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(54) Title: COMPOSITIONS AND METHODS FOR INHIBITING BIOFILM DEPOSITION AND PRODUCTION

(57) Abstract: The invention provides a method for combating biofilm, said method comprising contacting a biofilm with a composition comprising effective amounts of one or more biofilm degrading enzymes in combination with antimicrobial agents or antimicrobial essential oils. The biofilm may be on an animate or inanimate surface and both medical and non-medical uses and methods are provided. In a preferred aspect the invention provides a composition and method for use in the treatment or prevention of a biofilm infection in a subject, particularly in the oral cavity.



Compositions and Methods for Inhibiting Biofilm Deposition and Production

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10 This application claims priority to PCT/US2017/032437 filed May 12, 2017, the entire disclosure being incorporated herein by reference as though set forth in full.

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Field of the Invention

15 The present invention relates to the fields of biofilm deposition and the treatment of disease. More specifically, the invention provides compositions and methods useful for the treatment of dental caries and other oral diseases. The invention also provides methods for coating biomedical devices for inhibiting undesirable biofilm deposition thereon.

Background of the Invention

20 Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated by reference herein as though set forth in full.

Biopharmaceuticals produced in current systems are prohibitively expensive and are not affordable for large majority of the global population. The cost of protein drugs (\$140 billion in 25 2013) exceeds GDP of >75% of countries around the globe [Walsh 2014], making them

unaffordable. One third of the global population earns <\$2 per day and can't afford any protein drug (including the underprivileged, elderly and lower socio-economic groups in the US). Such high costs are associated with protein production in prohibitively expensive fermenters, purification, cold transportation/storage, short shelf life and sterile delivery methods [Daniell et al 2015, 2016].

Biofilms are formed by a complex group of microbial cells that adhere to the exopolysaccharide matrix present on the surface of medical devices. Biofilm-associated infections associated with medical device implantation pose a serious problem and adversely affects the function of the device. Medical implants used in oral and orthopedic surgery are fabricated using alloys such as stainless steel and titanium. Surface treatment of medical implants by various physical and chemical techniques has been attempted in order to improve surface properties, facilitate biointegration and inhibit bacterial adhesion as bacterial adhesion is associated with surrounding tissue damage and often results in malfunction of the implant.

Many infectious diseases in humans are caused by biofilms, including those occurring in the mouth [Hall-Stoodley et al., 2004; Marsh, et al 2011]. For example, dental caries (or tooth decay) continue to be the single most prevalent biofilm-associated oral disease, afflicting mostly underprivileged children and adults in the US and worldwide, resulting in expenditures of >\$81 billion annually [Beiker and Flemmig, 2011; Dye et al., 2015; Kassebaum et al, 2015]. Caries-causing (cariogenic) biofilms develop when bacteria accumulate on tooth-surfaces, forming organized clusters of bacterial cells that are firmly adherent and enmeshed in a extracellular matrix composed of polymeric substances such as exopolysaccharides (EPS) [Bowen and Koo, 2011]. Current topical antimicrobial modalities for controlling cariogenic biofilms are limited. Chlorhexidine (CHX) is considered the 'gold standard' for oral antimicrobial therapy, but has adverse side effects including tooth staining and calculus formation, and is not recommended for daily therapeutic use [Jones, 1997; Autio-Gold, 2008]. As an alternative, several antimicrobial peptides (AMPs) have emerged with potential antibiofilm effects against caries-causing oral pathogens such as *Streptococcus mutans* [da Silva et al., 2012; Guo et al., 2015].

Antimicrobial peptides (AMP) are an evolutionarily conserved component of the innate immune response and are naturally found in different organisms, including humans. When compared with conventional antibiotics, development of resistance is less likely with AMPs. They are potentially active against bacteria, fungi and viruses and can be tailored to target specific pathogens by

fusion with their surface antigens (Lee et al 2011; DeGray et al 2001; Gupta et al 2015). Linear AMPs have poor stability or antimicrobial activity when compared to AMPs with complex secondary structures. For example, retrocyclin or protegrin have high antimicrobial activity or stability when cyclized (Wang et al 2003) or when forming a hairpin structure (Chen et al 2000) via disulfide bond formation. RC 101 is highly stable at pH 3, 4, 7 and temperature 25°C to 37°C as well as in human vaginal fluid for 48 hours (Sassi et al 2011a), while its antimicrobial activity was maintained for up to six months (Sassi et al 2011b). Likewise, protegrin is highly stable in salt or human fluids (Lai et al 2002; Ma et al 2015) but lost potency when linearized. These intriguing characteristics of antimicrobial peptides with complex secondary structures may facilitate development of novel therapeutics. However, the high cost of producing sufficient amounts of antimicrobial peptides is a major barrier for their clinical development and commercialization.

Summary of the Invention

In accordance with the present invention, a multi-component composition comprising at least one antimicrobial peptide (AMP) and at least one biofilm degrading enzyme which act synergistically to degrade biofilm structures and inhibit biofilm deposition is provided. In certain embodiments, the AMP is selected from protegrin 1, RC-101 and the AMPs listed in Table 1. The biofilm degrading enzyme, includes, for example, mutanase, dextranase, glucoamylase, deoxyribonuclease I, DNAase, dispersin B, glycoside hydrolases and the enzymes provided in Table 2. In certain embodiments, the coding sequences for these enzymes are codon optimized for expression in a plant chloroplast. In a particularly preferred embodiment, the at least one AMP and at least one biofilm degrading enzyme are produced recombinantly. In a particularly preferred embodiment the AMP and biofilm degrading enzyme(s) are expressed as a fusion protein. In some embodiments, the enzymes are chemically synthesized, obtained from a microorganism or obtained from a commercial supplier. When the composition is for the treatment of oral diseases, the composition may optionally further comprise an antibiotic, fluoride, CHX, essential oils, or all of the above. The composition may be contained within chewing gum, hard candy, or within an oral rinse. Preferred fusion proteins of the invention include, without limitation, PG-1-Mut, PG-1-Dex, PG-1-Mut-Dex, RC-101-Mut, RC-101-Dex, RC-101-Mut-Dex for use alone or in combination for the degradation of biofilms. Notably any

of the AMPs listed in Table 1 can replace either PG-1 or RC-101 in the aforementioned fusion proteins to alter or improve the bacteriocidal action of the fusion protein. To alter the degradation activity of the fusion proteins, the enzymes listed above and hereinbelow may replace Mut, Dex or both in the fusion proteins of the invention. In another embodiment, when
5 two different EPS enzymes are employed in the compositions, such enzymes may be delivered at different ratios, e.g., 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8 etc. When Mut and Dex are delivered together in a gum or oral rinse for example, a preferred ratio is 5:1 Dex:Mut.

In another aspect, the invention provides a method of degrading and/or removing biofilm comprising contacting a surface harboring said biofilm with the compositions described above,
10 the composition having a bactericidal effect, and reducing or eliminating said biofilm comprising one or more undesirable microorganisms, wherein when said biofilm is present in or on an animal subject in need of said reduction or elimination. In certain embodiments, the biofilm is present in the mouth. In other embodiments, the biofilm is present on an implanted medical device. The method may also be used to remove biofilms present in an internal or external body
15 surface is selected from the group consisting of a surface in a urinary tract, a middle ear, a prostate, vascular intima, heart valves, skin, scalp, nails, teeth and an interior of a wound.

