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**Trueheart et al.**

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(54) **USE OF THERMOPHILIC NUCLEASES FOR DEGRADING NUCLEIC ACIDS**

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(51) **Int. Cl.**

**C12P 7/22** (2006.01)

**C12N 9/22** (2006.01)

**C12Q 1/68** (2018.01)

**C12N 1/08** (2006.01)

**C12P 23/00** (2006.01)

(52) **U.S. Cl.**

CPC ..... **C12N 1/08** (2013.01); **C12N 9/22** (2013.01); **C12P 23/00** (2013.01); **C12Y 301/21004** (2013.01)

(58) **Field of Classification Search**

CPC ..... **C12N 9/22**; **C12Q 1/683**

See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to the use of a thermophilic nuclease for degrading nucleic acids in vivo and/or in situ, wherein the thermophilic nuclease is heterologous to the host cell and is produced by the host rather than being added exogenously. The present invention further relates to a genetically modified cell which was produced according to the above method. The present invention is particularly beneficial in inactivating the biological activity of recombinant DNA in biomass or biomass-derived products.

**37 Claims, 10 Drawing Sheets**

**Specification includes a Sequence Listing.**

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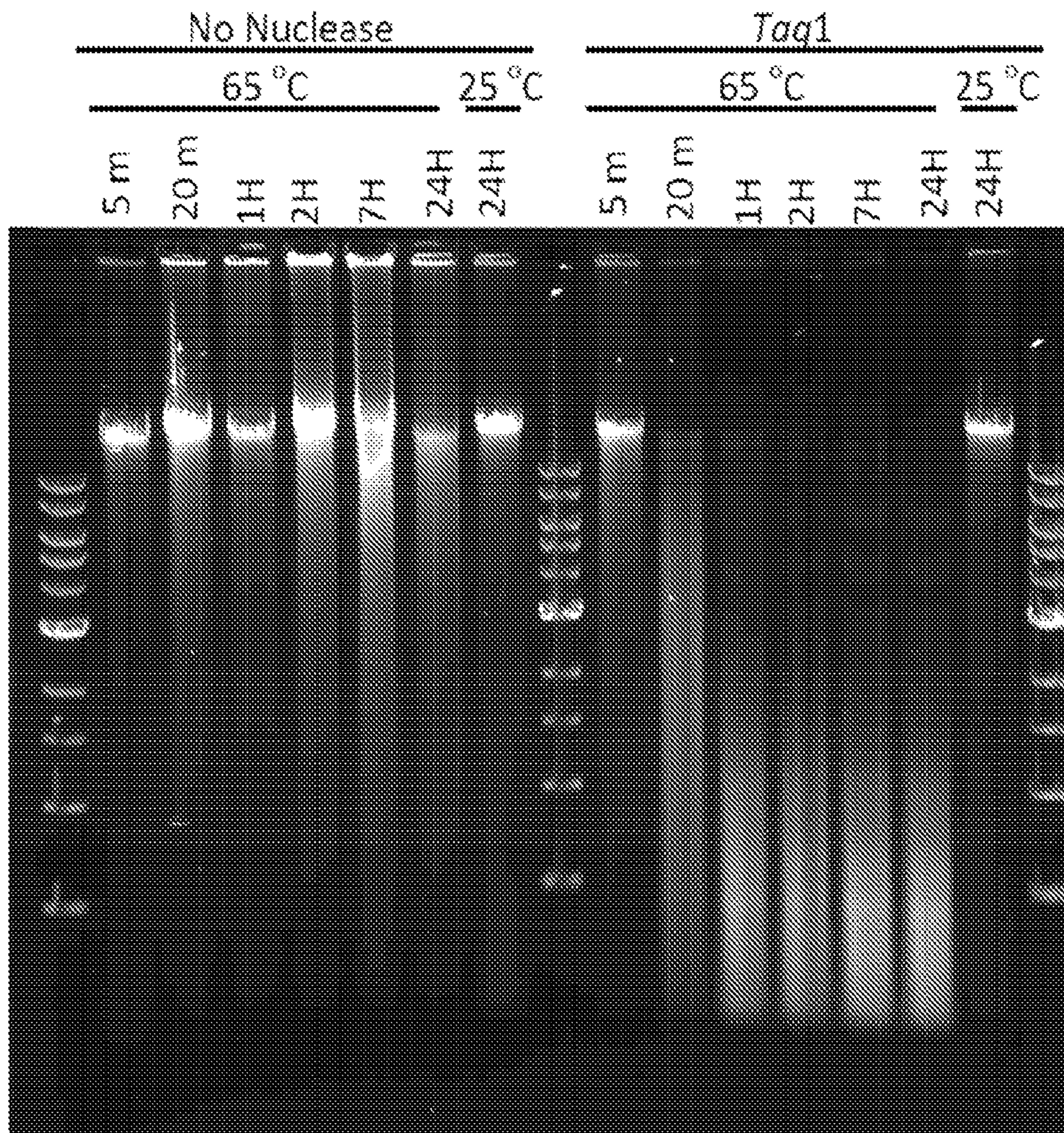


Figure 1.



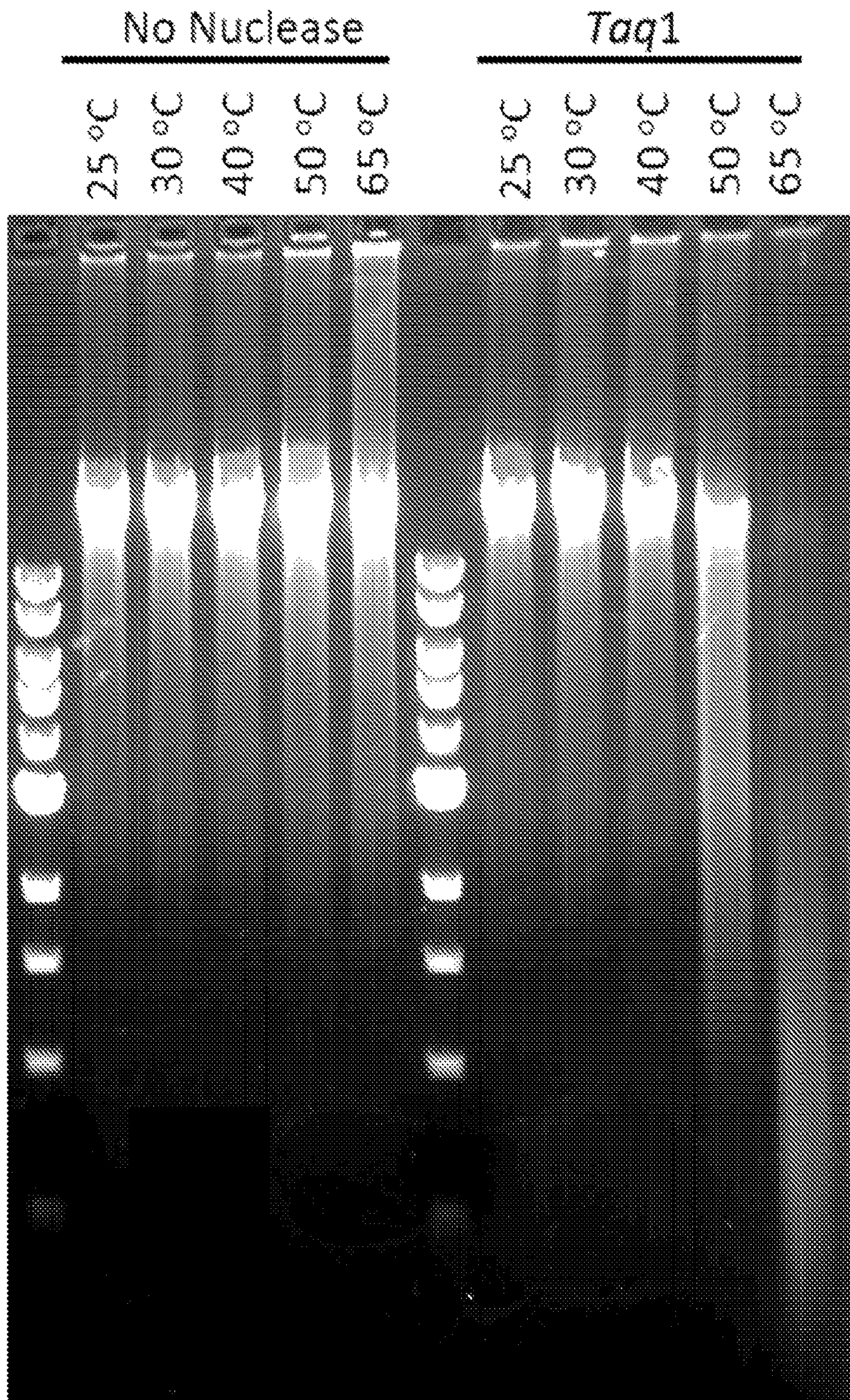


Figure 2.



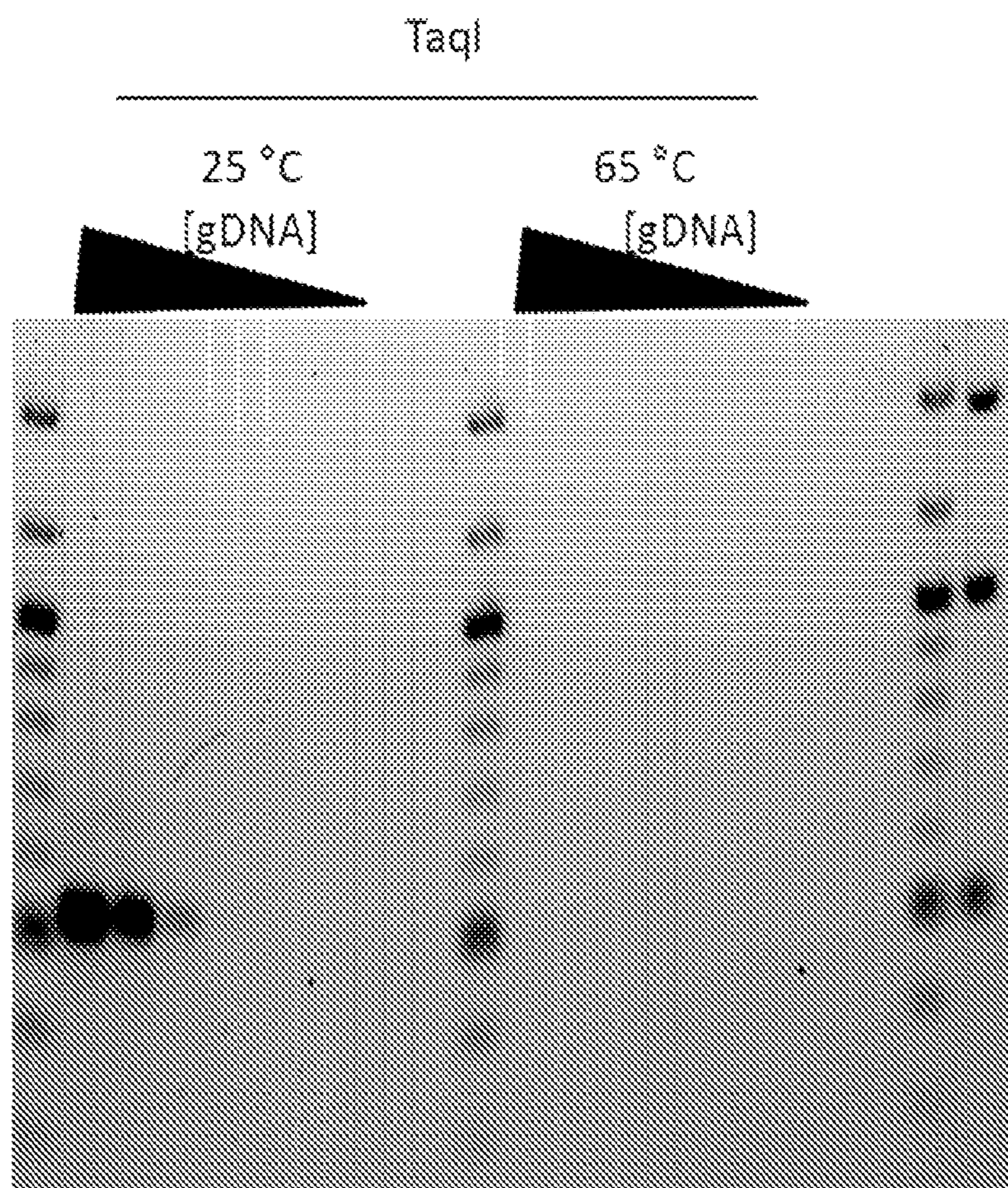


Figure 3.

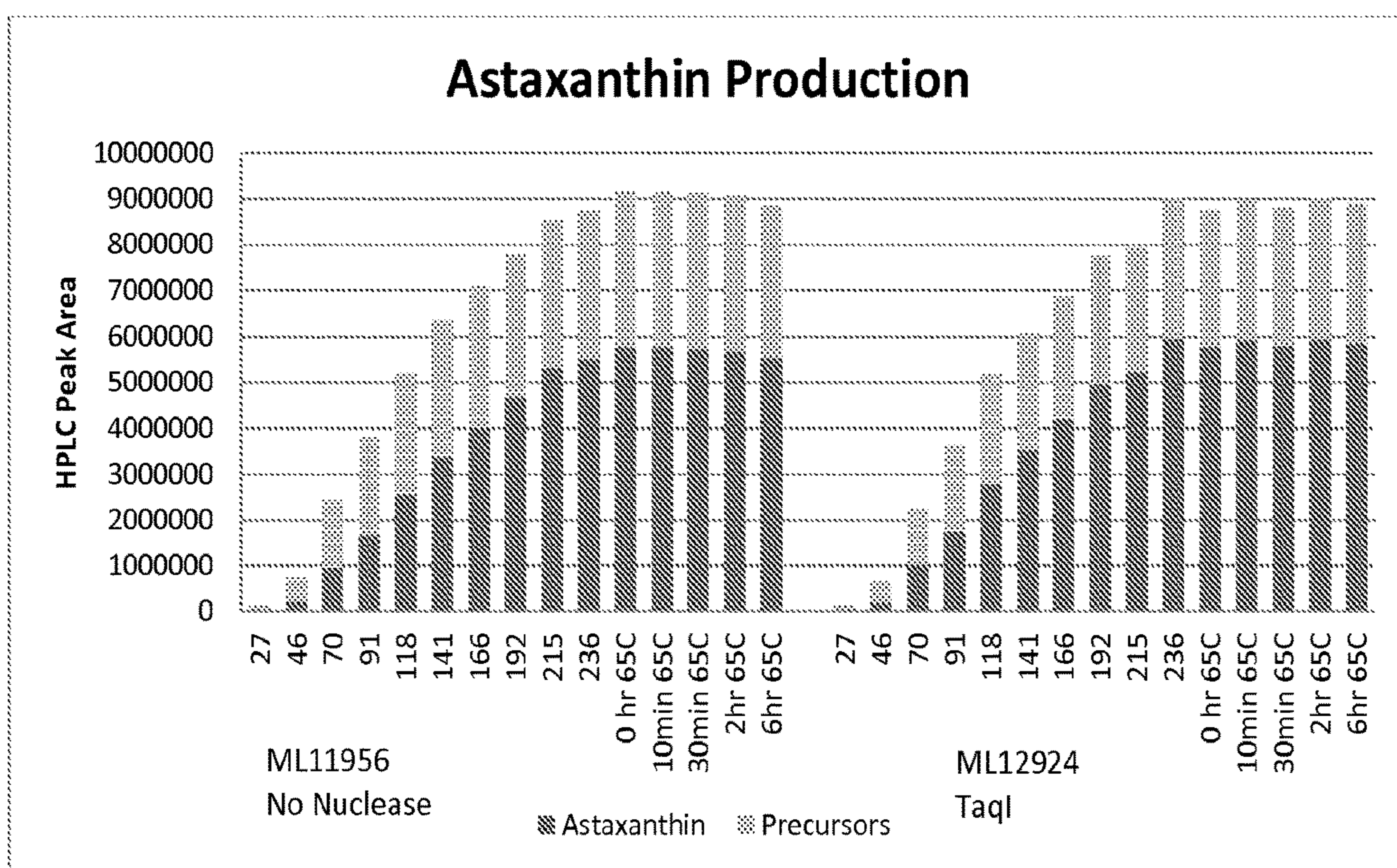


Figure 4.



