEQ ID NO:223)

Fragment 3:

AATTGTGTTLKGKTVSLNISSESGKYVLRDLSKPTGTQIITYDLQNREYNLPGLTVSSTTNQFTT
 SSQRAAVDAHYNLGKVYDYFYQKFNRNSYDNKGGKIVSSVHYGSRYNNAAWIGDQMIYGDGDGGSF
 FSPLSGSMDVTAHEMTHGVTQETANLNYENQPGALNESFSDFVFGYENDTEDWDIGEDITVSQPALRS
 (SEQ ID NO:224)

Fragment 4 (Deduced based on size):

AATTGTGTTLKGKTVSLNISSESGKYVLRDLSKPTGTQIITYDLQNREYNLPGLTVSSTTNQFTT
 SSQRAAVDAHYNLGKVYDYFYQKFNRNSYDNKGGKIVSSVHYGSRYNNAAWIGDQMIYGDGDGGSF
 FSPLSGSMDV (SEQ ID NO:225)

Fragment 5:

LSNPTKYGQPDNFKNYKNLPNTDAGDYGGVHTNSGIPNKAAYNTITKIGVNKAEQIYYRALTVYL
 TPSSTFKDAKAALIQSARDLYGSQDAASVEAAWNAVGL (SEQ ID NO:226)

FIG. 35