In yet another embodiment, the composition of the invention comprising said at least one AMP and said at least one biofilm degrading enzyme are produced in a plant plastid. The plant may be a tobacco plant and the sequences encoding said AMP and enzyme is codon optimized
20 for expression in a plant plastid. In a preferred embodiment, the AMP and biofilm degrading enzyme are expressed in a lettuce plant as a fusion protein under the control of endogenous regulatory elements present in lettuce plastids.

In another aspect of the invention, a composition comprising one or more essential oils and at least one biofilm degrading enzyme which act synergistically to degrade biofilm structures
25 and inhibit biofilm deposition, in a biologically acceptable carrier for delivery is disclosed. In some embodiments, the essential oils comprise one or more of menthol, thymol, eucalyptol and methyl salicylate and the at least one biofilm degrading enzyme is selected from mutanase, dextranase and glucoamylase. In other embodiments, the essential oils are menthol, thymol, eucalyptol and methyl salicylate, and the biofilm degrading enzymes are mutanase and
30 dextranase. In other embodiments, the mutanase and dextranase are present in a 5:1 ratio. In certain embodiments, the enzymes are obtained commercially, purified from biological sources

or produced in a plant plastid. In certain embodiments, the essential oils are present in a carrier is selected from the group consisting of mouthwash, mouth rinse, mouth spray, toothpaste, chewing gum, tooth gel, sub-gingival gel, mousse, foam, chewable tablet, dentifrice, lozenge, and dissolvable strip. In certain embodiments, the carrier is a mouthwash. Examples of

5 commercially available mouthwashes comprising essential oils such as menthol, thymol, eucalyptol and/or methyl salicylate include alcohol-free mouthwashes, e.g. those sold under the Listerine®Zero™ brand and antiseptic mouthrinses, e.g. those sold under the Listerine® brand.

The invention also provides a composition comprising an effective amount of one or more biofilm degrading enzymes selected from mutanase, dextranase and glucoamylase and one

10 or more essential oils selected from the group consisting of group consisting of menthol, thymol, eucalyptol, methyl salicylate, and combinations of two or more thereof in a carrier suitable for oral delivery, wherein the carrier is selected from the group consisting of mouthwash, mouth rinse, mouth spray, toothpaste, chewing gum, tooth gel, sub-gingival gel, mousse, foam, chewable tablet, dentifrice, lozenge, and dissolvable strip. In certain embodiments, the carrier is

15 a liquid carrier such as a mouthwash, mouth rinse, or mouth spray. In certain embodiments the carrier is a solid or semi-solid carrier such as a chewing gum, chewable tablet, lozenge, or dissolvable strip. In certain embodiments, the carrier is a toothpaste.

In yet another aspect, a method of degrading and/or removing unwanted biofilm from the oral cavity is disclosed. One exemplary method comprises administering an effective amount of

20 one or more biofilm degrading enzymes selected from the group consisting of mutanase, dextranase, glucoamylase, and combinations of two or more thereof, and one or more essential oils selected from the group consisting of menthol, thymol, eucalyptol, methyl salicylate, and combinations of two or more thereof, to a surface of the oral cavity having said biofilm. In certain embodiments said essential oils comprise a combination of menthol, thymol, eucalyptol

25 and methyl salicylate. Another exemplary method comprises contacting a surface of the oral cavity having said biofilm with an effective amount of a pretreatment composition comprising biofilm degrading enzymes in a suitable carrier, followed by treatment with an antimicrobial oral rinse, wherein said contacting and treating exert a bactericidal effect, thereby synergistically reducing or eliminating said unwanted biofilm. In certain aspects the antimicrobial oral rinse

30 comprises at least two essential oils selected from menthol, thymol, eucalyptol and methylsalicylate. In other aspects the essential oils are present in Listerine® mouthwash.

Notably, the aforementioned methods are effective to selectively kill pathogenic *S. mutans* without adversely affecting commensal bacteria, *A. naeslundii* and *S. oralis*.

The invention also provides a prophylactic method for inhibiting biofilm deposition on the surface in the oral cavity, comprising contacting a surface of the oral cavity susceptible to
 5 biofilm deposition with an effective amount of one or more biofilm degrading enzymes selected from the group consisting of mutanase, dextranase, glucoamylase, and combinations of two or more thereof, and one or more essential oils selected from the group consisting of menthol, thymol, eucalyptol, methyl salicylate, and combinations of two or more thereof, to a surface of the oral cavity having said biofilm. In certain embodiments the essential oils comprise a
 10 combination of menthol, thymol, eucalyptol and methyl salicylate. Another exemplary method comprises contacting a surface of the oral cavity susceptible to biofilm deposition with an effective amount of a pretreatment composition comprising biofilm degrading enzymes in a suitable carrier, followed by treatment with an antimicrobial oral rinse, wherein said contacting and treating exert a bactericidal effect, thereby synergistically inhibiting deposition of the
 15 unwanted biofilm. In certain aspects the antimicrobial oral rinse comprises at least two essential oils selected from menthol, thymol, eucalyptol and methylsalicylate. In other aspects the essential oils are present in Listerine®. Notably, the aforementioned methods are effective to selectively kill pathogenic *S. mutans* without adversely affecting commensal bacteria, *A. naeslundii* and *S. oralis*.

20

Brief Description of the Drawings

Figures 1A- 1D – Purification of GFP fused Retrocyclin (RC101) and Protegrin (PG1)

expressed in tobacco chloroplasts – Fig. 1A. Western blot analysis of purified GFP-RC101 fusion using Anti-GFP antibody. Fig. 1B. Native fluorescence gel of purified GFP-RC101
 25 fusion. Fig. 1C. Western blot of purified GFP-PG1 fusion using Anti-GFP antibody. Fig. 1D. Native fluorescence gel of purified GFP-PG1. Note – All the samples for Fig 1A -1D were loaded based on total protein values obtained from the Bradford method. Densitometry using Image J software was done to determine GFP concentration Expression level, purity and yield. Expression level and yield were calculated from GFP concentrations relative to total protein
 30 values. Yield was determined by multiplying GFP concentration with recovered volume after

purification. Individual peptide yield was determined by dividing GFP yield with molar factor 14 (ratio of GFP MW to peptide MW). The fold enrichment was calculated by dividing % purity with % expression in plant crude extracts.

Figures 2A -2E. Antimicrobial activity of AMPs (GFP-PG1 and GFP-RC101) against

5 ***Streptococcus mutans* and other oral microbes.** Cell viability was determined by absorbance ($A_{600\text{nm}}$) and counting colony forming units (CFU) over-time. (Fig. 2A) Time-killing curve of *S. mutans* treated with different concentrations of GFP-PG1 and synthetic PG1 ($A_{600\text{nm}}$). (Fig. 2B) Viable cells (CFU/ml) of *S. mutans* treated with GFP-PG1 and synthetic PG1 at each time point. (Fig. 2C) Time-killing curve of *S. mutans* treated with GFP-RC101 at different
10 concentrations ($A_{600\text{nm}}$). (Fig. 2D) Viable cells (CFU/ml) of *S. mutans* treated with GFP-RC101 at each time point. (Fig. 2E) Viable cells (CFU/ml) of *S. gordonii*, *A. naeshundii* and *C. albicans* treated with GFP-PG1 at 10 $\mu\text{g/ml}$ for 1 h and 2 h.