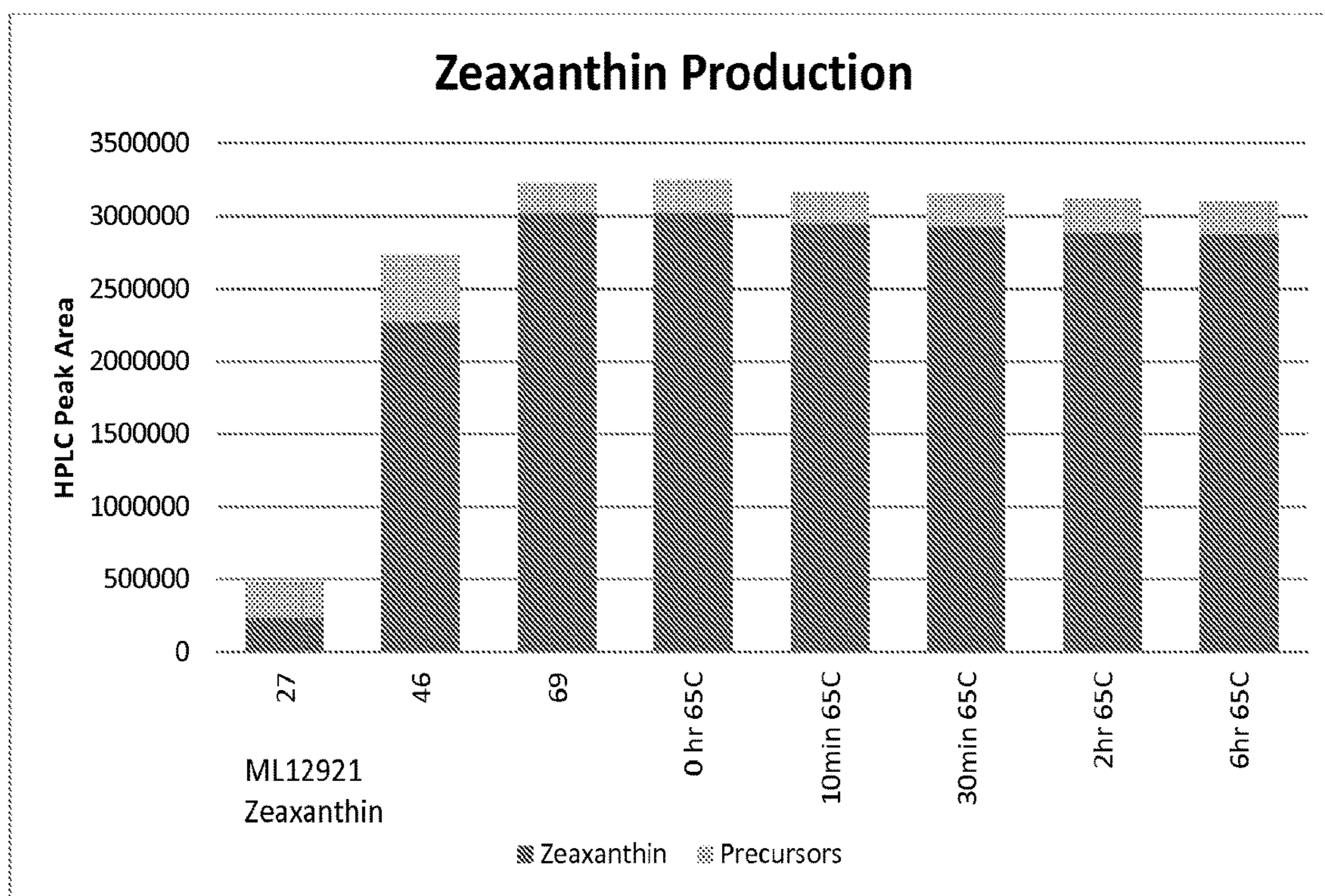


Figure 5.



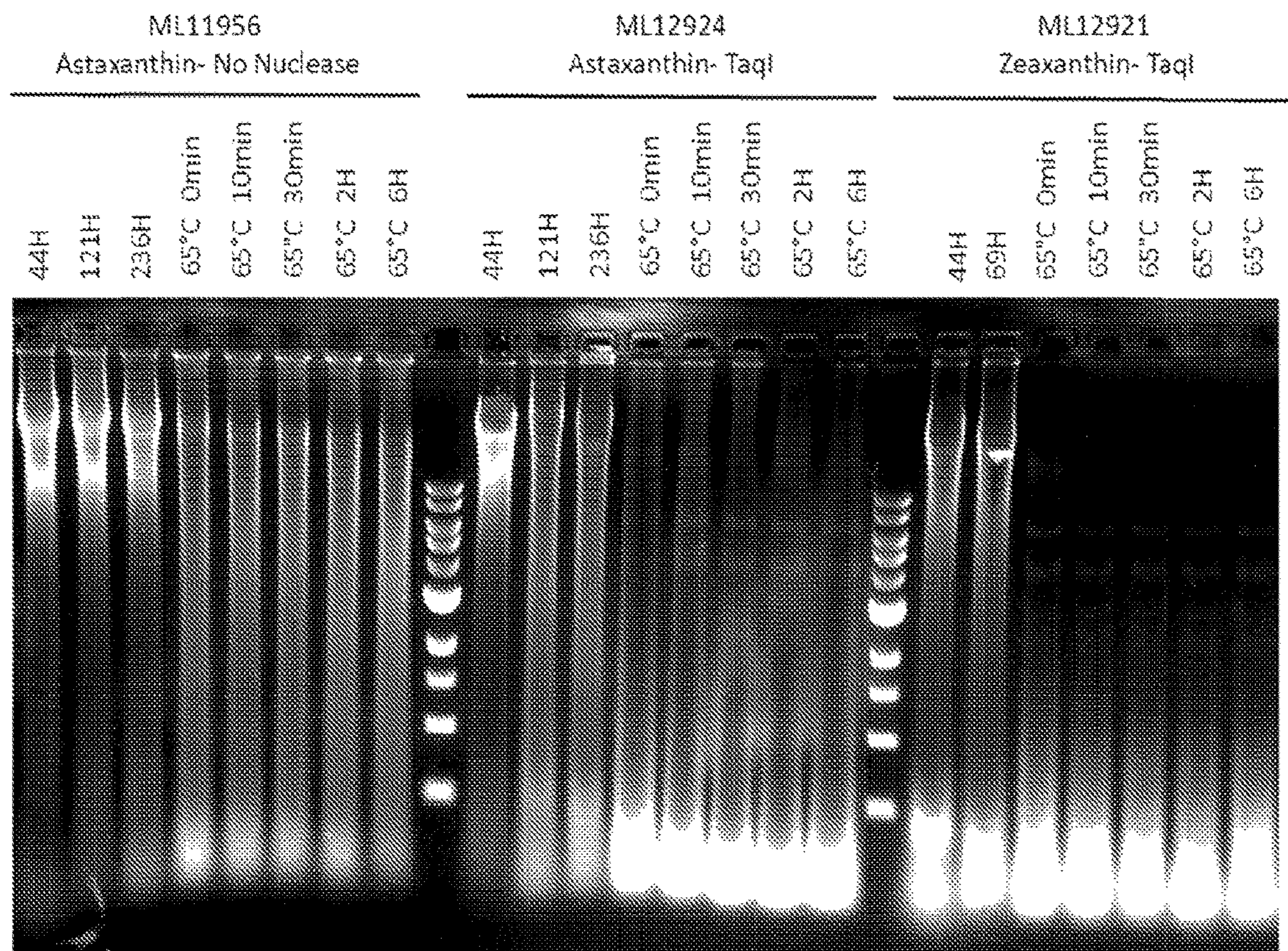


Figure 6.



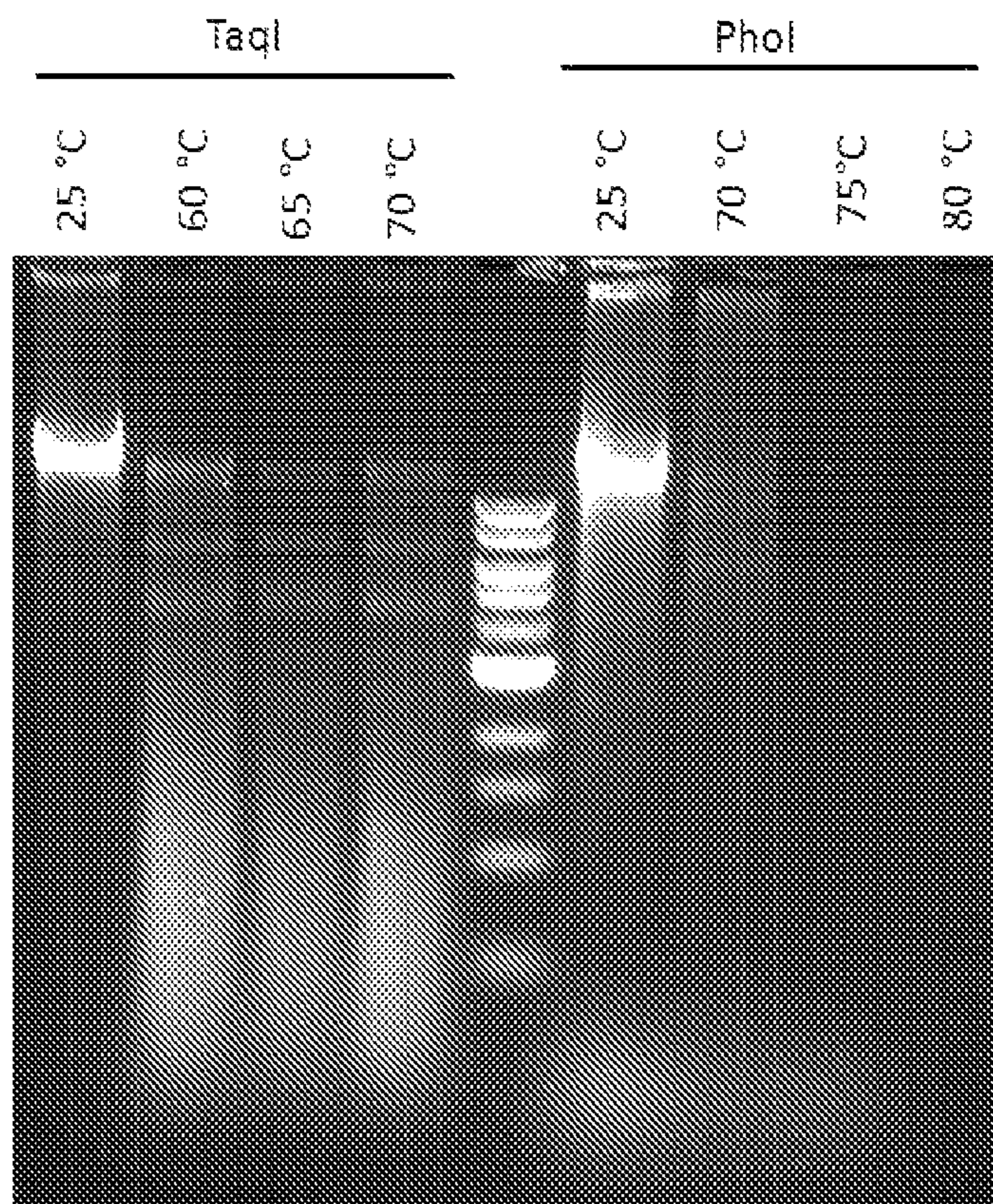


Figure 7.



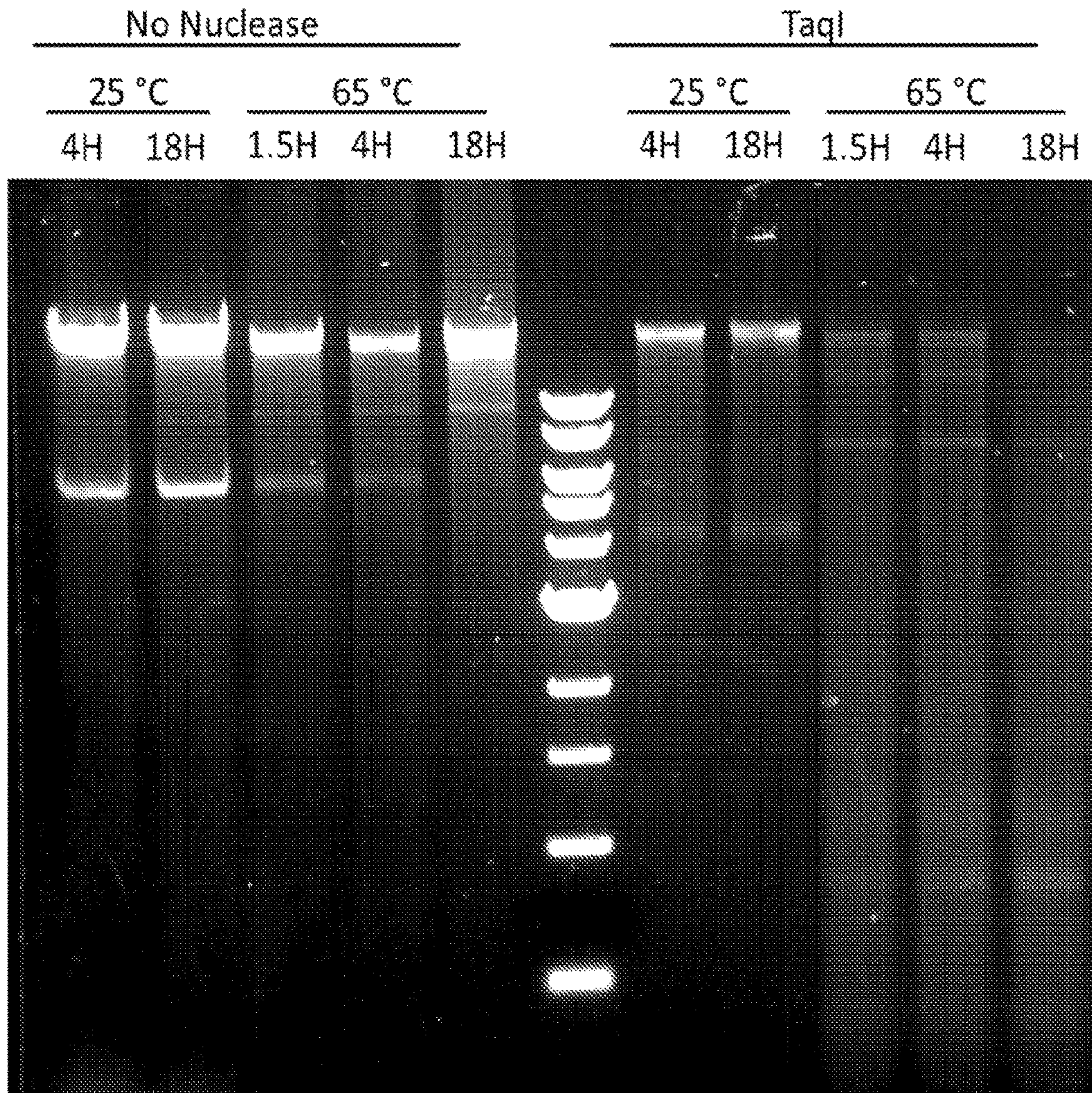


Figure 8.







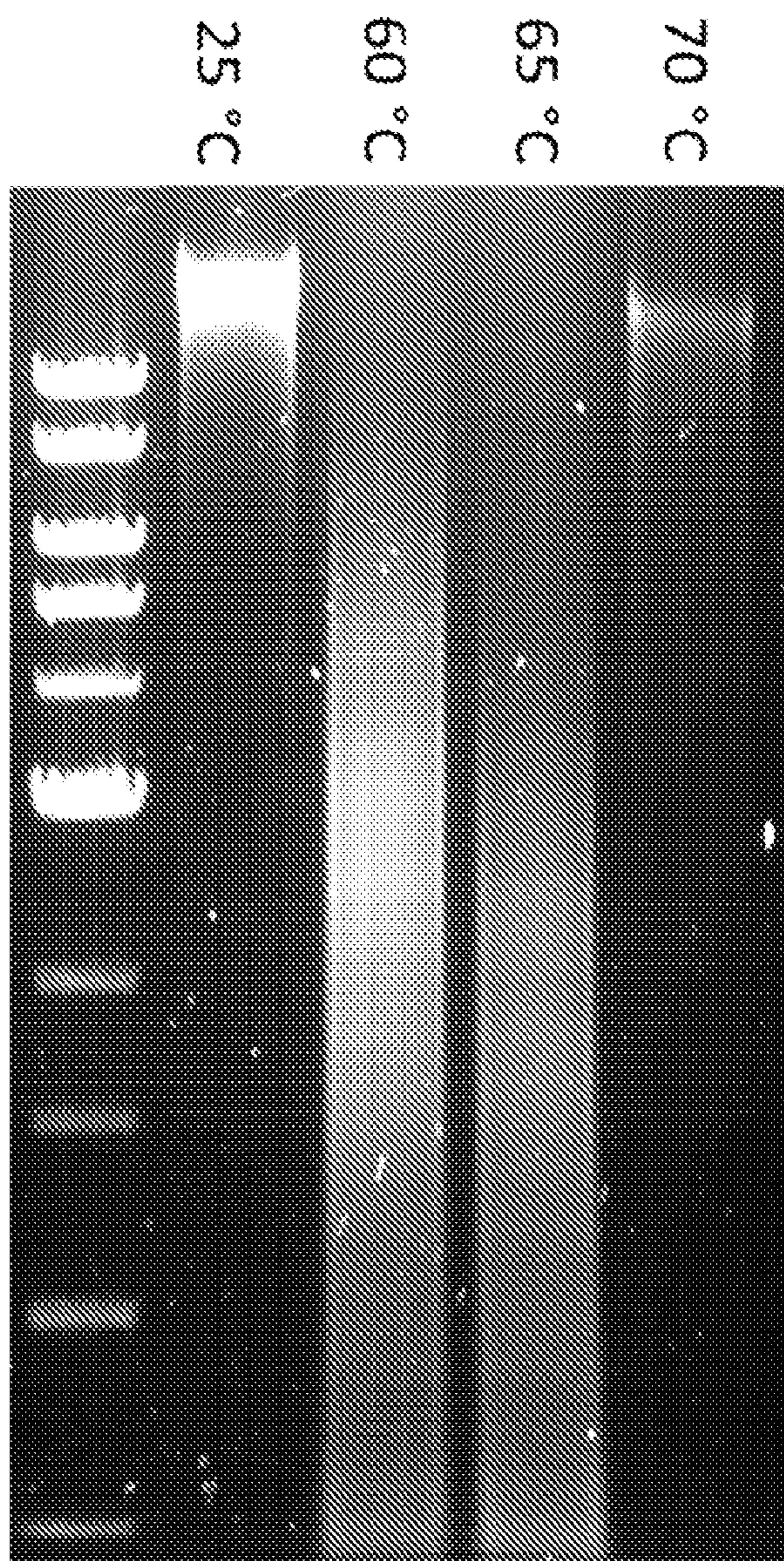


Figure 10.