Figures 3A – 3C. Bacterial killing by GFP-PG1 as determined via confocal fluorescence and SEM imaging

15 (Fig. 3A) Time-lapse killing of *S. mutans* treated with GFP-PG1 at 10 $\mu\text{g/ml}$. The control group (Fig. 3B) consisted of *S. mutans* cells treated with buffer only. Propidium iodide (PI) (in red) was used with confocal microscopy to determine the bacterial viability over time at single-cell level. PI is cell-impermeant and only enters cells with damaged membranes; in dying and dead cells a bright red fluorescence is generated upon binding of PI to DNA. GFP-PG1 is shown in green. (Fig. 3C) Morphological observations of *S. mutans* subjected
20 to GFP-PG1 at a concentration of 10 $\mu\text{g/ml}$ for 1 h using scanning electron microscopy. Red arrows show dimpled membrane and extrusion of intracellular content.

Figures 4A -4C Inhibition of biofilm formation by a single topical treatment of GFP-PG1.

This figure displays representative images of three-dimensional (3D) rendering of *S. mutans* biofilm. Bacterial cells were stained with SYTO 9 (in green) and EPS were labeled with Alexa
25 Fluor 647 (in red). Saliva-coated hydroxyapatite (sHA) disc surface was treated with a single topical treatment of GFP-PG1 with a short-term 30 min exposure (Fig. 4B). The control group (Fig. 4A) was treated with buffer only. Then, the treated sHA disc was transferred to culture medium containing 1% (w/v) sucrose and actively growing *S. mutans* cells (10^5 cfu/ml) and incubated at 37°C , 5% CO_2 for 19h. After biofilm growth, the biofilms were analyzed by two
30 photon confocal microscopy. (Fig. 4C) Quantitative analysis of proportion of live and dead *S.*

mutans cells via quantitative PCR (qPCR) with or without propidium monoazide (PMA) treatment (Klein et al., 2012). The combination of PMA and qPCR (PMA-qPCR) quantify viable cells with intact membrane. Before genomic DNA isolation and qPCR quantification, PMA is added to selectively cross-link DNA of dead cells, and thereby prevent PCR amplification (Klein et al., 2012). Asterisks indicate that the values from GFP-PG1 treatment are significantly different from control ($P < 0.05$).

Figure 5. EPS-degrading enzymes digesting biofilm matrix. Representative time-lapsed images of EPS degradation in *S. mutans* biofilm treated with combination of dextranase and mutanase. Bacterial cells were stained with SYTO 9 (in green) and EPS were labeled with Alexa Fluor 647 (in red). The white arrows show ‘opening’ of spaces between the bacterial cell clusters and ‘uncovering’ cells following enzymatic degradation of EPS.

Figures 6A -6C. Biofilm disruption by synthetic PG1 alone or in combination with EPS-degrading enzymes. (Fig. 6A) Time-lapse quantification of EPS degradation within intact biofilms using COMSTAT. (Fig. 6B) The viability of *S. mutans* biofilm treated with synthetic PG1 and EPS-degrading enzymes (Dex/Mut) either alone or in combination by ImageJ. (Fig. 6C) Antibiofilm activity of synthetic PG1 was enhanced by EPS-degrading enzymes (Dex/Mut). Asterisks indicate that the values for different experimental groups are significantly different from each other ($P < 0.05$).

Figure 7. *In vitro* uptake of purified fused protein CTB-GFP, PTD-GFP, Protegrin-1-GFP (PG1-GFP) and Retrocyclin101-GFP (RC101- GFP) in different human periodontal cell lines: human periodontal ligament stem cells (HPDLS), maxilla mesenchymal stem cells (MMS), human head and neck squamous cell carcinoma cells (SCC), gingiva-derived mesenchymal stromal cells (GMSC), adult gingival keratinocytes (AGK) and osteoblast cell (OBC) with confocal microscopy. 2×10^4 cells of human cell lines HPDLS, MMS, SCC, GMSC, AGK and OBC were cultured in 8-well chamber slides (Nunc) at 37°C for overnight, followed by incubation with purified GFP fusion proteins: CTB-GFP (8.8 µg), PTD-GFP (13 µg), PG1-GFP (1.2 µg), RC101-GFP (17.3 µg) in 100 µl PBS supplemented with 1% FBS, respectively, at 37°C for 1 hour. After fixing with 2% paraformaldehyde at RT for 10 min and washing with PBS for three times, the cells were stained with antifade mounting medium with DAPI. For negative control, cells were incubated with commercial GFP (2 µg) in PBS with 1% FBS and

processed in the same condition. All fixed cells were imaged using confocal microscope. The green fluorescence shows GFP expression; the blue fluorescence shows DAPI labeled cell nuclei. The images were observed under 100x objective, and at least 10-15 GFP-positive cells or images were observed in each cell line. Scale bar represent 10 μ m. All images studies have been analyzed in triplicate.

Figure 8. Downstream processing of GFP fusions from transplastomic tobacco: Flow diagram illustrating the different steps involved in generation of purified GFP fusions from transplastomic tobacco plants grown in greenhouse.

Figures 9A – 9B. Vectors and codon optimized sequences for mutanase (Fig. 9A) and dextranase (Fig. 9B). Codon optimized mutanase: SEQ ID NO: 1. Codon Optimized dextranase: SEQ ID NO: 2.

Figure 10. A schematic diagram of a chloroplast vector expressing tandem repeats of AMPs fused with GVGVP for use alone or for expressing fusion proteins comprising the EPS proteins in Figure 9.

Figure 11. Novel purification strategy: inverse temperature cycling purification process demonstrates high yield.

Figures 12A – 12B: Expression of functional codon optimized mutanase in E. coli. Fig. 12 shows western blots showing mutanase expression in E. coli. Fig. 12B shows E. coli spread on 0.5% blue dextran plates. Transformed clones are able to produce recombinant dextranase normally made in S. mutans and able to clear a blue halo around the colony. Fig. 12C represents a gel diffusion assay comparing the degradation activity of recombinant dextranase present in the crude lysate (Total Protein loading) from the transformed E. coli against blue dextran as compared to commercially purified enzyme from Penicillin.

Figure 13. A flow diagram of the steps for engineering lettuce plants for AMP/biofilm degrading enzyme production.

Figure 14. Chewing gum tablet preparation is shown. While GFP is exemplified herein, chewing gum comprising the AMP-enzyme fusion proteins (e.g., those provided in Figs. 9 and 10) is also within the scope of the invention.

Figure 15. Gum tables were evaluated via fluorescence, and by western blot to ascertain

the concentration of GFP. Quantification of the GFP release from chewing gum based on (i) Western blotting (ii) Fluorometer (Fluoroskan Ascent™ Microplate Fluorometer – Thermo; λ_{ex} 485nm; λ_{em} 538nm). Commercial GFP (Vector Laboratories, Cat# MB-0752) was used as standard. The chewing gum was ground in the protein extraction buffer.

Figure 16. A chewing simulator is shown which uses artificial saliva for assessing release kinetics of biofilm degrading agents from the gum tablets of the invention.

Figure 17. A graph showing quantification of GFP released from chewing gum. Gum tablets comprising increasing concentrations of GFP expressed in lettuce leaves were assessed in a chewing simulator in the presence of artificial saliva to determine GFP release kinetics.

Figure 18. A graph demonstrating that crude extracts comprising enzymes expressed from chloroplast vectors are as efficacious for inhibiting CFU formation as commercial enzymes, when mixed with Listerine® mouthwash. Enzymatic degradation of *in vitro* *S. mutans* biofilms using *E. coli* derived Mutanase and Dextranase (ratio 1:5) supplemented with listerine. Commercial Mutanase (from *Bacillus* sp., Amano) and Dextranase (from *Penicillium* sp., Sigma) was used as positive control while the crude *E. coli* extract served as negative control. CFU/ml is expressed as mean $\pm$ standard deviation (n = 2). ***, P < 0.001 versus *E. coli* extract.

Figures 19A – 19E Glucanohydrolases enhance antimicrobial killing efficacy in *S. mutans* biofilm with optimum activity ratio. (Fig. 19A) Saliva-coated hydroxyapatite (sHA) biofilm model used in this study. (Fig. 19B) Schematic of treatment regimen and hypothesis of EPS-degrading/Antimicrobial (EDA) approach on preformed *S. mutans* biofilms. (Fig. 19C) Effect of dextranase/mutanase treatments on antimicrobial killing efficacy. 19-h preformed *S. mutans* biofilms on sHA were topically treated with different combinations of glucanohydrolases for 120min and then immediately exposed to antimicrobial (EOs) for 1min. Antimicrobial killing efficacy was determined based on CFU recovery. The most potent killing was consistently

achieved using 8.75U/mL dextranase and 1.75U/mL mutanase before antimicrobial exposure (5:1 enzymatic activity ratio, ~3-log more effective versus antimicrobial alone). Data are shown as mean $\pm$ standard deviation (n=4). NS, not significantly different; *, p<0.05; **, p<0.01; ***, p<0.001 (Student t test). (Fig. 19D) Screening of combinatory effect of dextranase and mutanase on EPS degradation. Representative images of checkerboard microdilution assay. (Fig. 19E) Combinatory effect (biomass reduction) of dextranase and mutanase determined by checkerboard microdilution assay. Red arrow, optimum combination (8.75U/mL Dex and 1.75U/mL Mut, 5:1 activity ratio) selected for further experiments in this study.

Figures 20A -20F. EPS-degrading enzymes dismantle biofilm matrix *in situ* and facilitate

antimicrobial targeting within biofilms. (Fig. 20A) Confocal microscopy showing morphology of 19-h *S. mutans* biofilm after matrix degradation by glucanohydrolase(s) for 120min. Green, bacteria cells stained by SYTO9; Red, Exopolysaccharides (EPS) labelled by Alexa Fluor 647; White arrow, bacterial dispersion induced by dual-enzyme treatment. (Fig. 20B) Time-lapse killing assay performed using real-time bacterial live/dead staining and imaging. Preformed (19-h) *S. mutans* biofilms were treated with 8.75U/mL dextranase + 1.75U/mL mutanase or vehicle control for 120min and were both challenged with antimicrobial (EOs). Images of a single microcolony were acquired at 0min, 1min and 5min after antimicrobial challenge. Green, live cells (SYTO 9); magenta, dead cells (propidium iodide); red, EPS (Alexa Fluor 647). White arrows, in vehicle-treated group, the antimicrobial readily killed bacteria close to the surface of the microcolony while cells residing inside remained mostly vital. (Fig. 20C) Total biomass (dry weight) per biofilm after matrix degradation by glucanohydrolase(s) for 120min. (Fig. 20D) Biochemical properties of exopolysaccharides of *S. mutans* biofilm after matrix degradation by glucanohydrolase(s) for 120min. (Fig. 20E) Synergistic antibiofilm effect of glucanohydrolases and antimicrobial. D, Dextranase; M, Mutanase; D+M, Dextranase+Mutanase. Data are shown as mean $\pm$ standard deviation (n=4). NS, not significantly different; ***, p<0.001 (Student t test). (Fig. 20F) Three-enzyme combination with dextranase, mutanase and glucoamylase further enhances antimicrobial killing efficacy. G: 20U/mL Glucoamylase; D+M: 8.75U/mL dextranase + 1.75U/mL mutanase; D+M+G: Dextranase+Mutanase+Glucoamylase. *, p<0.05; ***, p<0.001 (Student t test).

Figures 21A -21E. Dynamics of enzymatic EPS-matrix dismantling and its associated protective mechanism of recalcitrance.

Preformed (19-h) *S. mutans* biofilms (EPS labelled with Alexa Fluor 647) were imaged in 0.1M sodium acetate buffer solution containing 5µM SYTO9 and 30µM propidium iodide for continuous labeling and real-time visualization of live and dead bacterial cells over time. Glucanohydrolases were added to the buffer solution to yield a final concentration of 8.75U/mL dextranase and 1.75U/mL mutanase while the same volume of buffer solution was used as vehicle control. (Fig. 21A and Fig. 21B) 4-dimentional time-lapse confocal imaging of matrix degradation. (Fig. 21C) and (Fig. 21D) Final image of the microcolony acquired after the 1-min antimicrobial killing (EOs). Green, live cells (SYTO 9); magenta, dead cells (propidium iodide); red, EPS (Alexa Fluor 647). Black dashed circle, the center core of vehicle-treated microcolony comprised of mostly live bacteria after antimicrobial attack. White arrows, EPS located inside the microcolony was efficiently degraded by glucanohydrolases. Inserted dashed boxes, live bacteria in the outer layers after antimicrobial exposure. (Fig. 21E) EPS degradation facilitates antimicrobial killing of bacterial cells inside microcolony (Time-lapse images of dead bacteria only). Magenta, dead cells(propidium iodide).

Figures 22A-22C. Mechanical stability and integrity of the biofilm scaffold is damaged by EPS-degradation.

(Fig. 22A) Time-lapse imaging showing “implosion-like” collapse of the physical structure of *S. mutans* biofilm accompanied with extensive cellular dispersion caused by matrix degradation (8.75U/mL dextranase and 1.75U/mL mutanase) within 120min. Green, bacteria cells stained by SYTO9; Red, EPS labelled by Alexa Fluor 647. (Fig. 22B) Time-resolved EPS degradation (red squares) and microcolony spatial displacement (green circle) curves. The biovolume of EPS in the biofilm was algorithmically analyzed using COMSTAT2. The movement of each microcolony was computationally quantified as cumulative displacement using TrackMate. Data of cumulative displacement are shown as median ± interquartile range. (Fig. 22C) Confocal image of glucan formation on sHA beads with/without glucanohydrolase pretreatment. Grey: hydroxyapatite surface; Red: EPS glucan.