## USE OF THERMOPHILIC NUCLEASES FOR DEGRADING NUCLEIC ACIDS

This application is a 371 of International Patent Application No. PCT/US2014/026379 filed Mar. 13, 2014, which claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application No. 61/928,080 filed Jan. 16, 2014, and to U.S. Provisional Patent Application Ser. No. 61/794,400 filed Mar. 15, 2013, the entire contents of each of which are hereby incorporated by reference.

### FIELD OF THE INVENTION

This invention relates to a method for degrading the nucleic acids of a host cell in vivo and/or in situ, in particular when the host cell comprises a recombinant DNA, using a heterologous thermophilic nuclease. The present invention is beneficial in inactivating the biological activity of recombinant DNA in a biomass. The inactivation of the biological activity of recombinant DNA helps to prevent active recombinant DNA molecules from remaining in the end product isolated from the biomass or in the biomass itself. In addition, the inactivation of the biological activity of recombinant DNA helps to prevent active recombinant DNA molecules from being released into the environment.

### BACKGROUND OF THE INVENTION

Biotechnological production processes are increasingly employed to obtain biological compounds and fine chemicals. The progress of molecular biology techniques makes possible the mass production of a wide variety of biological compounds and fine chemicals such as proteins, antibodies, polysaccharides, antibiotics, amino acids, vitamins, alcohols, etc. Often, in order to increase the efficiency of the production, the gene(s) producing the desired end product(s) is (are) genetically modified and/or introduced into a heterologous organism. The production of the desired end product(s) then takes place in fermenters controlled by modern control techniques. The end products are either the cells themselves, extracted from the cells, or collected from the cell culture broth if the compounds are found therein (either by active or passive secretion processes or cell lysis).

It is common to see that the biomass produced at the end of the fermentation process contains the desired end product(s) but also active DNA molecules. Often, the active DNA molecules are recombinant DNAs. If left untreated, the recombinant DNA molecules could remain in the isolated end product and could also be released into the environment. There have been great concerns by the general public about the possible adverse effect of the remnant recombinant DNA materials in crops and food products on human health. In addition, concerns about the potential impact of the recombinant DNA on the environment have caused the authorities and institutions in most countries to issue statutory requirements and regulations calling for inactivation of the waste materials produced from the fermentation process before being released into the environment.

There are several ways to inactivate nucleic acids. For example, nucleic acids can be inactivated physically, and most commonly, by heat. U.S. Pat. No. 5,417,862 reports a method of inactivating the biological activity of DNA by heating the DNA to 60-100° C. in the presence of an acid. A similar method of degrading DNA by a combination of heat and acid is reported in U.S. Pat. No. 5,118,603. The heating methods require considerable amounts of energy, which add to the costs of the end product in large scale

fermentation processes. Furthermore, depending on the nature of the end product, the harsh heat (and/or acidic) conditions could be detrimental to the integrity and activity of said product.

The nucleic acids can also be inactivated by other physical means. U.S. Pat. No. 6,165,711 reports a method using laser beams for disintegrating nucleic acids in a biologically active proteinaceous material. As another example, a method for inactivating microorganisms using high-intensity pulsed polychromatic light is reported in U.S. patent application Ser. No. 09/818,256, now abandoned. This method requires complicated light-generating devices to be used and constantly maintained, and thus is cost-intensive. The light beams may disintegrate not only nucleic acids, but also other biological substances or active compounds which will cause them to lose their desired properties.

The nucleic acids can also be inactivated chemically by acids or alkali. For example, in U.S. Pat. No. 7,435,567, a method using hypochlorous acids for induction of autodigestion of nucleic acids in a microorganism is reported. U.S. Pat. Nos. 5,417,862 and 5,118,603 described above use other types of acid for the degradation of nucleic acids. While these methods cause the disruption of nucleic acids, the acid and alkali conditions are severe. The severe conditions may cause the unwanted denaturation of certain biological compounds such as proteins. The amount of the acid and alkali used can be relatively large, which makes the method disadvantageous from the industrial production viewpoint as well.

Attempts have been made to inactivate nucleic acids of an organism enzymatically, such as by using a nuclease. U.S. patent application Ser. No. 13/127,825 reports a method for degrading host cell nucleic acids associated with vaccine production, where the method comprises a step of nucleic acid degradation by adding purified nuclease into the cell culture. In U.S. patent application Ser. No. 10/607,903, the construction of a transgenic bacterial strain expressing a heterologous nuclease gene in an amount effective to degrade nucleic acids is reported. While the method of adding nuclease in vitro such as the one reported in U.S. patent application Ser. No. 13/127,825 causes disruption of nucleic acids, the cost is high since large amounts of nuclease are required, and the efficiency of degradation is low because the cell wall of the host cell blocks the access to nucleic acids by the nuclease when added in vitro. The transgenic approach such as the one reported in U.S. patent application Ser. No. 10/607,903 allows the nuclease to be co-expressed with the host cell and thus gain access to the host nucleic acids in vivo. However, co-expressing a nuclease in a cell without any protection mechanism, such as methylation, will significantly stress the cell, resulting in weakened cell growth and reduced production of end product.

The problem underlying the present invention is therefore to provide a cost-effective way to degrade the nucleic acids of a host cell that produces biological compounds and fine chemicals, especially on an industrial production scale. A further problem underlying the present invention is to provide a method in which the nucleic acid degradation process is controllable and does not impede the production of the desired end product(s) in the host cell. The above problems are solved according to the invention by the subject matter of the present claims.

### SUMMARY OF THE INVENTION

We have now surprisingly found that thermophilic nucleases can be used for degrading nucleic acids in vivo and/or



in situ in a controlled manner. This invention relates to a novel method for degrading nucleic acids of a host organism where the host organism is modified or transformed with one or more heterologous thermophilic nuclease genes. The thermophilic nucleases disclosed in this invention are latent at temperatures at which the cell culture is normally grown to produce the desired product, but can be selectively activated at a higher temperature and thus degrade the nucleic acids of the host organism, and/or the recombinant nucleic acids integrated in the host organism, once activated. The activation can be triggered at any desired time point, such as the harvest time of the cell culture when product formation is already complete. In addition to easy control, the application of this inventive process has the benefit of acting in vivo and/or in situ and thus improving the efficiency of the enzymatic reaction and avoids the drawbacks and problems of the existing physical or chemical methods. It has thus been made possible, by application of the disclosed invention, to obtain biological compounds and fine chemicals in the form of intact cells where the active nucleic acids content of the cell, especially recombinant DNAs, is degraded and inactivated.

The practice of this invention is broadly applicable to both microorganisms and higher eukaryotic cell cultures, and particularly industrial strains used in a large scale production of commercial end products.

One aspect of the invention relates to a method for degrading the nucleic acids of a host cell in vivo and/or in situ, wherein the host cell comprises a heterologous nucleic acid sequence encoding a thermophilic nuclease gene, wherein the method comprises: a) growing the host cell at a temperature at which the thermophilic nuclease is latent; and b) degrading the nucleic acids of the host cell by changing the temperature in step (a) to a temperature at which the thermophilic nuclease is active.

The present invention also provides a genetically modified cell which comprises heterologous nucleic acid sequences encoding one or more of the thermophilic nuclease genes described above. In one embodiment, the genetically modified cell produces one or more end products, and the end products produced by the genetically modified cell are not the above thermophilic nucleases. In one embodiment, the genetically modified cell is created by introducing one or more heterologous thermophilic nuclease genes described above into a host cell. This host cell can be either a cell which has not been genetically modified, or a cell which has previously been genetically modified. This host cell, before being modified with the one or more heterologous thermophilic nuclease genes, produces one or more end products wherein said one or more end products are not the thermophilic nucleases. In another embodiment, the above mentioned host cell may be modified first with one or more thermophilic nuclease genes to create a genetically modified cell, and the genetically modified cell is subsequently genetically engineered to produce one or more other end products.

Another aspect of the invention relates to the use a thermophilic nuclease for degrading the nucleic acids of a host cell in vivo and/or in situ, wherein the thermophilic nuclease is heterologous to the host cell.

Yet another aspect of the invention relates to process for the production of a biomass product which is free of active nucleic acid molecules, wherein the biomass product is the product of a host cell comprising a heterologous nucleic acid sequence encoding a thermophilic nuclease gene, wherein the process comprises: a) fermenting the host cell at a temperature at which the thermophilic nuclease is latent; b)

degrading the nucleic acids of the cell by changing the temperature in step (a) to a temperature at which the thermophilic nuclease is active; and c) recovering the biomass product, wherein the order for performing steps b) and step c) may be exchanged.

In one embodiment, the nuclease mentioned in the above aspects of invention is a DNA-degrading nuclease and the nucleic acids of the host cell are DNA. In one specific embodiment, the DNA of the host cell contains recombinant DNA. In another embodiment, the nuclease is a RNA-degrading nuclease and the nucleic acids the host cell are RNA.

In one embodiment, the temperature in step (a) of the above process invention and the method invention is optimal for the growth of the host cell. In one embodiment, the degradation is conducted at a temperature that is within about  $\pm 5^\circ$  C. of the optimum temperature of said thermophilic nuclease. In a specific embodiment, the degradation is conducted at the optimum temperature of said thermophilic nuclease. In another embodiment, the degradation is conducted during the pasteurization of the host cell. In another embodiment, the degradation is conducted in a temperature range between  $40^\circ$  C. and  $100^\circ$  C.

In one embodiment, the DNA-degrading nuclease is TaqI nuclease. In one embodiment, the degradation is conducted at a temperature ranging between about  $60^\circ$  C. and about  $70^\circ$  C. In a specific embodiment, the degradation is conducted at  $65^\circ$  C. In one embodiment, the TaqI nuclease comprises a polypeptide having an amino acid sequence of SEQ ID NO:3. In another embodiment, the gene of TaqI nuclease comprises a nucleic acid sequence encoding a polypeptide having an amino acid sequence of SEQ ID NO:3.

In one embodiment, the DNA-degrading nuclease is PhoI nuclease. In one embodiment, the degradation is conducted at a temperature ranging between about  $70^\circ$  C. and about  $80^\circ$  C. In a specific embodiment, the degradation is conducted at  $75^\circ$  C. In one embodiment, the PhoI nuclease comprises a polypeptide having an amino acid sequence of SEQ ID NO:10. In another embodiment, the gene of PhoI nuclease comprises a nucleic acid sequence encoding a polypeptide having an amino acid sequence of SEQ ID NO:10.

In one embodiment, the host cell is from a plant. In another embodiment, the host cell is from an animal. In yet another embodiment, the host cell is from a microorganism. In one embodiment, the microorganism is selected from a group consisting of: yeast, fungi, algae, bacteria, and archaea. In another embodiment, the microorganism is selected from a group consisting of: *Yarrowia*, *Bacillus*, *Escherichia*, *Pseudomonas*, *Paracoccus*, *Corynebacterium*, *Candida*, *Hansenula*, *Saccharomyces*, *Mortierella*, *Schizosaccharomyces*, *Aspergillus*, *Fusarium*, *Trichoderma*, *Cryptocodium*, *Schizochytrium*, and *Thraustochytrium*. In a specific embodiment, the *Yarrowia* species is *Yarrowia lipolytica*.

In one embodiment, the host cell produces one or more end products that is not the thermophilic nuclease. In one embodiment, the end product is selected from the group consisting of: phytoene, lycopene, beta-carotene, alpha-carotene, beta-cryptoxanthin, lutein, zeaxanthin, astaxanthin, canthaxanthin, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, adonirubin, violaxanthin, adonixanthin, ubiquinone, vitamin K, vitamin E, retinol, retinal, retinoic acid, retinol palmitate, and modified forms thereof.

In one embodiment, the gene of the thermophilic nuclease is codon optimized to match the codon usage bias of the host cell. In one embodiment, the codon optimized thermophilic nuclease gene comprises the nucleic acid sequence of SEQ



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ID NO:1. In another embodiment, the codon optimized thermophilic nuclease gene comprises the nucleic acid sequence of SEQ ID NO:8.

In one embodiment, more than one thermophilic nuclease gene is introduced into the host cell. In another embodiment, the recombinant DNA is optimized to include additional sites of the thermophilic nuclease. In one embodiment, the recombinant DNA is optimized to include one cleavage site every 500 or fewer nucleotides.

In one embodiment, the heterologous thermophilic nuclease genes used in the present invention do not affect the production efficiency of the end product of the host cell.

## LIST OF SEQUENCES

The nucleic acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviation for nucleotide bases. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand. In the accompanying sequence listing:

SEQ ID NO:1 is the DNA sequence encoding TaqI nuclease from *Thermus aquaticus*, as optimized for expression in *Yarrowia lipolytica*, and includes restriction sites for cloning and a 5' ribosomal binding site.

SEQ ID NO:1:

gctagccacaaaaatggcttccactcaggctcagaaggctcttgagact  
 ttcgagcgatttctcgcttctcttgacctggagctctaccagcagaagt  
 accgacctcaagactgttgagcaggaccttccccgagagctgaaccc  
 ccttcccagacctgtacgagcactactggaaggcccttgaggataacccc  
 tccttccctgggcttcgaggagttctttgacctggtgggagaagcgac  
 ttcgacccctcgacgagttcattcgaaagtacttttggggttgctccta  
 cgctttgttcgacttgcccttgaggctcgactgtaccgaactgcccgtt  
 tccatctggactcagtttcaactctgctaccgatggaacgcctcctgcg  
 agcttccccttgaggccgccccgagcttgacgcccagggcattgacgc  
 cctgattcacacttccggttccctctaccggaatccagatcaagaaggag  
 acttaccgatccgaggccaagtccgagaaccgatttctccgaaagcagc  
 gaggcaccgcccctcatcgagattccctacaccttcagactcccagagga  
 gcttgaggagaaggccaagcgagcccaggttaacggagagacttaccga  
 ctttgggccaagggttgctcaccaccttgaccgacttgagaacggttttg  
 tcattttccgagagctctacgttaagtctattgagcttttccctccagaa  
 gaacgctcccaccctttctggactcattcgatgggaccgagttgctcag  
 gaggccctcaccgccccctaaacgcgt

SEQ ID NO:2 is the non-optimized DNA sequence encoding TaqI nuclease, from *Thermus aquaticus*.

SEQ ID NO:2:

atggcttccacacaagcccagaaagcgctcgaaacttttgagcggtttct  
 cgcaagcttggacctcgagtcctaccagcaaaagtaccgcccctatcaaaa  
 cggttgaacaagacctgcttagggagctgaacccgcttccggacctgtac  
 gagcattattggaagcgcttgaggataacccttccctcctgggcttcca

6

-continued

agagttctttgacctggtgggaaaagcgctacggcccttgacgagct  
 tcatacgcaataacttttggggatgctcctacgcgtttgttcgctgggg  
 5 ctcgaggctaggctgtaccgaacagcgtttccatctggactcagtttca  
 cttctgctaccgctggaacgcctcctgcgagcttccctctagaagctgccc  
 cagaactcgacgccccaggatagacgcgctgattcatacaagcgggtcc  
 10 tcaacaggaatccagatcaaaaaggaaacttaccggttccgaggccaagag  
 cgagaaccgctttttaaggaagcaaaagaggcaccgcccctcatcgagattc  
 cctacacctgcagacaccagaggagctcgaagaaaaagccaaacgggca  
 15 agagtgaacggagaaacctaccgtctatgggccaagggtgcacaccattt  
 ggaccgtctagaaaacggattcgtcatttttccgggaaagttatgtgaaaa  
 gcattgagctttttctccagaaaaacgctcctaccctatctgggctcacc  
 20 cgctgggacaggggtggcccaggaagccctcaccgccccgtga

SEQ ID NO:3 is the amino acid sequence of TaqI nuclease, as deduced from SEQ ID NO:1.