Figures 23A – 23F. EDA locally degrade EPS for enhanced targeting of EPS-producing pathogen in mixed-species biofilms.

(Fig. 23A) Schematic of treatment regimen and hypothesis of EDA approach on preformed mixed-species biofilms. (Fig. 23B) Ecological shift in the mixed-species biofilm model used in this study. Dashed boxes, EDA approach was tested at 43h

(the early stage) and 67h (the late stage). (Fig. 23C) Impact of EDA on microbial ecology in early mixed-species biofilm (43h). Top, CFU of different bacterial species recovered from the mixed-species biofilm after EDA treatment. Bottom, proportion of different species. (Fig. 23D) Impact of EDA on microbial ecology in late mixed-species biofilm (67h). Top, CFU of different bacterial species recovered from the mixed-species biofilm after EDA treatment. Bottom, proportion of different species. (Fig. 23E) Fluorescence *in situ* hybridization (FISH) showing spatial distribution of EPS, *S. mutans* and commensals in late mixed-species biofilms (67h). a. overview of mixed-species biofilm on sHA; b. cross-sectional magnified image(merged); c. *S. mutans* only (green); d. EPS only (red); e. *S. oralis* only (yellow); f. *A. naeslundii* only (cyan). (Fig. 23F) Dynamics of localized degradation of EPS inside the mixed-species biofilm exposing the embedded bacteria(yellow arrows). Grey, all bacteria stained by SYTO9; Red, Exopolysaccharides (EPS) labelled by Alexa Fluor 647. D, Dextranase; M, Mutanase; D+M, Dextranase+Mutanase. Data are shown as mean $\pm$ standard deviation (n=4). NS, not significantly different; *, p<0.05 ***, p<0.001 (Student t test)

Figures 24A- 24F. EPS-degrading enzymes in experimental salivary pellicle inhibit *S. mutans* biofilm formation *in situ*. (Fig. 24A) Schematic of treatment regimen and hypothesis for *S. mutans* biofilm prevention by EPS-degrading enzyme pretreatment. (Fig. 24B) Representative confocal images showing synergistic inhibition of biofilm formation by glucanohydrolases in experimental salivary pellicle. sHA discs were topically treated with EPS-degrading enzymes (8.75U/mL dextranase and/or 1.75U/mL mutanase) or vehicle control for 60 min before inoculum and were incubated to allow *S. mutans* biofilm formation for 19h before imaging. Green, bacteria cells stained by SYTO9; Red, EPS labelled by Alexa Fluor 647; White arrow, biofilms formed on single enzyme pretreated sHA showed formation of microcolony-like, albeit altered structures. (Fig. 24C) Glucanohydrolases in salivary pellicle reduce biomass(dry weight) accumulation. (Fig. 24D) CFU of the biofilm formed on glucanohydrolase-pretreated salivary pellicle. (Fig. 24E) CFU recovered from the biofilm on glucanohydrolase-pretreated salivary pellicle followed by antimicrobial killing at 19h. *S. mutans* biofilms formed on sHA pretreated with EPS-degrading enzymes or vehicle were challenged with antimicrobial (EOs) for 1min at 19h and antibiofilm efficacy was assayed by determining the CFU recovered from the biofilm. (Fig. 24F) Dose-dependent inhibitory effect of dextranase and/or mutanase on soluble and insoluble glucan synthesis by purified GtfB. Purified GtfB(10U) was mixed with dextranase

or/and mutanase, and incubated with ($[^{14}\text{C}]$ glucosyl)-sucrose substrate for 4h at 37°C to allow glucan synthesis. Insoluble glucans were collected by centrifugation (13,400g, 4°C, 10min). Soluble glucans were collected from the supernatant after precipitation with ethanol (final concentration: 70%) for 18h at -20°C. The amount of radiolabeled insoluble and soluble glucans were quantified by scintillation counting. D, Dextranase; M, Mutanase; D+M, Dextranase+Mutanase. Data are shown as mean $\pm$ standard deviation (n=4). NS, not significantly different; *, p<0.05 ***, p<0.001 (Student t test)

Figures 25A – 25E. EDA selectively prevents early colonization of *S. mutans* in mixed-species biofilms. (Fig. 25A) Schematic of treatment regimen and hypothesis for *S. mutans*

biofilm prevention by EPS-degrading enzyme pretreatment. (Fig. 25B) Microbial population of mixed-species biofilms after early colonization on sHA. sHA discs were topically treated with EPS-degrading enzymes (8.75U/mL dextranase and 1.75U/mL mutanase) or vehicle control for 60 min before inoculum and were incubated to allow mixed-species biofilm formation. (Fig. 25C) FISH image of early-colonizing mixed-species community. Green, *S. mutans*; Yellow, *S. oralis*; Cyan, *A. naeslundii*; Red, EPS. (Fig. 25D) Enzyme pretreatment potentiates overall killing efficacy and helps eliminate *S. mutans* in mixed-species biofilm. 43h Mixed-species biofilms formed on sHA pretreated with EPS-degrading enzymes or vehicle were challenged with antimicrobial (EOs) for 1min and antibiofilm efficacy was assayed by determining the CFU of different bacterial species recovered from the biofilm. (Fig. 25E) Bacterial adhesion assayed by ^3H -thymidine radioisotope tracing spectroscopy. sHA beads were pretreated with either vehicle or EPS-degrading enzymes before GtfB immobilization and glucans were synthesized *in situ* in the presence of sucrose substrate. The beads were incubated with 1.0×10^9 cells/mL radiolabeled *S. mutans*, *S. oralis* and *A. naeslundii*, respectively and were washed to remove unbound bacteria. The number of adhered bacterial was quantified by scintillation counting. D, Dextranase; M, Mutanase; D+M, Dextranase+Mutanase. NS, not significantly different; *, p<0.05; **, p<0.01 ***, p<0.001 (Student t test)

Detailed Description of the Invention

Many infectious diseases in humans are caused by virulent biofilms, including those occurring within the mouth (e.g. dental caries and periodontal diseases). Dental caries (or tooth decay) continues to be the single most costly and prevalent biofilm-associated oral disease in the

US and worldwide. It afflicts children and adults alike, and is a major reason for emergency room visits leading to absenteeism from work and school. Unfortunately, the prevalence of dental caries is still high (>90% of US adult population) and it remains the most common chronic disease afflicting children and adolescents, particularly from a poor socio-economic background. Furthermore, poor oral health often leads to systemic consequences and impacts overall health. Importantly, the cost to treat the ravages of this disease (e.g. carious lesions and pulpal infection) exceeds \$40 billion/yr in the US alone. Fluoride is the mainstay of dental caries prevention. However, its widespread use offers incomplete protection against the disease. Fluoride is effective in reducing demineralization and enhancing remineralization of early carious lesions, but has limited effects against biofilms. Conversely, current antimicrobial modalities for controlling caries-causing biofilms are largely ineffective.