SEQ ID NO:3:

25 MASTQAQKALETFERFLASLDLESYQQKYRPIKTVEQDLPRELNPLPDLY  
 EHYWKALEDNPSFLGFEEFFDHWWEKRLRPLDEFIRKYFWGCSYAFVRLG  
 30 LEARLYRTAVSIWTQFHFYRWNASCLEPLEAAPELDAQGIDALIHTSGS  
 STGIQIKKETYRSEAKSENFRKQRTALIEIPYTLQTPPEELEEKAKRA  
 RVNGETYRLWAKVAHHLDRLENGFVIFRESYVKSIELFLQKNAPTLISGLI  
 35 RWDRVAQEALTAP

SEQ ID NO:4 is the DNA sequence of the heterologous carB open reading frame harbored by a *Yarrowia lipolytica* strain.

SEQ ID NO:4: carB gene

40 atgtccaagaacacattgtcattatcgggtgctggcgtgggtggcacgg  
 ctacagctgctcgtttggcccgcgaaggcttcaaggctcactgtggtgga  
 45 gaaaaacgactttggtggcggccgctgctccttgatccatcaccagggc  
 catcgctttgatcagggcccgtcgtctacctgatgcccagtaactttg  
 aggacgcctttgcccgatctggacgagcgcattcaagaccacctggagct  
 50 gctgcatgacgacaacaactacaagggtgcaacttgacgacgggtgagtcg  
 atccagctgtcgtctgacttgacacgcatgaaggctgaattggaccgcg  
 tggagggcccccttgggtttggccgattcctggatttcatgaaagagac  
 55 acacatccactacgaaagcggcaccctgatgctgctcaagaagaatttc  
 gaatccatctgggacctgattcgcacatcaagtacgctccagagatctttc  
 gcttgacacctgtttggcaagatctacgaccgcttccaagtacttcaa  
 60 gaccaagaagatgctgcatggcattcacgtttcagaccatgtatggggc  
 atgtcgccctacgatgctgctgctctacagcctggtgacgacacccg  
 agttcgctgaaggcatctggatccccgtggcggcttcaacatggtggt  
 tcagaagctagaggcgattgcaaaagcaaaagtacgatgcccagtttatc  
 65 tacaatgctgctgttgccaagattaacaccgatgatgccaccaaacaag



-continued

tgacaggtgtaaccttgaaaaatggccacatcatcgatgccgatgcggt  
 tgtgtgtaacgcagatctggtctatgcttatcacaatctggtgcctccc  
 tgccgatggacgcaaaacacactggcttccaagaaattgacgtcttctt  
 ccatttcccttctactggtccatgtccaccaaggtgctcaattggacgt  
 gcacaacatcttttggccgaggttatcaggagagctttgacgaaatc  
 ttcaaggactttggcctgcttctgaagcctccttctacgtcaatgtgc  
 cctctcgcatcgatccttctgctgctcccagcggcaaggactctgtcat  
 tgtcttgggtgcctattggtcatatgaagagcaagacgggcatgcttcc  
 accgagaactacccggccatggtggacaaggcacgcaagatggtgctgg  
 ctgtgattgagcgtcgtctgggcatgtcgaatttcgcccacttgattga  
 gcgatgagcaagtcaatgatcccgtgtatggcagagcaagttcaatctg  
 ttgagaggctcaattctgggtttgtctcatgatgtgcttcagggtgctgt  
 ggttccgtcccagcacaaggattctaccggctcgttatgataacctatt  
 ctttgtgggtgcaagcacgcacatcccggaaactgggtgtcccattgtcctt  
 gcaggaagcaagctcacctctgaccaagttgtcaagacgtttggaaaga  
 cgccaagccaagaaagatcgagatggagaacacgcaagcacctttgga  
 ggagcctgatgctgaatcgacattccctgtgtggttctggttgcgcgct  
 gccttttgggtcatgtttatgttctttacttcttccctcaatccaatg  
 gccaaacgcccgcacatctttatcaataatttggttacctgaagtatccg  
 cgttcataactctaattgcatttaa

SEQ ID NOs:5 and 6 are the 5' and 3' PCR primers for a 529 bp fragment within SEQ ID NO:4.

SEQ ID NO:5: primer MO4641:

caatctgttgcctccctgc

SEQ ID NO:6: primer MO4642:

atcctttgtgctgggacgg

SEQ ID NO:7 is the DNA sequence of the heterologous crtZ-Dc open reading frame harbored by a *Yarrowia lipolytica* strain.

SEQ ID NO:7: crtZ-Dc gene

atgcttgcgttgtggaataccgggatcgtgctactgactatcatcatca  
 tggaaaggggtggcaacgttcgcacacaagtagatcatgacggctgggg  
 atggggctggcatcattcgaccatccccgcgcaaaagggcggtttgag  
 cgtaacgatctctatgcggtggtgtttgcgctactggccattgcgctga  
 tttacgcgggcagcgaagggtactggccgcttcagtggattggcgcggg  
 aatgacgggctacggcgtgatctactttatcgttcacgatggtcttgtc  
 caccagcgtggccttccgttacgtgcccgcgcccggctatctgcgcc  
 gcctctacatggcacaccggctgcatcacgcggtgccccggcgcaagg  
 gtgctctccttcgggtttatctacgccccaccgggtggacaagctgcag  
 gcggtgctgcgcgaacgtaacggcagacccgccagcggcgctgcca

-continued

gaggtgcggatcgcgcgccggccagctcgccttccgggaagccatcgcc  
 tgcttcgcccggaaataa

5 SEQ ID NO:8 is the DNA sequence encoding PhoI nuclease from *Pyrococcus horikoshii* OT3, as optimized for expression in *Yarrowia lipolytica*, and includes restriction sites for cloning and a 5' ribosomal binding site.

10 SEQ ID NO:8:

tgctagccacaaaaatggagatgtacaagggttggtcgatacctcgttga  
 ttccctccagatttacttccccgcttctcttgagatccaggaggactt  
 15 attaacaacggcttctacggtccccgatctcctgatcgaagggtttcta  
 tgccattcccatgtttactccgattttggtggcaggttattctat  
 tgagcactcattcctcccagtggttgagatttctcccagcagctc  
 20 ggttgggaggagacttaccttgagaacaagcagggtttcaagctcccta  
 aggaggaggtttacggttgatgtttctatttctaacgattccattttt  
 cgagcttgacgtaagaactaccaccttgagcgaacctccattcgaggc  
 25 atcaacctgagaagtggagaactgggttatggtttacattgatctca  
 agtacgttgatgagttcattaacgctcttcgagagcacatccctgctt  
 cgagaacaagaccgagttatcagagagaagcagcaggggtggcaaggag  
 30 gttacttactacgtaagggttaacgtcaagaacttctctctttgtctcg  
 gttgtttcgatctcgtcagcgataccttcagatcaaggctaaggagca  
 ctgtaacatttaccctgggttctcccacctgtaacgattctctctetaag  
 35 ctcaagctccgacttgagtacgatccctccattaccacctttgctaagg  
 ttggtattgccaagatttctggttaagcagccccagatcatggttaagct  
 cacctctaccgagactaagaccattcgaggtattctcaagcctgagatt  
 40 aagggttaaggcccagggtaagctcgtttactgtgatcccgagagaagc  
 gacagtacattgctcttgacctcttcgatttttacaaggccctcgtttc  
 tactaagaagtacgagggtaagctccctaccgatgattaaacgcgt

45 SEQ ID NO:9 is the non-optimized DNA sequence encoding PhoI nuclease, from *Pyrococcus horikoshii* OT3.

SEQ ID NO:9:

atggaaatgataaagggtggaagataccttgttgattcccttcaatata  
 50 ttttcccgttcaactagagatccaggaggagcttataaataatggcttct  
 acgtaccagagagcccagatagaaaagt aagcatgccaatccaatagta  
 tactccgattttggaggggaggttatatctatgagagggttaatacctcc  
 55 ggagtggcttgaatctcgccagagcaattagggtgggaagaaacttatt  
 tagagaacaaacgtgggttttaactacctaaagaagaggtctacgtggat  
 gttagtataagcaacgattccataatattcgagctagacgtgaaaaatta  
 60 tcacctgaaaggacatccattagaggcatcaatcctgaaaaatggaaga  
 attggtgatgttttatattgatttgaagtacgtagatgagttcattaac  
 gctttaagggaacatatcccagcttccgaaaaataaaacaagggttattcg  
 65 tgaaaagcagcaagggggcaaggaagttacatattatgctaaagttaacg



9

-continued

tcaagaatttcagcttatgcttaggatgcttcgatctcgctcaaaggtat  
 cttcaaatcaaagctaaagagcactgtaacatatatccaggtcaccaac  
 atgtaacgattctctaagcaaatataaattaaggcttgaatacgatccat  
 ccattactacctttgctaaagttggaatagccaagatctcaggcaagaga  
 ccccagatcatgggttaaattaacctctacggaaactaaaactataagggg  
 aataacttaagccagaaataaaaggttaaggcgcgtggtaaaacttgtatatt  
 gcgatcacagagaaaagagacagtatatagctttggacttgtttgatttt  
 tacaaggccttggttaagcactaagaagtatgaaggttaagctaccgactga  
 tgattaa

SEQ ID NO:10 is the amino acid sequence of PhoI nuclease, as deduced from SEQ ID NO:8.

SEQ ID NO:10:

MEMYKVGRYLVDSLQIYFPASLEIQEELINNGFYVPRSPDRKVSMPPIPIV  
 YSDFGGRVISIERLIPPEWLEISPEQLGWEETYLENKRGFKLPKEEVYVD  
 VSISNDSIIFELDVKNYHLERTSIRGINPEKWKNWVMFYIDLKYVDEFIN  
 ALREHIPAFENKTRVIREKQQGGKEVTTYAKVNVKNFSLCLGCFDLAORY  
 LQIKAKEHCNIYPGSPTCNDSLKLRLEYDPSITTFKAVGIAKISGKR  
 PQIMVKLTSTETKTIRGILKPEIKGKARGKLVYCDHREKRQYIALDLDFD  
 YKALVSTKKYEGKLPTDD

SEQ ID NO:11 is the DNA sequence encoding TaqI nuclease from *Thermus aquaticus*, as optimized for expression in *Escherichia coli*, and includes restriction sites for cloning.

SEQ ID NO:11:

CCATGGCATCAACCCAAGCACAAAAAGCCCTGGAAACCTTCGAACGCTTC  
 CTGGCGAGCCTGGACCTGGAATCATAACAGAAATACCGTCCGATTAA  
 AACCGTGGAACAGGATCTGCCCGTGAACCTGACCCGCTGCCGGACCTGT  
 ATGAACATTACTGGAAGCACTGGAAGATAATCCGTCTTTCTGGGCTTC  
 GAAGAATTTTTCGATCACTGGTGGGAAAAACGCTGCGCCCGCTGGACGA  
 ATTTATTCGCAAATATTTCTGGGGCTGCAGCTACGCGTTTGTTCGTCTGG  
 GTCTGGAAGCGCTGTATCGCACCCGCTCAGCATCTGGACGCAATTT  
 CATTTCTGTACCGCTGGAACGCTCTTGTGAACGCGCTGGAAGCAGC  
 ACCGGAACCTGGATGCACAGGCATTGACGCTCTGATCCACACAGTGGCA  
 GCTCTACGGGTATCCAAATCAAAAAAGAAACCTACCGTAGTGAAGCAAAA  
 TCCGAAAATCGTTTTCTGCGCAAACAGCGTGGTACGGCTCTGATTGAAAT  
 CCCGTATAACCTGCAAACGCCGGAAGAACTGGAAGAAAAAGCAAAACGTG  
 CTCGCGTCAACGGCGAAACCTACCGTCTGTGGGCGAAAGTGGCCCATCAC  
 CTGGATCGCCTGGAATGGTTTTGTGATTTTCCGTGAATCATAACGTTAA  
 ATCGATCGAACTGTTCTGCAGAAAAACGCCCGACCCCTGTCCGGGCTGA  
 TTCGTTGGGACCGTGTGCACAAGAAGCACTGACCGCTCCGTGAGGTACC

10

SEQ ID NO:12 and 13 are the 5' and 3' PCR primers used to amplify taqI using pMB6736 as template for expression in *Corynebacterium glutamicum*. Primers include restriction sites for cloning.

5 SEQ ID NO:12: primer MO9315:

CACACACCATGGCTTCCACTCAGGCTCAG

10 SEQ ID NO:13: primer MO9316:

CACACAGGTACCTTAGGGGGCGGTGAGGGCCTCC

15 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the time course of DNA degradation activity in *Y. lipolytica* strains with or without the taqI nuclease gene.

20 FIG. 2 shows DNA degradation activity at different temperatures in *Y. lipolytica* strains with or without the taqI nuclease gene.

FIG. 3 shows the completeness of DNA degradation based on PCR result.

25 FIG. 4 shows the production of astaxanthin and its precursors in *Y. lipolytica* strains with and without the taqI nuclease gene.

FIG. 5 shows the production of zeaxanthin and its precursors in a *Y. lipolytica* strain which has the taqI nuclease gene.

30 FIG. 6 shows the time course for degradation of DNA in *Y. lipolytica* strains producing astaxanthin and zeaxanthin in fermentors.

35 FIG. 7 shows the temperature range around the optimum temperature of the TaqI and PhoI nucleases, respectively, in *Y. lipolytica*.

FIG. 8 shows the nuclease activity of TaqI nuclease in *E. coli* expressing TaqI nuclease gene.

40 FIG. 9 shows the nuclease activity of TaqI nuclease in *C. glutamicum* expressing TaqI nuclease gene.

FIG. 10 shows the temperature range around the optimum temperature of the TaqI nuclease in *C. glutamicum*.

#### 45 DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of degrading nucleic acids in vivo and/or in situ using a heterologous thermophilic nuclease. The method is intended to degrade the deoxyribonucleic acids of an organism, recombinant DNA in particular, and thus inactivating the biological activity of the deoxyribonucleic acids at any stage during the production of the end product(s). According to the invention, a heterologous thermophilic nuclease gene is introduced into the organism in which nucleic acids are intended to be degraded. The thermophilic nuclease is latent at the normal growth temperature of the organism, but can be activated by raising the temperature, preferably to a range around the optimum temperature of the thermophilic nuclease.

60 In the present invention, the thermophilic nuclease is used to inactivate the biological activity of the nucleic acids of the host organism. The term "inactivate the biological activity of nucleic acids" is considered to refer to the degradation of nucleic acids by the present invention. When nucleic acid is cleaved such that it can no longer accomplish the roles of replication, transcription or translation, it is considered to be biologically inactivated. Preferably, the nucleic acid is



recombinant DNA. Preferably, the nucleic acid will be cleaved into units which are 500 base pairs or less.

In the present invention, the thermophilic nuclease is produced by the host cell rather than being added exogenously, and thus degrades the nucleic acids of the host cell in vivo and/or in situ. The term "in vivo" refers to the degradation of nucleic acids inside the host cell by the thermophilic nuclease produced by the cell, which may or may not be viable at the time of degradation. The term "in situ" refers to the degradation of nucleic acids by the thermophilic nuclease produced by the host cell after the cell wall of the host cell is broken (fully or partially). For example, an in situ degradation can be performed at the end of the fermentation process where cells are lysed to release their contents. Degradation can be performed immediately after the cells are lysed or after some additional processing of the lysed broth has already occurred.

In connection with the present invention, the term "nucleic acid" denotes single-stranded, double-stranded, or partially-double stranded deoxyribonucleic acid (DNA) or ribonucleic acid (RNA).

The term "gene", as used herein, generally refers to a nucleic acid encoding a polypeptide, optionally including certain regulatory elements that may affect expression of one or more gene products (i.e., RNA or protein). For example, a thermophilic nuclease gene according to the invention refers to the open reading frame (ORF) encoding a polypeptide of the thermophilic nuclease, and also to nucleic acid sequences which encode the ORF of the thermophilic nuclease together with certain promoters which are placed upstream of the ORF to affect the expression of the nuclease gene, and/or a region for termination of transcription, and/or other regulatory elements.

The term "heterologous", as used herein, refers to a gene or a polypeptide that does not naturally occur in the organism in which it is being expressed. It is understood that, where a heterologous polypeptide is to be expressed in a host cell, it will often be desirable to utilize nucleic acid sequences encoding the polypeptide that have been adjusted to accommodate codon preferences of the host cell and/or to link the encoding sequences with regulatory elements active in the host cell. For example, when the host cell is a *Yarrowia* strain (e.g., *Yarrowia lipolytica*), it will often be desirable to alter the gene sequence encoding a given polypeptide such that it conforms more closely with the codon preferences of such a *Yarrowia* strain. In certain embodiments, a gene sequence encoding a given polypeptide is altered to conform more closely with the codon preference of a species related to the host cell. Such embodiments are advantageous when the gene sequence encoding a given polypeptide is difficult to optimize to conform to the codon preference of the host cell due to experimental (e.g., cloning) and/or other reasons. In certain embodiments, the gene sequence encoding a given polypeptide is optimized even when such a gene sequence is derived from the host cell itself (and thus is not heterologous). For example, a gene sequence encoding a polypeptide of interest may be codon optimized for expression in a given host cell even though such a gene sequence is isolated from the host cell strain. In such embodiments, the gene sequence may be further optimized to account for codon preferences of the host cell. Alternately, the (non-heterologous) gene sequence might not be codon optimized but instead be linked to a regulatory element other than its own (whether that regulatory element comes from another gene in the host or from another species). Those of ordinary skill in the art will be aware of host cell codon preferences and will be able to employ the

inventive methods and compositions disclosed herein to optimize expression of a given polypeptide in the host cell.