There is an urgent need to develop efficacious therapies to control virulent oral biofilms. In accordance with the present invention, methods for low-cost production and delivery of therapeutically effective plant-expressed biopharmaceuticals superior to current antibiofilm/anti-caries modalities are provided. In certain embodiments, the antimicrobials and enzymes are obtained from commercial sources.

Definitions:

As used herein, antimicrobial peptides are small peptides having any bacterial activity. “RC-101” is an analogue of retrocyclin, a cyclic octadecapeptide, which can protect human CD4+ cells from infection by T- and M-tropic strains of HIV-1 in vitro and prevent HIV-1 infection in human cervicovaginal tissue. The ability of RC-101 to prevent HIV-1 infection and retain full activity in the presence of vaginal fluid makes it a good candidate for other topical microbicide applications, especially in oral biofilms. The sequence of RC-101 is provided in Plant Biotechnol J. 2011 Jan; 9(1): 100–115 which is incorporated herein by reference.

“C16G2” is a novel synthetic antimicrobial peptide with specificity for *S. mutans*,

“Protegrin-1 (PG)” is a cysteine-rich, 18-residue β -sheet peptide. It has potent antimicrobial activity against a broad range of microorganisms, including bacteria, fungus, virus, and especially some clinically relevant, antibiotic-resistant bacteria. For example, bacterial pathogens *E. coli* and fungal opportunist *C. albicans* are effectively killed by PG in laboratory testing. The sequence of PG-1 is provided in Plant Biotechnol J. 2011 Jan; 9(1): 100–115 which is incorporated herein by reference.

Additional antimicrobial peptides include those set forth below in Table 1 below.

TABLE 1.		
Peptide sequences (single-letter amino acid code) of CSP, CSP _{C16} -containing STAMPs, and STAMP components		
Peptide	Amino acid sequence ^a	Molecular wt (observed)
CSP	SGSLSTFFRLFNRSFTQALGK	2,364.9
CSP _{C16}	TFFRLFNRSFTQALGK (SEQ ID NO: 3)	1,933.3
G2	KNLRIIRKGIHIIKKY ^b (SEQ ID NO: 4)	1,993.5
C16G2	TFFRLFNRSFTQALGK <u>GGG</u> KNLRIIRKGIHIIKKY ^b (SEQ ID NO: 5)	4,079.0
CSP _{M8}	TFFRLFNRSFTQALGK (SEQ ID NO: 6)	1,100.6
M8G2	TFFRLFNRSFTQALGK <u>GGG</u> KNLRIIRKGIHIIKKY ^b (SEQ ID NO: 7)	3,246.9
S6L3-33	FKKFWKWFRRF (SEQ ID NO: 8)	1,677.5
C16-33	TRRRLFNRSFTQALGK <u>SGGG</u> FKKFWKWFRRF (SEQ ID NO: 9)	3,849.0
M8-33	TFFRLFNRSFTQALGK <u>SGGG</u> FKKFWKWFRRF (SEQ ID NO: 10)	3,016.9

- 5
- ^a Linker regions between targeting and killing peptides are underlined.
 - ^b Peptide C-terminal amidation.

10 An “antimicrobial” as used herein, includes without limitation, CHX, triclosan/copolymer dentrifices, clindamycin, doxycycline gels, minocycline powder, macrolides, sulfonamides, penicillin and derivatives thereof, tetracycline, quinolones, levofloxacin, ciprofloxacin and fluconazole.

15 “Essential oils” may include one or more selected from the group consisting of menthol, thymol, eucalyptol, methyl salicylate and combinations of two or more thereof. In certain embodiments, the essential oils comprises menthol. In certain embodiments, the essential oils comprises thymol. In certain preferred embodiments, the essential oils comprise a combination of menthol, thymol, eucalyptol, and methyl salicylate.

A “biofilm” is a complex structure adhering to surfaces that are regularly in contact with water, consisting of colonies of bacteria and usually other microorganisms such as yeasts, fungi, and protozoa that secrete a mucilaginous protective coating in which they are encased. Biofilms can form on solid or liquid surfaces as well as on soft tissue in living organisms, and are typically resistant to conventional methods of disinfection. Dental plaque, the slimy coating that fouls pipes and tanks, and algal mats on bodies of water are examples of biofilms. Biofilms are generally pathogenic in the body, causing such diseases as dental caries, cystic fibrosis and otitis media.

“Biofilm degrading enzymes” include, without limitation, exo-polysaccharide degrading enzymes such as dextranase, mutanase, DNase, endonuclease, deoxyribonuclease I, dispersin B, and glycoside hydrolases, such as 1→3) - α -D-glucan hydrolase, although use of chloroplast codon optimized sequences encoding dextranase and mutanase are preferred, the skilled person is well aware of many different biofilm degrading enzymes in the art. Additional enzyme sequences for use in the fusion proteins of the invention are provided below. As noted above, suitable enzymes can also be purified from the bacteria which produce them or obtained from commercial sources.

As used herein, the terms “administering” or “administration” of an agent, drug, or peptide to a subject includes any route of introducing or delivering to a subject a compound to perform its intended function. The administering or administration can be carried out by any suitable route, including orally, topically, intranasally, parenterally (intravenously, intramuscularly, intraperitoneally, or subcutaneously), or rectally. Administering or administration includes self-administration and the administration by another. In one embodiment a single administration entails oral delivery of a single formulation comprising enzymes and antimicrobials. In other embodiments, the administration can be sequential. In a preferred embodiment, biofilm degrading enzymes are first administered, followed by delivery of antimicrobials. Enzyme treatment is performed for a suitable time to degraded the extracellular matrix where in the oral cavity, gums and teeth are exposed to enzymes for at least 5 minutes, at least 10 minutes, at least 15 minutes, at least 20 minutes, at least 25 minutes, at least 30 minutes, at least 45 minutes, at least 60 minutes. Preferably, the teeth and gums are treated with enzyme for between 30 and 120 minutes. Most preferably the enzyme treatment is performed for at least 30 minutes. After this period, an antimicrobial oral rinse is administered

for 20, 30, 40, 50, 60, 90, or 120 seconds. In a preferred embodiment, at least two or more of methol, eucalyptol, thymol and methylsalicylate are present in the rinse. In a particularly preferred embodiment, the rinse is Listerine®. The enzyme containing composition may be in a form selected from the group consisting of a mouthwash, mouth rinse, mouth spray, toothpaste, tooth gel, sub-gingival gel, mousse, foam, denture care product, chewable tablet, dentifrice, lozenge, dissolvable strip, and the like.

As used herein, the terms “disease,” “disorder,” or “complication” refers to any deviation from a normal state in a subject.

As used herein, by the term “effective amount” “amount effective,” or the like, it is meant an amount effective at dosages and for periods of time necessary to achieve the desired result. In certain embodiments, the amount is effective to act prophylactically to inhibit formation of biofilm. In other embodiments, the amounts are effective to disperse existing biofilms.

As used herein, the term “inhibiting” or “preventing” means causing the clinical symptoms of the disease state not to worsen or develop, e.g., inhibiting the onset of disease, in a subject that may be exposed to or predisposed to the disease state, but does not yet experience or display symptoms of the disease state.