As used herein, a "host cell" means any cell type that is amenable to introduction of a nucleic acid construct or expression vector. The means of introduction of the nucleic acid construct or expression vector can be in the form of transformation, transfection, transduction, and the like. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. In preferred embodiments, the host cells produce one or more end products which are desired biological compounds or fine chemicals. It is preferred that the host cell is an industrial strain. The host cell can be a genetically modified cell or a natural cell which was initially not genetically modified. A genetically modified host cell refers to a host cell which has been modified, engineered, or manipulated, often to overexpress certain biological compounds or fine chemicals.

The host cell may be any cell useful in the recombinant production of biological compounds and/or fine chemicals in a prokaryote (bacteria or archaea) or a eukaryote.

The term "biological compounds" is known in the art and includes compounds which are found in nature in living matter. Examples of biological compounds include, but are not restricted to: proteins, polypeptides, amino acids, nucleic acids, nucleotides, carbohydrates, and lipids.

The term "fine chemical" is known in the art and includes compounds which are used in various branches of industry such as, for example but not restricted to, the pharmaceutical industry, the agriculture, cosmetics, food and feed industries. These compounds include organic acids such as, for example, tartaric acid, itaconic acid and diaminopimelic acid, lipids, saturated and unsaturated fatty acids (e.g., arachidonic acid), diols (e.g., propanediol and butanediol), aromatic compounds (e.g., aromatic amines, vanillin and indigo), carotenoids, vitamins and cofactors.

Higher animals have lost the ability to synthesize vitamins, carotenoids, cofactors and nutraceuticals and therefore need to take them in, although they are widely synthesized by other organisms such as bacteria. These molecules are either biologically active molecules per se or precursors of biologically active substances which serve as electron carriers or intermediates in a number of metabolic pathways. These compounds have, besides their nutritional value, also a significant industrial value as coloring agents, antioxidants and catalysts or other processing aids. The term "vitamin" is known in the art and includes nutrients which are required by an organism for normal functioning, but cannot be synthesized by this organism itself. The group of vitamins may include cofactors and nutraceutical compounds. The term "cofactor" includes non-protein compounds which are necessary for the occurrence of normal enzymatic activity. These compounds may be organic or inorganic; the cofactor molecules of the invention are preferably organic. The term "nutraceutical" includes food additives which promote health in organisms and animals, especially in humans. Examples of such molecules are vitamins, antioxidants and likewise certain lipids (e.g., polyunsaturated fatty acids).

Preferred fine chemicals or biosynthetic products which can be produced in organisms of the genus *Yarrowia* are: carotenoids such as, for example, phytoene, lycopene, beta-carotene, alpha-carotene, beta-cryptoxanthin, lutein, zeaxanthin, astaxanthin, canthaxanthin, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, adonirubin, violaxanthin, adonixanthin, and/or modified forms thereof (including, but not limited to isomerization, esterification, glycosylation, and breakdown products of these com-



pounds); quinone derived compounds such as, for example, ubiquinone (including, but not limited to, coenzymes Q10 and Q5 to Q9 quinone compounds), vitamin K compound, and vitamin E compound; and retinolic compounds such as, for example, retinol, retinal, retinoic acid, and retinol palmitate.

The prokaryotic host cell may be any gram-positive or gram-negative bacterium. Gram-positive bacteria include, but are not limited to, *Bacillus*, *Brevibacillus*, *Clostridium*, *Corynebacterium*, *Geobacillus*, *Lactobacillus*, *Lactococcus*, *Paenibacillus*, and *Streptomyces*. Gram-negative bacteria include, but are not limited to *Escherichia coli*, *Pseudomonas* and *Paracoccus*.

The bacterial host cell may be any Bacillales cell including, but not limited to, *Bacillus amyloliquefaciens*, *Brevibacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lentus*, *Bacillus licheniformis*, *Geobacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis* cells.

The bacterial host cell may also be any *Streptomyces* cell including, but not limited to, *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, and *Streptomyces lividans* cells.

The bacterial host cell may also be any *Paracoccus* cell including, but not limited to *Paracoccus denitrificans*, *Paracoccus versutus*, *Paracoccus carotinifaciens*, *Paracoccus marcusii*, and *Paracoccus zeaxanthinifaciens* cells.

The host cell may also be a eukaryote, such as a mammalian, insect, plant, algal, or fungal cell.

The algal host cell may be a *Cryptocodinium*, *Schizochytrium*, and *Thraustochytrium* cell such as a *Cryptocodinium cohnii*, *Schizochytrium* sp. or *Thraustochytrium* sp. cell.

The host cell may be a fungal cell. "Fungi" as used herein include the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth et al., *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth et al., 1995, supra, page 171) and all mitosporic fungi (Hawksworth et al., 1995, supra).

The fungal host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth et al., 1995, supra). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

The filamentous fungal host cell may be an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolyposcladium*, *Trametes*, or *Trichoderma* cell.

For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermisporea*, *Chrysosporium inops*, *Chrysosporium keratino-*

*philum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

The fungal host cell may also be a yeast cell. "Yeast" as used herein includes ascosporegenous yeast (Endomycetales), basidiosporegenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeasts shall be defined as described in *Biology and Activities of Yeast* (Skinner, F. A., Passmore, S. M., and Davenport, R. R., eds, *Soc. App. Bacteriol. Symposium Series No. 9*, 1980).

The yeast host cell may be a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell such as a *Kluyveromyces lactis*, *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, *Saccharomyces oviformis*, *Schizosaccharomyces pombe* or *Yarrowia lipolytica* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known to those of ordinary skill in the art. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238023 and Yelton et al., 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474. Suitable methods for transforming *Fusarium* species are described by Malardier et al., 1989, *Gene* 78: 147-156, and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarante, In Abelson, J. N. and Simon, M. I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, *J. Bacteriol.* 153: 163-168; and Hinnen et al., 1978, *Proc. Natl. Acad. Sci. USA* 75: 1929-1933.

For constructing a host cell strain with a heterologous thermophilic nuclease gene, the recombinant thermophilic nuclease gene construct is inserted into a host specific vector which allows optimal gene expression in the host. Vectors are well known in "Cloning Vectors" (Pouwels et al., Eds., Elsevier, Amsterdam-New York-Oxford, 1985). Vectors are to be understood as meaning not only plasmids, but all other vectors known to the skilled worker such as, for example, phages, viruses, such as SV40, CMV, baculovirus and adenovirus, transposons, IS elements, plasmids, cosmids, and linear or circular DNA. These vectors can be replicated autonomously in the host organism or chromosomally.

The vectors according to the invention allow the generation of recombinant organisms which are transformed, for example, with at least one vector according to the invention



and which can be employed for producing the thermophilic nuclease. The above-described recombinant constructs according to the invention are advantageously introduced into the genome of the host organism and expressed. It is preferred to use usual cloning and transfection methods known to the skilled worker in order to bring about expression of the thermophilic nucleic acids in the expression system in question. Suitable systems are described, for example, in *Current Protocols in Molecular Biology*, F. Ausubel et al., Eds., Wiley Interscience, New York 1997.

In some embodiments, the modified host cell contains a plasmid carrying the heterologous thermophilic nuclease gene and the plasmid replicates autonomously in the host organism.

DNA is considered to be "recombinant" if it results from the application of Recombinant DNA Techniques. Examples of recombinant techniques include but are not limited to cloning, mutagenesis, transformation, etc. Recombinant DNA Techniques are disclosed, for example, in Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y. (1989) and in Ausubel F M, et al., *Current Protocols in Molecular Biology*, Wiley, New York (1998).

The term "nuclease" denotes an enzyme that effects hydrolytic cleavage of the ester bond between the 5'-phosphate group of a nucleotide and the 3'-hydroxy group of the adjacent nucleotide in a nucleic acid and therefore accomplishes the degradation of a DNA or RNA molecule. Nucleases are known from numerous organisms. Nucleases cleave either RNA or DNA to smaller units or even to their monomers.

In a preferred embodiment, the thermophilic nuclease according to the present invention is a DNA-degrading nuclease, and the nucleic acid which the thermophilic nuclease degrades is either double-stranded or single-stranded DNA. In a specific embodiment, the DNA being degraded is a recombinant DNA.

Although the preferred embodiment of the present invention relates to inactivation of DNA, inactivation of any nucleic acid molecule can be achieved. This includes inactivation of RNA, DNA-RNA hybrids, or nucleic acid-protein conjugates.

"DNA-degrading nuclease" means a nuclease which is able to cleave DNA molecules into smaller units or monomers. Such enzyme is also termed "DNase" in the state of art. "RNA-degrading nuclease" is a nuclease that is able to cleave RNA molecules into smaller units or monomers. Such enzymes are also termed "RNase" in the state of art.

The DNA or RNA nuclease can be an endonuclease or an exonuclease. An endonuclease is an enzyme that degrades a nucleic acid chain from a position inside the chain. Conversely, an exonuclease is an enzyme that degrades a nucleic acid chain from one or both ends of the chain. It is preferred for the nuclease to be an endonuclease.

"Thermophilic nuclease" means a nuclease which has its optimal activity at a temperature of 45° C. or higher. Said optimal activity is observed at a temperature that is optimal for the activity of the nuclease. The optimal temperature may be determined for the nuclease in an in vitro assay, or in an in vivo assay where the nuclease is expressed in the recombinant host according to the present invention, using a suitable substrate and under assay conditions (in terms of medium composition, assay time, etc.) that are relevant for the intended application, as determined by a person skilled in the art. As an example, the temperature optimum of a nuclease such as TaqI is typically determined in vitro using the purified TaqI nuclease. A fixed quantity of enzyme can

be incubated with one microgram of lambda DNA in a fifty microliter reaction held at a range of temperatures (typically from 25 to 85° C.) for one hour. The optimum temperature is the one at which the most digestion is observed (for example, as assayed by gel electrophoresis of the resulting lambda DNA TaqI restriction fragments). Alternately, one unit of enzyme is defined as the amount of protein required to fully digest one microgram of lambda DNA in a fifty microliter reaction in one hour at the optimum temperature. Therefore, the optimum temperature is the temperature at which the quantity of protein required to achieve full digestion is the lowest. To define the optimum temperature within the cell, a defined ratio of enzyme to DNA substrate (as used in in vitro assays) cannot be guaranteed. Instead, a more pragmatic approach is taken. Given that the optimum temperature in vivo is likely to be similar to in vitro, the in vitro defined optimum can be used as a guide. Nuclease acid digestion by the nuclease is examined at and around the in vitro optimal temperature. The optimal temperature is the one at which the most digestion is observed. For some nucleases, the in vivo optimal temperature is a range of temperatures rather than a single point, as complete digestion of genomic DNA can be observed throughout the temperature range.

The thermophilic nucleases disclosed in the present application include both native thermophilic nucleases and any homologue or variant thereof which has thermophilic nuclease activity. Means to obtain thermophilic nucleases used in the present invention include but are not limited to: isolation from thermophiles, modification of a homologous nuclease to acquire thermophilic activity and/or increase thermostability by rational enzyme engineering and/or directed evolution. Unlike the majority of cells, thermophiles are heat-loving organisms with an optimum growth temperature of 50° C. or higher. In contrast, most cells grow at a lower temperature and cannot survive at the high temperature preferred by thermophiles. For example, common microorganisms grow at a temperature between 25 and 40° C., mammalian cell cultures grow at around 37° C., and plant and insect cultures grow at their respective low temperatures which are known in the art. Thermophilic nucleases lose activity outside their optimum temperature range and have lower activity at the temperature at which common microorganisms and cell cultures grow. The levels of nuclease activity of each thermophilic nuclease at a low temperature are different and thus have to be determined individually. In the present invention, a thermophilic nuclease is considered latent at a low temperature if the nuclease activity is reduced to less than 20% of the peak activity at the optimum reaction temperature of the nuclease.

The method of the invention is amenable to a wide range of thermophilic nucleases. Any nuclease which is latent at the normal growth temperature of the host microorganisms or eukaryotic cell cultures but is (highly) active at its own optimum reaction temperature is suitable for use in the present invention.

Some thermophilic nucleases are less suited for the present invention than others. For example, some thermophilic nucleases have low-level nuclease activities at low temperatures between 25 and 37° C. Such residual activities at the growth temperature of the host cell may constrain the growth of the host cells and thus hinder the production of their end product. Some thermophilic nucleases may not have strong nuclease activity at their optimum temperature and thus are inefficient in degradation of nucleic acids. Thus,



a screening process may be performed to select those thermophilic nucleases which work best in the present invention.

The best thermophilic nuclease candidates are those whose nuclease activities are low enough at the normal growth temperature so as not to impose any significant stress on the host organism, and are high enough at the optimum reaction temperature to perform efficient degradation of the nucleic acids of the host.

Owing to the property of displaying nucleic acid degradation activity at a temperature that is outside the temperature range in which a normal host organism grows, the thermophilic nuclease according to the invention can be used advantageously in degrading nucleic acids of the host cell in a controlled manner. For example, in one embodiment, the present invention discloses that a heterologous thermophilic nuclease gene is introduced into a host cell and thus is used to degrade the nucleic acids of a host cell. According to the invention, the thermophilic nuclease gene is introduced into a host cell whose nucleic acids are to be degraded. The host cell is grown at its optimum growth temperature at which the heterologous thermophilic nuclease is latent. After the host cell is grown for a period of time to a certain stage, such as at the harvest time of the cell, the activity of the thermophilic nuclease in the host cell can be activated by switching the temperature to one which is optimal for the nuclease activity. By activating the thermophilic nuclease, the DNA and/or RNA of the host cell are degraded by the nuclease, thus eliminating activities of the DNA and/or RNA of the host cell.

According to the present invention, any homologues or variants of a native thermophilic nuclease may be used in exchange for the native thermophilic nuclease as long as the homologue or variant has thermophilic nuclease activity.

In another embodiment, instead of using a heterologous thermophilic nuclease gene, the method according to the present invention could use a homologue or variant of a host cell nuclease which has been made thermophilic using methods such as rational enzyme engineering and/or directed evolution.

In one embodiment, one thermophilic nuclease is introduced into a host cell and thus is used to degrade the nucleic acids of the host cell. In another embodiment, two or more thermophilic nucleases are introduced into a host cell. The introduction of additional thermophilic nucleases may, for example, enhance the efficiency of the degradation of the nucleic acid.

In another embodiment, the sequence of the recombinant DNA to be degraded is optimized to include additional cleavage sites of the thermophilic nuclease. The addition of the cleavage sites helps to ensure the complete degradation of the recombinant DNA. The recombinant DNA to be degraded can include the nuclease gene itself. In a specific embodiment, the sequence of the recombinant DNA is engineered to include one cleavage site every 500 or fewer nucleotides. The cleavage site may be designed to be recognized by a single nuclease or multiple nucleases. In another specific embodiment, the sequence of the recombinant DNA is engineered to include cleavage sites in a manner in which the amino acid sequence of the resulting protein is not altered.

The above method of nucleic acid degradation can be carried out at any scale ranging from lab bench to commercial scale fermentation. As described in the background section of this application, DNA of the host cell, especially recombinant DNA, is preferred to be degraded prior to product recovery (for example, at the end of the fermenta-

tion process). The present invention provides a process for degrading the recombinant DNA in the biomass and thus inactivating any recombinant DNA. The biomass can be, for example, cells harvested from cultivation of microorganism or higher eukaryotic cell culture.

In a specific embodiment, the invention provides a fermentation process by which the recombinant DNA in the biomass is inactivated. The methodological steps for such process are exemplified as follows: First, the gene encoding the thermophilic nuclease according to the invention is introduced into the host cell. Integration of a nucleic acid construct containing the gene of a thermophilic nuclease into the host cell can take place intrachromosomally or extrachromosomally. Second, the host cell is grown in a fermentor at a temperature which is optimal for the growth of the host cell and/or the production process. Third, at the end of the fermentation process, the temperature of the fermentor is raised to the optimum temperature of the thermophilic nuclease and thus activates the nuclease. The higher temperature is maintained for a period of time to allow sufficient degradation of the DNA (recombinant or not) in the host cell. Subsequently, the biomass is collected and optionally is further processed to purify the end product (if not the biomass itself).

In another embodiment, instead of degrading DNA in the biomass, the method disclosed in the present invention can also be used for degrading DNA in partially purified products. For example, in the fermentation process described above, instead of activating the thermophilic nuclease before collection of biomass, the activation of the nuclease can be withheld until after the biomass is collected and further processed to obtain partially purified end products. The thermophilic nuclease is then activated to degrade the DNA contained in the partially purified end products, which can then be further purified, if desired. In the initial (partial) purification process, it is essential to maintain the activities of the nuclease. In fact, as long as the activity of the thermophilic nuclease is preserved during the post biomass collection process, the degradation of DNA of the host cell can be performed at any stage after the biomass is collected.