As used herein, the term “expression” in the context of a gene or polynucleotide involves the transcription of the gene or polynucleotide into RNA. The term can also, but not necessarily, involves the subsequent translation of the RNA into polypeptide chains and their assembly into proteins.

A plant remnant may include one or more molecules (such as, but not limited to, proteins and fragments thereof, minerals, nucleotides and fragments thereof, plant structural components, etc.) derived from the plant in which the protein of interest was expressed. Accordingly, a composition pertaining to whole plant material (e.g., whole or portions of plant leafs, stems, fruit, etc.) or crude plant extract would certainly contain a high concentration of plant remnants, as well as a composition comprising purified protein of interest that has one or more detectable plant remnants. In a specific embodiment, the plant remnant is rubisco.

In another embodiment, the invention pertains to an administrable composition for treating or preventing biofilm formation in situ (e.g., in the mouth) and on biomedical devices useful for surgical implantation such as stents, artificial joints, and the like. In this embodiment, the devices are coated with the composition to inhibit unwanted biofilm deposition on the device.

The composition comprises a therapeutically-effective amount of one or more antimicrobial peptides (AMP) and one or more enzymes having biofilm degrading activity in combination, each of said AMP and enzyme thereof having been expressed by a plant and a plant remnant and acting synergistically to degrade said biofilm. In certain embodiments the AMP(s) and
5 enzymes(s) are expressed from separate plastid transformation vectors. In other embodiments, the plastid transformation vectors comprising polycistronic coding sequences where both the AMP and the enzymes are expressed from a single vector.

In other embodiments, the antimicrobial peptides are optional and one or more enzymes are employed in combination with one or more antimicrobials, and/or essential oils.

10 Proteins expressed in accord with certain embodiments taught herein may be used in vivo by administration to a subject, human or animal in a variety of ways. The pharmaceutical compositions may be administered orally, topically, subcutaneously, intramuscularly or intravenously, though oral topical administration is preferred.

Oral compositions produced by embodiments of the present invention can be
15 administered by the consumption of the foodstuff that has been manufactured with the transgenic plant producing the plastid derived therapeutic protein. The edible part of the plant, or portion thereof, is used as a dietary component. The therapeutic compositions can be formulated in a classical manner using solid or liquid vehicles, diluents and additives appropriate to the desired mode of administration. Orally, the composition can be administered in the form of
20 tablets, capsules, granules, powders, gums, and the like with at least one vehicle, e.g., starch, calcium carbonate, sucrose, lactose, gelatin, etc. The therapeutic protein(s) of interest may optionally be purified from a plant homogenate. The preparation may also be emulsified. The active ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, e.g., water, saline, dextrose,
25 glycerol, ethanol or the like and combination thereof. In addition, if desired, the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants. In a preferred embodiment the edible plant, juice, grain, leaves, tubers, stems, seeds, roots or other plant parts of the pharmaceutical-producing transgenic plant is ingested by a human or an animal thus providing a very inexpensive means of treatment of
30 disease.

In a specific embodiment, plant material (e.g. lettuce material) comprising chloroplasts expressing AMPs and biofilm degrading enzymes and combinations thereof, is homogenized and encapsulated. In one specific embodiment, an extract of the lettuce material is encapsulated. In an alternative embodiment, the lettuce material is powderized before encapsulation. As
5 mentioned previously, the biofilm degrading proteins may also be purified from the plant following expression.

In alternative embodiments, the compositions may be provided with the juice of the transgenic plants for the convenience of administration. For said purpose, the plants to be transformed are preferably selected from the edible plants consisting of tomato, carrot and apple,
10 among others, which are consumed usually in the form of juice.

According to another embodiment, the subject invention pertains to a transformed chloroplast genome that has been transformed with a vector comprising a heterologous gene that expresses a combination of peptides as disclosed herein.

Of particular present interest is a transformed chloroplast genome transformed with a
15 vector comprising a heterologous gene that expresses one or more AMP and biofilm degrading enzyme or a combination thereof, polypeptide. In a related embodiment, the subject invention pertains to a plant comprising at least one cell transformed to express a peptide as disclosed herein.

Reference to genetic sequences herein refers to single- or double-stranded nucleic acid
20 sequences and comprises a coding sequence or the complement of a coding sequence for polypeptide of interest. Degenerate nucleic acid sequences encoding polypeptides, as well as homologous nucleotide sequences which are at least about 50, 55, 60, 65, 60, preferably about 75, 90, 96, or 98% identical to the cDNA may be used in accordance with the teachings herein polynucleotides. Percent sequence identity between the sequences of two polynucleotides is
25 determined using computer programs such as ALIGN which employ the FASTA algorithm, using an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2. Complementary DNA (cDNA) molecules, species homologs, and variants of nucleic acid sequences which encode biologically active polypeptides also are useful polynucleotides.

Variants and homologs of the nucleic acid sequences described above also are useful
30 nucleic acid sequences. Typically, homologous polynucleotide sequences can be identified by hybridization of candidate polynucleotides to known polynucleotides under stringent conditions,

as is known in the art. For example, using the following wash conditions: 2 X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, room temperature twice, 30 minutes each; then 2X SSC, 0.1% SDS, 50⁰ C. once, 30 minutes; then 2X SSC, room temperature twice, 10 minutes each homologous sequences can be identified which contain at most about 25-30% basepair mismatches. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.

Species homologs of polynucleotides referred to herein also can be identified by making suitable probes or primers and screening cDNA expression libraries. It is well known that the T_m of a double-stranded DNA decreases by 1-1.5°C with every 1% decrease in homology (Bonner et al., J. Mol. Biol. 81, 123 (1973). Nucleotide sequences which hybridize to polynucleotides of interest, or their complements following stringent hybridization and/or wash conditions also are also useful polynucleotides. Stringent wash conditions are well known and understood in the art and are disclosed, for example, in Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed., 1989, at pages 9.50-9.51.

The following materials and methods are provided to facilitate the practice of Examples I and II.

Microorganisms and growth conditions

Streptococcus mutans UA159 serotype c (ATCC 700610), *Actinomyces naeshlundii* ATCC 12104, *Streptococcus gordonii* DL-1 and *Candida albicans* SC5314 were used in present study.

These strains were selected because *S. mutans* is a well-established virulent cariogenic bacteria [Ajdić D et al, 2002]. *S. gordonii* is a pioneer colonizer of dental biofilm, and *A. naeshlundii* is also detected during the early stages of dental biofilm formation and may be associated with development of root caries [Dige I et al, 2009]. *C.albicans* is a fungal organism that colonizes human mucosal surfaces, and it is also detected in dental plaque from toddlers with early childhood caries [Hajeshengallis E et al, 2015]. All strains were stored at –80°C in tryptic soy broth containing 20% glycerol. Blood agar plates were used for cultivating *S.mutans*, *S.gordonii* and *A.naeshlundii*. Sabouraud agar plates were used for *C.albicans*. All these strains were grown in ultra-filtered (10 kDa molecular-weight cut-off membrane; Prep/Scale, Millipore, MA) buffered tryptone-yeast extract broth (UFTYE; 2.5% tryptone and 1.5% yeast extract, pH 7.0) with 1% glucose to mid-exponential phase (37°C, 5% CO₂) prior to use.