The process according to the invention can be used in many other technical fields in addition to fermentation. For example, the process according to the invention can be used for inactivating the DNA of the host cell during vaccine production.

Cell culture-based methods of producing vaccine containing virus or viral antigens are increasingly used in commercial production of viral vaccines. However, it is known in the art that one of the risks associated with using cell culture for vaccine production is the exposure of vaccine recipients to contaminating residual cellular DNA of the host cell. There is a concern that any host cell DNA be removed from the final formulation of a vaccine manufactured in the host cell system, thus eliminating any potential oncogene from the final product. The process according to the invention can be carried out to degrade the host cell nucleic acid associated with a virus or a viral antigen thereof produced by cell culture. The process comprises the steps of: (a) introducing the gene of a thermophilic DNA nuclease into a host cell culture and thus creating a new host strain; (b) inoculating the population of the new host strain with a virus; (c) culturing the population of host cells so as to allow the virus to replicate, wherein the culturing is conducted at a temperature at which the thermophilic nuclease is latent; (d) degrading the DNA of the host cells by changing the temperature in step (c) to a temperature at which the thermophilic DNA nuclease is active; (e) collecting the



produced virus thereby providing a viral harvest, and (f) isolating the virus. The order of steps (d) through (f) can be exchangeable. The virus can be, for example, influenza virus. However, any virus or viral antigen can be produced according to the invention.

The process according to the invention can also be used for inactivating the nucleic acid of a genetically modified crop. For example, a thermophilic nuclease according to the invention can be introduced into a genetically modified crop. The crop is grown in the field at temperatures which normally do not exceed 50° C. After the crop is harvested, it can be, for example, processed at the optimal temperature of the above thermophilic nuclease in order to degrade the crop nucleic acid.

The process according to the invention can also be used for reducing viscosity in a cell lysate. It is known in the art that when cells are lysed, DNA is released from the cell and thus causes the cell lysate to become viscous. This hinders the further processing of cell lysate. The thermophilic DNA degrading nuclease works by cleaving DNA into small oligonucleotide fragments and thus removing the viscosity caused by the DNA content in a controlled manner.

The thermophilic nuclease that is used according to the invention can be, for example, TaqI endonuclease from *Thermus aquaticus*. TaqI has an optimum temperature of 65° C. Other thermophilic nucleases can be tested for their suitability of being used in the present invention. Examples of additional thermophilic nucleases include: Tsp509I which has an optimum temperature of 65° C., MwoI which has an optimum temperature of 60° C., PhoI which has an optimum temperature of 75° C., BsaJI which has an optimum temperature of 60° C., and BspQI which has an optimum temperature of 50° C.

Basically, any existing thermophilic nuclease known in the state of art or any thermophilic nuclease yet to be identified can be used according to the invention, as long as such thermophilic nuclease has high nucleic acid cleavage activity at its optimum reaction temperature but is latent at the lower temperature at which the host microorganisms or cell cultures normally grow.

Within the scope of the present invention it is preferred that the temperature for degrading nucleic acid is the temperature known to have the optimal activity of the specific thermophilic nuclease. For example, one preferred temperature for degrading nucleic acids of the host cell using thermophilic nuclease TaqI is 65° C. because TaqI is known to be most active at 65° C. However, the optimum temperature according to the present invention is not limited to a single temperature point. According to the present invention, it is also preferred that the temperature for degrading nucleic acids is any temperature which is within a range of  $\pm 5^\circ$  C. of the known optimum temperature of the thermophilic nuclease. In the case of TaqI, for example, a preferred temperature is any temperature which falls between 60° C. and 70° C. In another example, PhoI is known to be most active at 75° C. A preferred temperature for PhoI is any temperature which falls between 70° C. and 80° C. It is particularly preferred that a temperature which falls within  $\pm 1^\circ$  C.,  $\pm 2^\circ$  C.,  $\pm 3^\circ$  C., or  $\pm 4^\circ$  C. of the known optimum temperature of the thermophilic nuclease is the temperature for activating the thermophilic nuclease. Thus, in one embodiment, the temperature used for activating the thermophilic nuclease activity is within  $\pm 5^\circ$  C. of the known optimum temperature of the thermophilic nuclease.

It is clear to one skilled in the art that the temperature used for activating the thermophilic nuclease activity is not only limited to a temperature range that is within  $\pm 5^\circ$  C. of the

known optimum temperature of a selected thermophilic nuclease. As an alternative, the temperature used for activating the thermophilic nuclease activity can be any temperature at which the host cell does not actively grow but the thermophilic nuclease has active enzyme activity. For example, the temperature used for activating TaqI can be at 75° C. where the host cell does not have active growth but the thermophilic nuclease still has enzyme activity. In one embodiment, the temperature used for activating the thermophilic nuclease activity is a temperature or temperature range at which the thermophilic nuclease has 20% or more of its optimal enzyme activity. In another embodiment, the temperature used for activating the thermophilic nuclease activity is a temperature or temperature range at which the host cell grows at 20% or less of its optimal growth rate or has no growth and the thermophilic nuclease has 20% or more of its optimal enzyme activity. In another embodiment, the temperature used for activating the thermophilic nuclease activity is a temperature between 40° C. and 100° C.

To the extent where the degradation of nucleic acids can be performed in a range of temperatures without jeopardizing its efficiency, a preferred temperature is any temperature within the temperature range that strikes the optimum balance with other considerations, including but not limited to end-product titer, end-product stability, end-product purity, end-product yield, ease of handling of the material for subsequent processing steps, etc.

Thus, it is within the scope of the present invention that any temperature which permits efficient degradation of the nucleic acids may be chosen to activate the thermophilic nuclease activity. Sometimes such temperature may not provide the best thermophilic nuclease activity, but in combination with some additional factors, it may provide best overall efficiency. For example, heat pasteurization may be used in place of the heat activation step of thermophilic nuclease. By heating up the biomass gradually during pasteurization, both pasteurization of the host cell and activation of thermophilic nuclease activity in the cell can be performed in one step, and thus result in cost savings. In such case, the optimum temperature or temperature range is crossed on the trajectory to the pasteurization temperature.

The nucleic acid degradation process according to the present invention can be carried out relatively quickly. According to an embodiment of the invention, the process of nucleic acid degradation is carried out at the optimum temperature of the thermophilic nuclease for the duration of less than 48 hours, less than 24 hours, less than 1 hour, less than 40 minutes, less than 30 minutes, or less than 20 minutes. The optimal time length can be determined experimentally for each thermophilic nuclease and under specific experimental conditions.

The present invention can be used in any host cell. Examples of host cells include microorganisms and higher eukaryotic cells such as agricultural crops or animal cells. According to the invention, the microorganisms can be for example prokaryotic cells, such as bacteria or archaeobacteria, or eukaryotic cells, such as yeasts, lower or higher fungi, algae, or protozoa. According to a preferred embodiment, microorganisms are bacterial cells, fungal cells or yeast, or microalgal cells.

In certain embodiments of the invention, the organism whose nucleic acids can be degraded according to the method of the present invention is a microorganism from a genus such as, but not limited to, *Yarrowia*, *Bacillus*, *Corynebacterium*, *Escherichia*, *Pseudomonas*, *Paracoccus*, *Candida*, *Hansenula*, *Saccharomyces*, *Mortierella*, *Schi-*



*zosterales*, *Aspergillus*, *Fusarium*, *Trichoderma*, *Cryptococcus*, *Schizochytrium*, and *Thraustochytrium*.

In one embodiment of the invention, the host cell in accordance with the present invention is *Yarrowia lipolytica*. Advantages of *Y. lipolytica* include, for example, tractable genetics and molecular biology, availability of genomic sequence, suitability to various cost-effective growth conditions, ability to grow to high cell density, and history of safe use. There is already extensive commercial experience with *Y. lipolytica*.

*Escherichia coli* is useful because it is the most commonly used bacterial strain. It has been regularly used as a carrier strain for recombinant DNA.

*Saccharomyces cerevisiae* is also a useful host cell in accordance with the present invention, particularly due to its experimental tractability and the extensive experience that researchers have accumulated with this organism.

The host cell in accordance with the present invention also includes higher eukaryotic cells. The term "higher eukaryotic cell" means a eukaryotic cell of a high state of development, such as those which occur for example in animal or plant organisms. Higher eukaryotic cells do not perform all vital biochemical and metabolic functions independently and thus may be grown as cell culture. The higher eukaryotic cell in accordance with the present invention comprises, inter alia, all cells from a mammal, a plant, an insect, or an avian. According to one embodiment of the invention, the higher eukaryotic cell is from a mammal. According to another embodiment of the invention, the higher eukaryotic cell is from a plant. According to another embodiment of the invention, the higher eukaryotic cell is from an insect.

According to one embodiment of the invention, the degradation of the nucleic acids of the host cell is carried out with no or minimal impact to the other cellular components of the host cell. In a preferred embodiment, the degradation of the nucleic acids of the host cell is carried out with no or minimal impact to the production of the biological compounds and/or fine chemicals which the host cell is engineered to produce. In one embodiment, the host cell is a *Yarrowia* strain and the biological compounds which are genetically engineered to produce include: phytoene, lycopene, beta-carotene, alpha-carotene, beta-cryptoxanthin, lutein, zeaxanthin, astaxanthin, canthaxanthin, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, adonirubin, violaxanthin, adonixanthin, ubiquinone, vitamin K, vitamin E, retinol, retinal, retinoic acid, retinol palmitate, and/or modified forms thereof (including, but not limited to isomerization, esterification, glycosylation, and breakdown products of these compounds). In one embodiment, the *Yarrowia* strain is a genetically modified *Yarrowia* strain. In a specific embodiment, the genetically modified *Yarrowia* strain cell introduced with a thermophilic nuclease gene is *Yarrowia lipolytica* strain ML12924 and the biological compound produced is astaxanthin. In another embodiment, the genetically modified *Yarrowia* strain cell introduced with a thermophilic nuclease gene is *Yarrowia lipolytica* strain ML12921 and the biological compound produced is zeaxanthin. In another embodiment, the genetically modified *Yarrowia* strain cell introduced with a thermophilic nuclease gene is *Yarrowia lipolytica* strain ML12805 and the biological compound produced is lycopene.

In one embodiment of the invention, codon optimization is performed in order to enhance the expression of the heterologous nuclease gene in the host cell. It is known in the art that the expression of non-native genes is hampered by the existence of variation in their respective codon usage

pattern compared to the host organism. To overcome these problems, codon optimization according to the present invention is performed on the thermophilic nuclease gene in order to match the host codon usage before the gene is introduced into the host cell. This process encompasses the replacement of rare codons within the DNA sequence of the nuclease gene in order to closely match the host codon usage bias while retaining 100% identity or near 100% identity to the original amino acid sequence. This process of codon optimization also allows for the simultaneous modification of predicted mRNA secondary structures that could result from changes in the GC content.

In the thermophilic nuclease of the present invention, the codon usage pattern is altered from that typical of the thermophilic nuclease gene to modify the codons without altering the encoded amino acid sequence. The nucleic acid sequences for many thermophilic nuclease genes are known. In cases where the sequence is not initially known, the information can be derived by methods known to those of ordinary skill in the art (including, but not limited to DNA and RNA sequencing). In accordance with this invention, thermophilic nuclease gene segments were converted to sequences having identical translated sequences but with alternative codon usage.

In one embodiment of the invention, the codon usage pattern of the native thermophilic nuclease TaqI (SEQ ID NO:2) is optimized to the codon bias of *Y. lipolytica*. The resulting codon modified TaqI gene is specified in SEQ ID NO:1. In another embodiment, the codon usage pattern of the native thermophilic nuclease TaqI (SEQ ID NO:2) is optimized to the codon bias of *E. coli*. The resulting codon modified TaqI gene is specified in SEQ ID NO:11. The amino acid sequence encoded by SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:11 is specified in SEQ ID NO:3.

In one embodiment of the invention, the codon usage pattern of the native thermophilic nuclease PhoI (SEQ ID NO:9) is optimized to the codon bias of *Y. lipolytica*. The resulting codon modified phoI gene is specified in SEQ ID NO:8. The amino acid sequence encoded by SEQ ID NO:8 and SEQ ID NO:9 is specified in SEQ ID NO:10.

The codon modified taqI and phoI genes illustrated in SEQ ID NO:1 and SEQ ID NO:8 serve only as examples of many possible codon modified taqI and phoI genes, or other thermophilic nuclease genes. It is clear to a skilled person that any suitable codon modification method could be used to modify the thermophilic nuclease gene according to the present invention, and the resulting sequence from using different codon modification methods may vary. Having now generally described this invention, it will become more readily understood by referencing the following specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

The following examples illustrate the invention.

## EXAMPLES

Table 1 below describes certain *Yarrowia lipolytica* strains used in the following exemplification:

TABLE 1

| <i>Yarrowia lipolytica</i> strains. |                                                                  |                                                     |
|-------------------------------------|------------------------------------------------------------------|-----------------------------------------------------|
| Strain Number                       | Genotype                                                         | How Constructed                                     |
| ML8195                              | MATA erg9-4789::ura3<br>HMG-tr GGS carB<br>carRP(E78G) ura3 ade1 | Classical and standard molecular genetic techniques |



TABLE 1-continued

| <i>Yarrowia lipolytica</i> strains. |                                                                            |                                                     |
|-------------------------------------|----------------------------------------------------------------------------|-----------------------------------------------------|
| Strain Number                       | Genotype                                                                   | How Constructed                                     |
| ML12805                             | ML8195 [pMB6722]                                                           | Untargeted transformation                           |
| ML11956                             | MATB erg9-4789::URA3HMG-tr GGS carB carRP crtW crtZ (prototrophic)         | Classical and standard molecular genetic techniques |
| ML12924                             | ML11956 [pMB6736]                                                          | Untargeted transformation                           |
| ML11218                             | MATB erg9-4789::URA3 HMG-tr GGS carB carRP crtW crtZ-Xa crtZ-Dc crtW-Δ6180 | Classical and standard molecular genetic techniques |
| ML12921                             | ML11218 [pMB6736]                                                          | Untargeted transformation                           |
| ML13270                             | ML11218 [pMB6868]                                                          | Untargeted transformation                           |

*Yarrowia* strains ML8195, ML12805, ML11956, ML12924, ML11218, ML12921 and ML13270 were constructed by the introduction of heterologous genes under the control of constitutive promoters (for example, TEFL), coupled with several generations of crossbreeding, starting with ML350 and ATCC201249 as described in U.S. Pat. No. 7,851,199 B2. The GGS gene and the truncated HMG gene (“HMG-tr”) were derived from *Yarrowia lipolytica* sequences corresponding to native geranylgeranyl pyrophosphate synthase and hydroxymethylglutaryl-CoA reductase genes, respectively. The carRP and carB genes were derived from *Mucor circinelloides*, and they encode a bifunctional phytoene synthase/lycopene cyclase and a phytoene dehydrogenase, respectively. The crtW gene was synthesized to encode the carotene ketolase of *Parvularcula bermudensis*. The crtZ gene was amplified from *Xanthobacter autotrophicus* (Xa), or synthesized to encode the carotene hydroxylase from *Enterobacteriaceae* bacterium DC404 (Dc) (SEQ ID NO:7). These genes are sometimes but not always associated with auxotrophic markers (URA3, LEU2, URA2, LYS1, ADE1) or a loxP site, remnant of a Hyg<sup>R</sup> (hygromycin resistance) marker.

#### Example 1: Production of Plasmids for *Yarrowia* Strain Construction

Plasmids were generated for expression of TaqI thermophilic nucleases in *Yarrowia* as described in Table 2. A codon optimized taqI nuclease ORF sequence was synthesized de novo based on the sequence of the taqI nuclease gene of *Thermus aquaticus* (SEQ ID NO:2), using the *Y. lipolytica* codon bias as specified in SEQ ID NO:1. This codon-optimized taqI nuclease ORF was cleaved using NheI and MluI and ligated to pMB5082 cut with NheI and MluI to produce pMB6722. The resulting encoded TaqI protein of pMB6722 is specified in SEQ ID NO:3.

In a second plasmid, the same taqI nuclease gene sequence was excised from plasmid pMB6722 and cloned into a different plasmid backbone, pMB6157, to produce a new plasmid, pMB6736.

A plasmid was generated for expression of PhoI thermophilic nuclease as described in Table 2. A codon optimized phoI nuclease ORF sequence was synthesized de novo based on the sequence of the phoI nuclease gene of *Pyrococcus horikoshii* OT3 (SEQ ID NO:9), using the *Y. lipolytica* codon bias as specified in SEQ ID NO:8. This codon-optimized phoI nuclease ORF was cleaved using NheI and MluI and ligated to pMB6771 cut with NheI and MluI to produce pMB6868. The resulting encoded PhoI protein of pMB6868 is specified in SEQ ID NO:10.

Plasmid constructions were performed based on basic molecular biology and DNA manipulation procedures. All basic molecular biology and DNA manipulation procedures described in this and other examples are generally performed according to Sambrook et al. or Ausubel et al. (Sambrook J, Fritsch E F, Maniatis T (eds). 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press: New York; Ausubel F M, Brent R, Kingston R E, Moore D D, Seidman J G, Smith J A, Struhl K (eds). 1998. Current Protocols in Molecular Biology. Wiley: New York).

TABLE 2

| Plasmids |                |        |                                 |
|----------|----------------|--------|---------------------------------|
| Plasmid  | Backbone       | Insert | Oligos or source                |
| pMB6722  | pMB5082 (URA3) | taqI   | Synthesized NheI-MluI fragment  |
| pMB6736  | pMB6157 (HygR) | taqI   | NheI-MluI fragment from pMB6722 |
| pMB6868  | pMB6771 (HygR) | phoI   | Synthesized NheI-MluI fragment  |

#### Example 2: Demonstration of Nuclease Activity of *Y. lipolytica* Expressing TaqI Nuclease

In this example, the gene encoding TaqI nuclease was introduced into a host strain of *Y. lipolytica*. The TaqI nuclease activity in the host strain was tested.

Plasmid pMB6722 was cleaved with XbaI and transformed into host strain ML8195 (Table 1). *Yarrowia* strain ML8195 is a strain which was previously genetically modified to produce lycopene. Transformants introduced with the heterologous taqI nuclease gene were selected on YNB glutamate plates supplemented with 0.12 mM adenine; one such colony was selected and designated as *Yarrowia* strain ML12805. ML12805 was incubated for 3 days at 30° C. at 250 rpm in 20 mL YPD medium in a 125 mL flask. The cultures were split and 1.5 mL aliquots incubated at 65° C. or 25° C. for 5 min to 24 hr.

DNA from strains ML8195 and ML12805 was extracted and analyzed after incubation at 65° C. or 25° C. DNA was extracted using a “Smash and Grab” yeast genomic DNA extraction protocol. Essentially, 1.5 mL of a 3-day culture was pelleted, supernatant decanted and the cells resuspended in 0.5 mL water. Cells were pelleted for 10 seconds and supernatant decanted. The pellet was resuspended in 0.2 mL Smash and Grab Solution (1% SDS, 2% Triton-X100, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA) and 0.2 mL phenol-chloroform-isoamyl alcohol, and 0.3 g glass beads were added. The tubes were vortexed for 5 min using a multi-tube holder. To the mixtures, 0.2 ml, TE pH 8.0 (10 mM Tris, 1 mM EDTA) was added and centrifuged for 5 min. The aqueous phase was transferred to a new tube and 1.0 mL cold ethanol was added and mixed to precipitate the DNA. After centrifugation for 5 min, the supernatant was removed. The pellet was resuspended in 0.4 mL TE with 75 μg/mL RNaseA and incubated at room temperature for 5 min. 10 μL 4 M ammonium acetate and 1.0 mL ethanol was added. After incubation on ice for 5 minutes and centrifugation for 10 minutes, the supernatant was discarded. The pellet was washed with 0.5 mL 70% ethanol and centrifuged for 10 min. The supernatant was discarded and the pellet air



dried, then resuspended in 100  $\mu$ L TE. The resulting DNA from the above extraction process was analyzed by electrophoresis on 0.8% agarose gel in 1 $\times$  Tris-acetate-EDTA (TAE) running buffer at 100 V for 1.5 h.

As shown in FIG. 1, the host strain of *Y. lipolytica* with no taqI nuclease gene (ML8195) did not display DNA degradation activity at either 65 $^{\circ}$  C. or 25 $^{\circ}$  C. The host strain of *Y. lipolytica* that harbors the taqI nuclease gene (ML12805) displayed DNA degradation activity at 65 $^{\circ}$  C. but not at 25 $^{\circ}$  C. The degradation activity was observed as early as 20 minutes after the cell culture was incubated at 65 $^{\circ}$  C. The ML12805 strain did not display any DNA degradation activity at 25 $^{\circ}$  C. even after being incubated for 24 hours, showing that TaqI nuclease in the *Y. lipolytica* strain is latent at 25 $^{\circ}$  C.

#### Example 3: Temperature Activity Profile of TaqI

In this example, the activity of TaqI nuclease was examined at different temperatures.

*Y. lipolytica* strains ML12805 and ML8195 were grown for 3 days as described in Example 2. 1.5 mL aliquots of ML12805 and ML8195 cells were incubated at temperatures of 25 $^{\circ}$  C., 30 $^{\circ}$  C., 40 $^{\circ}$  C., 50 $^{\circ}$  C. and 65 $^{\circ}$  C. for 1.5 h and DNA was extracted and analyzed as described in Example 2.

As shown in FIG. 2, no DNA degradation was observed at temperatures of 25 $^{\circ}$  C., 30 $^{\circ}$  C., 40 $^{\circ}$  C., and 50 $^{\circ}$  C., in both strains ML12805 and ML8195. DNA degradation was observed at 65 $^{\circ}$  C., only in strain ML12805, as was shown in Example 2. The results show that TaqI nuclease is latent at temperatures equal to or below 50 $^{\circ}$  C.

#### Example 4: PCR Analysis of In Vivo TaqI-Digested gDNA

In this example, the degradation of the heterologous carB gene in a recombinant *Y. lipolytica* strain was examined.

The carB gene is 1740 nucleotides in length and encodes phytoene dehydrogenase, an enzyme in the carotenoid biosynthetic pathway. The carB gene has been introduced into *Y. lipolytica* to enable production of carotenoids. As described in Example 2, ML8195 is a lycopene-producing strain lacking any thermophilic nuclease, and ML12805 is a derivative of ML8195 containing the taqI nuclease gene; both strains therefore contain the carB gene.

The presence of the carB gene was examined in strains ML8195 and ML12805 at both 25 $^{\circ}$  C. and 65 $^{\circ}$  C. Serial dilutions of genomic DNA from ML12805 incubated at 25 $^{\circ}$  C. or 65 $^{\circ}$  C. for 24 hours were prepared. PCR was performed on the above samples using primers designed for amplifying a 529 bp fragment of the carB gene. The sequence of primer MO4641 is specified in SEQ ID NO:5. The sequence of primer MO4642 is specified in SEQ ID NO:6. The DNA fragment of the carB gene between oligos MO4641 and MO4642 contains two TaqI sites.

PCR was performed under the conditions described below. In each 25  $\mu$ L reaction, 1  $\mu$ L of diluted gDNA was combined with 0.5  $\mu$ L of each primer (10  $\mu$ M stock solution), 0.5  $\mu$ L water and 22.5  $\mu$ L of Platinum Taq Super Mix. The reaction parameters were 1 cycle at 94 $^{\circ}$  C. for 2 min followed by 45 cycles at 94 $^{\circ}$  C. for 30 s, 58 $^{\circ}$  C. for 30 s and 72 $^{\circ}$  C. for 60 s. A final single cycle of 72 $^{\circ}$  C. for 5 min finished the PCR in a MJ Research PTC-225 Peltier Thermal Cycler. The entire reaction plus 1 $\times$  loading dye was loaded onto a 2% agarose in 1 $\times$ TAE gel with 0.5  $\mu$ g/ml ethidium bromide and run at 100 V for 1.5 h.

As shown in FIG. 3, for the *Y. lipolytica* strain in which the taqI nuclease gene was introduced (ML12805), a 529 bp PCR product of carB was observed in the sample which was incubated at 25 $^{\circ}$  C. but not in the sample which was incubated at 65 $^{\circ}$  C. (serial dilutions of the PCR template were performed to demonstrate the sensitivity of the assay, as is known to those of ordinary skill in the art). These results show that the TaqI nuclease degraded the recombinant DNA (carB gene) in strain ML12805 when incubated at 65 $^{\circ}$  C. FIG. 3 also illustrates that foreign DNA in strain ML12805 can be targeted for degradation, and that the cleavage is complete, resulting in no detectable fragment of approximately 500 nucleotides.

#### Example 5a: Astaxanthin Production in a *Y. lipolytica* Strain Harboring the taqI Thermophilic Nuclease Gene

In this example, the production of astaxanthin in a strain of *Y. lipolytica* was examined, both before and after the taqI nuclease gene was introduced.

In this example, a strain of *Y. lipolytica* which produces astaxanthin (ML11956) was used. A second strain (ML12924) was constructed by introducing the taqI nuclease gene (MB6736) into strain ML11956. The amount of astaxanthin and its precursors in both strains was measured. Measurements were made at different time points during the cell growth period and after the host strains were incubated at 65 $^{\circ}$  C.

Strains ML11956 and ML12924 were grown in a fermentor using a fed-batch process. Samples were taken at different time points during the fermentation process and carotenoid analysis was performed according to the methods described previously in U.S. Pat. No. 7,851,199 B2.

FIG. 4 shows that the amount of astaxanthin increased as strain ML11956 grew and the production eventually reached a plateau. The amount of astaxanthin did not change after ML11956 was incubated at 65 $^{\circ}$  C. Strain ML12924 behaved in the same manner as strain ML11956. The amount of astaxanthin produced by strain ML12924 was the same as in strain ML11956 throughout the fermentation period and after incubation at 65 $^{\circ}$  C. The amount of astaxanthin precursors produced also remained unchanged in strains ML11956 and ML12924 before and after incubation at 65 $^{\circ}$  C. This result shows that neither the introduction of the taqI nuclease gene nor activation of its activity affected the amount or purity of astaxanthin produced by *Y. lipolytica*.

#### Example 5b: Zeaxanthin Production in a *Y. lipolytica* Strain Harboring the taqI Thermophilic Nuclease Gene

In this example, the production of zeaxanthin in a host strain of *Y. lipolytica* with the taqI nuclease gene was examined.

In this example, a strain of *Y. lipolytica* which produces zeaxanthin (ML11218) was transformed with a taqI nuclease gene (MB6736). In the resulting *Y. lipolytica* strain (ML12921), the amount of zeaxanthin and its precursors produced were measured. Measurements were made at different time points during the cell growth period and were also made after the host strain was incubated at 65 $^{\circ}$  C.

Strain ML12921 was grown in a fermentor using a fed-batch process. Samples were taken at different time points during the fermentation process and carotenoid analysis was performed according to the methods described previously in U.S. Pat. No. 7,851,199 B2.



FIG. 5 shows that the amount of zeaxanthin increased as strain ML12921 grew and the production eventually reached a plateau. The amount of zeaxanthin produced did not change after the host cells were incubated at 65° C. The amount of zeaxanthin precursors also remained the same after the host cells were incubated at 65° C. In the control strain ML11218 (not shown), zeaxanthin production was similar to ML12921 in all the conditions tested. This result shows that neither the presence of the TaqI nuclease nor its activation affect the production or purity of zeaxanthin in *Y. lipolytica*.

#### Example 5c: Demonstration of TaqI Nuclease Activity in Strains Producing Astaxanthin or Zeaxanthin

In this example, the TaqI nuclease activities of *Y. lipolytica* strains ML11956, ML12924, and ML12921 described in Examples 5a and 5b were examined.

Strains ML11956, ML12924, and ML12921 were grown in fermentors. For strains ML11956 and ML12924 (producing astaxanthin in a longer fermentation), samples were taken at 46, 121 and 236 h post inoculation. For strain ML12921 (producing zeaxanthin in a shorter fermentation), samples were taken at 46 and 69 h post inoculation. At the end of the fermentation process (236 h for astaxanthin strains and 69 h for zeaxanthin strains) the temperature was raised to 65° C. and strains were incubated at this temperature for 6 hours. Samples were taken at 0 min, 10 min, 30 min, 2 h and 6 h after the shift to the higher temperature. DNA was extracted and analyzed as described in Example 2.

As shown in FIG. 6, no DNA degradation was observed in strain ML11956 lacking the nuclease. In both strains ML12924 and ML12921, DNA degradation was observed after the cells were incubated at 65° C. The above result shows that the TaqI nuclease was latent at the fermentation temperature but was active at 65° C. in *Y. lipolytica* strains, allowing for DNA degradation without affecting the amount or purity of the desired end product (astaxanthin or zeaxanthin).

#### Example 6: Temperature Range for TaqI and PhoI Centered Around their Respective Optima

In this example, the activities of TaqI and PhoI nucleases were examined at and around their optimum temperatures.

As shown in FIG. 7, the strain of *Y. lipolytica* harboring the taqI nuclease gene (ML12921) displayed DNA degradation activity at 65° C. but not at 25° C. ML12921 further displayed DNA degradation activity at 60° C. and 70° C., i.e.,  $\pm 5^\circ$  C. of 65° C., the optimal temperature for TaqI nuclease activity. Similarly, the host strain of *Y. lipolytica* with the PhoI nuclease gene (ML13270) displayed DNA degradation activity at 75° C. but not at 25° C. ML13270 also displayed DNA degradation activity at 70° C. and 80° C., i.e.,  $\pm 5^\circ$  C. of 75° C., the optimal temperature for PhoI nuclease activity. The results show that in a *Y. lipolytica* host strain transformed with an exogenous nuclease gene, the genomic DNA of the host strain can be degraded within a temperature range of at least 10 degrees centered around the optimal temperature for the nuclease gene. This result was observed in two different nucleases where each nuclease has a different optimal temperature.

#### Example 7: Production of Plasmids for Bacterial Expression

A plasmid was generated for expression of TaqI thermophilic nuclease in *E. coli* as shown in Table 3. A codon

optimized taqI nuclease ORF sequence was synthesized de novo based on the sequence of the taqI nuclease gene of *Thermus aquaticus* (SEQ ID NO:2), using the *E. coli* codon bias as specified in SEQ ID NO:11. This codon-optimized taqI nuclease ORF was cleaved using NcoI and Acc65I and ligated to pMB4124 (Kan<sup>R</sup> oriP15) cut with NcoI and Acc65I to produce pMB7268 (trcP-taqI). The resulting encoded TaqI protein of pMB7268 is specified in SEQ ID NO:3.

For expression of TaqI thermophilic nuclease in *Corynebacterium glutamicum*, a fragment was generated by PCR with oligos MO9315 and MO9316 and pMB6736 as template, cleaved with NcoI and KpnI, and ligated to pMB4124 cut with NcoI and Acc65I to produce pMB7263 (trcP-taqI). The resulting encoded TaqI protein of pMB7263 is specified in SEQ ID NO:3. The sequence of primer MO9315 is specified in SEQ ID NO:12. The sequence of primer MO9316 is specified in SEQ ID NO:13.

TABLE 3

| Plasmids |                                    |        |                                                                      |  |
|----------|------------------------------------|--------|----------------------------------------------------------------------|--|
| Plasmid  | Backbone                           | Insert | Oligos or source                                                     |  |
| pMB4124  | KanR oriP15 lacI <sup>q</sup> trcP | lacZ   | Previously synthesized                                               |  |
| pMB7268  | pMB4124                            | taqI   | Synthesized NcoI-Acc65I fragment                                     |  |
| pMB7263  | pMB4124                            | taqI   | PCR amplified using oligos MO9315 and MO9316 and pMB6736 as template |  |

#### Example 8: Demonstration of Nuclease Activity of *E. coli* Expressing TaqI Nuclease

In this example, the gene encoding TaqI nuclease was introduced into a host strain of *E. coli*. The TaqI nuclease activity in the host strain was tested.

*E. coli* DH5a (F- $\Phi$ 80lacZ $\Delta$ M15  $\Delta$ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44  $\lambda$ -thi-1 gyrA96 relA1) harboring either pMB4124 (trcP-lacZ) or pMB7268 (trcP-taqI) was grown to exponential phase at 30° C., shifted to 37° C. for 1 hr, and induced with 500  $\mu$ M isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) for 2.5 hr. Cells were then incubated at 25° C. for 4 and 18 hr, or at 65° C. for 1.5, 4, and 18 hr. Total DNA was prepared from  $\sim 1 \times 10^8$  A<sub>600</sub> unit of cells using a Qiagen Blood and Tissue DNeasy Kit, following the manufacturer's recommendations, except that the incubation temperature for the Proteinase K digestion was changed to 22° C. As seen in FIG. 8, the cells harboring the trcP-taqI construct showed extensive DNA degradation at 65° C. but not at 25° C. The control cells lacking the trcP-taqI construct do not exhibit degradation at either temperature.

#### Example 9: Demonstration of Nuclease Activity of *C. glutamicum* Expressing TaqI Nuclease

In this example, the gene encoding TaqI nuclease was introduced into a host strain of *C. glutamicum*. The TaqI nuclease activity in the host strain was tested.

*C. glutamicum* strain MA428 was constructed by replacing the native hom(thrA)-thrB operon in ATCC 13032 with a feedback-resistant allele (G362E) of *Streptomyces coeli-*



*color* hom driven by the *C. glutamicum* *gpd* promoter (U.S. patent application Ser. No. 10/858,730, now abandoned). This strain was transformed with pMB7263 (*trcP-taqI*), and a Kan<sup>R</sup> colony was grown at 30° C. in BHI medium supplemented with 20 µg/ml kanamycin, and induced overnight with 250 µM IPTG. Cells were then incubated at 25, 60, 65 or 70° C., and DNA was extracted using a Qiagen Blood and Tissue DNeasy Kit, following the manufacturer's recommendations for gram-positive bacteria, except that the incubation temperature for the Proteinase K digestion was changed to 22° C. Cells harboring the *trcP-taqI* construct were incubated at 25° C. for 5 hrs and at 65° C. for 0.5, 1, 2.5 and 5 hrs. As seen in FIG. 9, cells containing the *trcP-taqI* construct showed extensive DNA degradation at

65° C. but not at 25° C. FIG. 10 depicts the temperature dependence of the activity (all incubations were for 5 hrs). The genomic DNA of the host strain *C. glutamicum* was degraded within a temperature range of at least 10 degrees centered around the optimal temperature for TaqI nuclease of 65° C.

All of the various aspects, embodiments, and options described herein can be combined in any and all variations.

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

## SEQUENCE LISTING

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<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Nucleotide sequence containing the codon optimized *taqI* gene

<400> SEQUENCE: 1

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gcaggacctt ccccagagac tgaaccccct tcccagctg tacgagcact actggaaggg      180
ccttgaggat aaccctcct tccctgggctt cgaggagttc tttgaccact ggtgggagaa      240
gcgacttoga cccctcgacg agttcattcg aaagtacttt tggggttgct cctacgcctt      300
tgttcgactt ggccttgagg ctogactgta ccgaactgcc gtttccatct ggactcagtt      360
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tgacgcccag ggcattgacg cccctgattca cacttccggt tcctctaccg gaatccagat      480
caagaaggag acttaccgat ccgaggccaa gtccgagaac cgatttctcc gaaagcagcg      540
aggcaccgcc ctcatcgaga ttccctacac ccttcagact cccgaggagc ttgaggagaa      600
ggccaagcga gcccagatta acggagagac ttaccgactt tgggccaagg ttgctcacca      660
ccttgaccga cttgagaacg gttttgtcat ttttcgagag tcttacgta agtctattga      720
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<210> SEQ ID NO 2

<211> LENGTH: 792

<212> TYPE: DNA

<213> ORGANISM: *Thermus aquaticus*

<220> FEATURE:

<223> OTHER INFORMATION: *taqI* gene

<400> SEQUENCE: 2

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agggagctga acccgcttcc ggacctgtac gagcattatt ggaaagcgct tgaggataac      180
ccttccttcc tgggcttcga agagttcttt gaccactggg gggaaaagcg cctacggccc      240
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ctcgaggcta ggctgtaccg aacagccggt tccatctgga ctcagtttca cttctgctac 360
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atagacgcgc tgattcatac aagcgggtcc tcaacaggaa tccagatcaa aaaggaaact 480
taccgttccg aggccaagag cgagaaccgc tttttaagga agcaaagagg caccgccttc 540
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agagtgaacg gagaaaccta cagtctatgg gccaaaggtg cacaccattt ggaccgtcta 660
gaaaaaggat tcgtcatttt tcgggaaagt tatgtgaaaa gcattgagct ttttctccag 720
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accgccccgt ga 792

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<212> TYPE: PRT
<213> ORGANISM: Thermus aquaticus
<220> FEATURE:
<223> OTHER INFORMATION: Amino Acid sequence of TaqI nuclease

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

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 <223> OTHER INFORMATION: carB

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cgctgctect tgatccatca ccagggccat cgctttgatc agggcccgtc gctctacctg    180
atgccaagt actttgagga cgcccttgcc gatctggacg agcgattca agaccacctg    240
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accaagaaga tgcgcatggc attcacgttt cagaccatgt atatgggcat gtgcacctac    600
gatgcgcctg ctgtctacag cctggttcag tacaccgagt tcgctgaagg catctggtat    660
ccccgtggcg gcttcaacat ggtggttcag aagctagagg cgattgcaa gcaaaagtac    720
gatgccgagt ttatctaaa tgcgcctggt gccaaagatta acaccgatga tgccacaaa    780
caagtgacag gtgtaacctt ggaaaatggc cacatcatcg atgccgatgc ggttgtgtgt    840
aacgcagatc tggctctatg ttatcacaat ctgttgctc cctgccgatg gacgcaaaac    900
aactggctt ccaagaaatt gacgtcttct tccatttct tctactggtc catgtccacc    960
aaggtgcctc aattggacgt gcacaacatc tttttggccg aggcttatca ggagagcttt   1020
gacgaaatct tcaaggactt tggcctgcct tctgaagcct ccttctacgt caatgtgccc   1080
tctcgcacg atccttctgc tgetcccagc ggcaaggact ctgtcattgt cttggtgcct   1140
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19



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<210> SEQ ID NO 7  
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 <212> TYPE: DNA  
 <213> ORGANISM: Yarrowia lipolytica  
 <220> FEATURE:  
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 cataccccgc gcaaaggggc gtttgagcgt aacgatctct atgcggtggt gtttgcgcta 180  
 ctggccattg cgctgattta cgcgggcagc gaagggactt ggccgcttca gtggattggc 240  
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 cggctgcata acgcggtgcg ggggcgcgaa ggggtcgtct ccttcggggt tatctacgcc 420  
 ccaccggtgg acaagctgca ggccggtgctg cgcgaacgta acggcagacc cgccagcgcg 480  
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 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Nucleotide sequence containing the codon optimized phoI gene

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 cccgatctcc tgatcgaaag gtttctatgc ccattcccat tgtttactcc gattttggtg 180  
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 tccctgcttt cgagaacaag acccgagtta ttcgagagaa gcagcagggt ggcaaggagg 540  
 ttacttacta cgctaagggt aacgtcaaga acttctctct ttgtctcggg tgtttcgatc 600  
 tcgctcagcg ataccttcag atcaaggcta aggagcactg taacatttac cctggttctc 660  
 ccacctgtaa cgattctctc tctaagctca agctccgact tgagtacgat ccctccatta 720  
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ccgatgatta aacgcgt 977

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<212> TYPE: DNA
<213> ORGANISM: Pyrococcus horikoshii
<220> FEATURE:
<223> OTHER INFORMATION: phoI gene

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attgagaggt taatacctcc ggagtggctt gaaatctcgc cagagcaatt aggttgggaa 240
gaaacttatt tagagaaca acgtggtttt aaactaccta aagaagaggt ctacgtggat 300
gttagtataa gcaacgattc cataatattc gagctagacg tgaaaaatta tcacctgaa 360
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gatttgaagt acgtagatga gttcattaac gctttaaggg aacatatccc agctttcgaa 480
aataaaaca gggttattcg tgaaaagcag caagggggca aggaagttac atattatgct 540
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cttcaaatca aagctaaaga gcactgtaac atatatccag gctcaccaac atgtaacgat 660
tctctaagca aattaaatt aaggcttgaa tacgatccat ccattactac ctttgctaaa 720
gttgaatag ccaagatctc aggcaagaga cccagatca tggttaaatt aacctctacg 780
gaaactaaaa ctataagggg aatacttaag ccagaaataa aaggtaaggc gcgtggtaaa 840
cttgatatt gcgatcacag agaaaagaga cagtatatag ctttgactt gtttgatttt 900
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<210> SEQ ID NO 10
<211> LENGTH: 318
<212> TYPE: PRT
<213> ORGANISM: Pyrococcus horikoshii
<220> FEATURE:
<223> OTHER INFORMATION: Amino acid sequence of PhoI nuclease

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<400> SEQUENCE: 10

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

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Val Tyr Val Asp Val Ser Ile Ser Asn Asp Ser Ile Ile Phe Glu Leu  
                   100                                  105                                  110  
 Asp Val Lys Asn Tyr His Leu Glu Arg Thr Ser Ile Arg Gly Ile Asn  
           115                                  120                                  125  
 Pro Glu Lys Trp Lys Asn Trp Val Met Phe Tyr Ile Asp Leu Lys Tyr  
       130                                  135                                  140  
 Val Asp Glu Phe Ile Asn Ala Leu Arg Glu His Ile Pro Ala Phe Glu  
 145                                  150                                  155                                  160  
 Asn Lys Thr Arg Val Ile Arg Glu Lys Gln Gln Gly Gly Lys Glu Val  
                   165                                  170                                  175  
 Thr Tyr Tyr Ala Lys Val Asn Val Lys Asn Phe Ser Leu Cys Leu Gly  
           180                                  185                                  190  
 Cys Phe Asp Leu Ala Gln Arg Tyr Leu Gln Ile Lys Ala Lys Glu His  
       195                                  200                                  205  
 Cys Asn Ile Tyr Pro Gly Ser Pro Thr Cys Asn Asp Ser Leu Ser Lys  
       210                                  215                                  220  
 Leu Lys Leu Arg Leu Glu Tyr Asp Pro Ser Ile Thr Thr Phe Ala Lys  
 225                                  230                                  235                                  240  
 Val Gly Ile Ala Lys Ile Ser Gly Lys Arg Pro Gln Ile Met Val Lys  
           245                                  250                                  255  
 Leu Thr Ser Thr Glu Thr Lys Thr Ile Arg Gly Ile Leu Lys Pro Glu  
           260                                  265                                  270  
 Ile Lys Gly Lys Ala Arg Gly Lys Leu Val Tyr Cys Asp His Arg Glu  
           275                                  280                                  285  
 Lys Arg Gln Tyr Ile Ala Leu Asp Leu Phe Asp Phe Tyr Lys Ala Leu  
       290                                  295                                  300  
 Val Ser Thr Lys Lys Tyr Glu Gly Lys Leu Pro Thr Asp Asp  
 305                                  310                                  315

<210> SEQ ID NO 11  
 <211> LENGTH: 800  
 <212> TYPE: DNA  
 <213> ORGANISM: Thermus aquaticus  
 <220> FEATURE:  
 <223> OTHER INFORMATION: DNA sequence encoding TaqI nuclease

<400> SEQUENCE: 11

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ccatggcatc aaccaagca caaaaagccc tggaaacctt cgaacgcttc ctggcgagcc      60
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cgcgtgaact gaaccgctg ccggacctgt atgaacatta ctggaaagca ctggaagata      180
atccgtcctt tctgggcttc gaagaatddd tcgatcactg gtgggaaaaa cgtctgcgcc      240
cgttggaaga atttattcgc aaatatttct ggggctgcag ctacgcgttt gttcgtctgg      300
gtctggaagc gcgtctgtat cgcaccgccg tcagcatctg gacgcaattt catttctgct      360
accgctggaa cgcctcttgt gaactgccgc tggaaagcag accggaactg gatgcacagg      420
gcattgagc tctgatccac accagtggca gctctacggg tatccaaatc aaaaaagaaa      480
cctaccgtag tgaagcaaaa tccgaaaatc gttttctgcg caaacagcgt ggtacggctc      540
tgattgaaat cccgtatacc ctgcaaacgc cggagaact ggaagaaaaa gcaaaactg      600
ctcgcgtcaa cggcgaacc tacctctgt gggcgaaagt ggccatcac ctggatcgcc      660
tggaaaatgg tttgtgatt ttccgtgaat catacgttaa atcgatcgaa ctgttctgc      720
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tgaccgctcc gtgaggtacc                                     800
  
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<210> SEQ ID NO 12  
 <211> LENGTH: 29  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Primer M09315

<400> SEQUENCE: 12

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29

<210> SEQ ID NO 13  
 <211> LENGTH: 34  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Primer M09316

<400> SEQUENCE: 13

cacacaggtta ccttagggg cggtgagggc ctcc

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What is claimed is:

1. A genetically modified yeast or fungal cell which comprises a heterologous nucleic acid sequence encoding a thermophilic TaqI or PhoI nuclease gene, wherein the thermophilic nuclease produced by said thermophilic TaqI or PhoI nuclease gene with an optimum temperature of 50° C. or higher and which is latent at the temperature at which the cell has normal growth, wherein the genetically modified cell produces one or more biological compounds or fine chemicals which are not the thermophilic nuclease, said biological compound being selected from the group consisting of: phytoene, lycopene, beta-carotene, alpha-carotene, beta-cryptoxanthin, lutein, zeaxanthin, astaxanthin, canthaxanthin, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, adonirubin, violaxanthin, adonixanthin, vitamin E, retinol, retinal, retinoic acid, retinyl palmitate, and modified forms thereof.

2. The cell of claim 1, wherein the nuclease is a DNA-degrading nuclease.

3. The cell of claim 2, wherein the DNA-degrading nuclease is TaqI nuclease.

4. The cell of claim 2, wherein the DNA-degrading nuclease is PhoI nuclease.

5. The cell of claim 1, wherein the yeast or fungal cell is selected from the group consisting of: *Yarrowia*, *Candida*, *Hansenula*, *Saccharomyces*, *Mortierella*, *Schizosaccharomyces*, *Aspergillus*, *Fusarium*, *Trichoderma*, and *Thraustochytrium*.

6. The cell of claim 5, wherein the *Yarrowia* species is *Yarrowia lipolytica*.

7. The cell of claim 1, wherein the gene of the thermophilic nuclease is codon optimized to match the codon usage bias of the yeast or fungal cell.

8. The cell of claim 3, wherein the TaqI nuclease comprises a polypeptide having an amino acid sequence of SEQ ID NO: 3.

9. The cell of claim 4, wherein the PhoI nuclease comprises a polypeptide having an amino acid sequence of SEQ ID NO: 10.

10. The cell of claim 1, wherein the yeast or fungal cell comprises a combination of more than one thermophilic nuclease gene.

11. A method for degrading the nucleic acids of a host cell in vivo and/or in situ, wherein the method comprises:

a) growing the host cell at a temperature at which the thermophilic nuclease is latent; and

b) degrading the nucleic acids of the host cell by changing the temperature in step (a) to a temperature at which the thermophilic nuclease is active,

wherein the host cell is a genetically modified yeast or fungal cell of claim 1.

12. The method of claim 11, wherein the nuclease is a DNA-degrading nuclease and the nucleic acids are DNA.

13. The method of claim 12, wherein the DNA of the host cell contains recombinant DNA.

14. The method of any of claim 13, wherein the temperature in step (a) is optimal for the growth of the host cell.

15. The method of any of claim 13, wherein the degradation is conducted at a temperature that is within about  $\pm 5^\circ$  C. of the optimum temperature of said thermophilic nuclease.

16. The method of claim 15, wherein the degradation is conducted at a temperature that is optimal for the activity of the thermophilic nuclease.

17. The method of claim 11, wherein the degradation is conducted during the pasteurization of the host cell.

18. The method of claim 11, wherein the degradation is conducted in a temperature range between 40° C. and 100° C.

19. The method of claim 12, wherein the DNA-degrading nuclease is TaqI nuclease.

20. The method of claim 19, wherein the degradation is conducted at a temperature ranging between about 60° C. and about 70° C.

21. The method of claim 19, wherein the degradation is conducted at 65° C.

22. The method of claim 12, wherein the DNA-degrading nuclease is PhoI nuclease.

23. The method of claim 22, wherein the degradation is conducted at a temperature ranging between about 70° C. and about 80° C.

24. The method of claim 23, wherein the degradation is conducted at 75° C.

25. The method of claim 11, wherein the host cell is selected from a group consisting of: *Yarrowia*, *Candida*,



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*Hansenula*, *Saccharomyces*, *Mortierella*, *Schizosaccharomyces*, *Aspergillus*, *Fusarium*, *Trichoderma*, and *Thraustochytrium*.

26. The method of claim 25, wherein the *Yarrowia* species is *Yarrowia lipolytica*.

27. The method of claim 11, wherein the gene of the thermophilic nuclease is codon optimized to match the codon usage bias of the host cell.

28. The method of claim 19, wherein the TaqI nuclease comprises a polypeptide having an amino acid sequence of SEQ ID NO:3.

29. The method of claim 22, wherein the PhoI nuclease comprises a polypeptide having an amino acid sequence of SEQ ID NO: 10.

30. The method of claim 11, wherein more than one thermophilic nuclease gene is introduced into the host cell.

31. The method of claim 13, wherein said recombinant DNA comprises cleavage sites of the thermophilic nuclease.

32. The method of claim 13, wherein said recombinant DNA comprises one cleavage site of the thermophilic nuclease every 500 or fewer nucleotides.

33. A process for the production of a biomass product which is free of active nucleic acid molecules, wherein the biomass product is the product of a host cell, wherein the process comprises:

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a) fermenting the host cell at a temperature at which the thermophilic nuclease is latent;

b) degrading the nucleic acids of the host cell by changing the temperature in step (a) to a temperature at which the thermophilic nuclease is active; and

c) recovering the biomass product;

wherein the order for performing steps b) and step c) may be exchanged, and wherein the host cell is a genetically modified yeast or fungal cell of claim 1.

34. The process of claim 33, wherein the nuclease is a DNA-degrading nuclease and the nucleic acids are DNA.

35. The process of claim 34, wherein the DNA of the host cell contains recombinant DNA.

36. The process of any of claims 33-35, wherein the temperature in step (a) is optimal for the growth of the host cell.

37. The process of any of claims 33-35, wherein the degradation is conducted at a temperature that is within about  $\pm 5^\circ$  C. of the optimum temperature of said thermophilic nuclease.

\* \* \* \* \*