Creation of transplastomic lines expressing different tagged GFP fusion proteins

The transplastomic plants expressing GFP fused with CTB, PTD, retrocyclin and protegrin were created as described in previous studies [Limaye et al 2006; Kwon et al 2013; Xiao et al 2016; Lee et al 2011]. Transplastomic lines expressing GFP fusion proteins were confirmed using Southern blot assay as described previously [Verma et al 2008]. Also, expression of GFP tagged proteins were confirmed by visualizing green fluorescence from the leaves of each construct under UV illumination.

Purification of tag-fused GFP proteins

Purification of GFP fusions Protegrin-1 (PG1) and Retrocyclin (RC101) from transplastomic tobacco was accomplished by organic extraction followed by hydrophobic chromatography done previously (Lee et al, 2011). About 0.2-1 gm of lyophilized leaf material was taken and reconstituted in 10-20 ml of plant extraction buffer (0.2M Tris HCl pH 8.0, 0.1M NaCl, 10mM EDTA, 0.4M sucrose, 0.2 % Triton X supplemented with 2% Phenylmethylsulfonylfluoride and 1 protease inhibitor cocktail). The resuspension was incubated in ice for 1 hour with vortex homogenization every 15 min. The homogenate was then spun down at 75000g at 4°C for 1 hour (Beckman LE-80K optima ultracentrifuge) to obtain the clarified lysate. The lysate was subjected to pretreatment with 70% saturated ammonium sulfate and 1/4th volume of 100% ethanol, followed by vigorous shaking for 2 min (Yakhnin et al, 1998). The treated solution was spun down at 2100 g for 3 min. The upper ethanol phase was collected and the process was repeated with 1/16th volume of 100% ethanol. The pooled ethanol phases were further treated with 1/3rd volume of 5M NaCl and 1/4th volume of 1-butanol, homogenized vigorously for 2 min and spun down at 2100 g for 3 min. The lowermost phase was collected and loaded onto a 7kDa MWCO zeba spin desalting column (Thermo scientific) and desalted as per manufacturer's recommendations.

The desalted extract was then subjected to hydrophobic interaction chromatography during the capture phase for further purification. The desalted extract was injected into a Toyopearl butyl – 650S hydrophobic interaction column (Tosoh bioscience) which was run on a FPLC unit (Pharmacia LKB-FPLC system). The column was equilibrated with 2.3 column volumes of salted buffer (10mM Tris-HCl, 10mM EDTA and 50% saturated ammonium sulfate) to a final 20% salt saturation to facilitate binding of GFP onto the resin. This was followed by a

column wash with 5.8 column volumes of salted and unsalted buffer mix and then eluted with unsalted buffer (10mM Tris-HCl, 10mM EDTA). The GFP fraction was identified based on the peaks observed in the chromatogram and collected. The collected fractions were subjected to a final polishing step by overnight dialysis. After dialysis the purified proteins were lyophilized (labconco lyophilizer) in order to concentrate the finished product and then stored in -20°C.

Quantification of purified GFP fusions

Quantification of GFP-RC101 and GFP-PG1 was done by both western blot and fluorescence based methods. The lyophilized purified proteins were resuspended in sterile 1X PBS and the total protein was determined by Bradford method. The purified protein was then quantified by SDS-PAGE method by loading denatured protein samples along with commercial GFP standards (Vector labs) onto a 12 % SDS gel and then western blotting was done using 1:3000 dilution of mouse Anti-GFP antibody (Millipore) followed by probing with 1:4000 dilution of secondary HRP conjugated Goat-Anti Mouse antibody (Southern biotech).

The purified proteins were also quantified using GFP fluorescence. The protein samples were run on a 12 % SDS gel under native conditions. After the run, the gel was placed under a UV lamp and then photographed. The GFP concentration in both western and native fluorescence methods was determined by densitometric analysis using Image J software with commercial GFP standards in order to obtain the standard curve. Purity was determined based on GFP quantitation with respect to total protein values determined in Bradford method.

Uptake of purified tag-fused GFP proteins by human periodontal cell lines

As previously described (Xiao, et al 2016), to determine the uptake of four tags, CTB, PTD, PG1 and RC101, in different human periodontal cell lines, including human periodontal ligament stem cells (HPDLS), maxilla mesenchymal stem cells (MMS), human head and neck squamous cell carcinoma cells (SCC-1), gingiva-derived mesenchymal stromal cells (GMSC), adult gingival keratinocytes (AGK) and osteoblast cells (OBC), briefly, each human cell line cells (2×10^4) were cultured in 8 well chamber slides (Nunc) at 37°C overnight, followed by incubation with purified GFP-fused tags: CTB-GFP (8.8 µg), PTD-GFP (13 µg), GFP-PG1 (1.2 µg) and GFP-RC101 (17.3 µg) in 100 µl PBS supplemented 1% FBS at 37°C for 1 hour. After fixing with 2% paraformaldehyde at RT for 10 min and washing with PBS for three times, all

cells were stained with antifade mounting medium with DAPI (Vector laboratories, Inc). For negative control, cells were incubated with commercial GFP (2 µg) in PBS with 1% FBS at 37°C for 1 hour. All fixed cells were imaged using confocal microscopy. The images were observed under 100x objective, and at least 10-15 GFP-positive cells were recorded for each cell line in three independent analysis.

Evaluation of antibacterial activity

The killing kinetics of AMPs (Gfp-PG1 and Gfp-RC101) against *S.mutans* were analyzed by time-lapse killing assay. *S.mutans* were grown to log phase and diluted to 10⁵ CFU/ml in growth medium. GFP-PG1 and GFP-RC101 were added to *S.mutans* suspensions at concentrations of 0 to 10 µg/ml and 0 to 80 µg/ml, respectively. At 0, 1, 2, 4, 8 and 24 h, samples were taken and serially diluted in 0.89% NaCl, then spread on agar plates and colonies were counted after 48 h. Absorbance at 600 nm was also checked at each time point. *S.gordonii*, *A.naeshlundii* and *C.albicans* suspensions were mixed with Gfp-PG1 at concentration of 10 µg/ml, and at 0, 1 and 2 h, aliquots were taken out for enumeration of CFU.

The effects of AMP on the viability of *S.mutans* cells were also assessed by time-lapsed measurements. *S. mutans* were grown to log phase and harvested by centrifugation (5500 g, 10min) and the pellet was washed once with sodium phosphate-buffered saline (PBS) (pH 7.2), re-suspended in PBS and adjusted to a final concentration of 1×10⁵ CFU/ml. GFP-PG1 was added to *S.mutans* suspensions at concentrations of 10 µg/ml and 2.5 µM propidium iodide-PI (Molecular Probe Inc., Eugene, OR, USA) was added for labeling dead cells. 5 µl of mixtures were loaded on an agarose pad for confocal imaging. Confocal images were acquired using Leica SP5-FLIM inverted single photon laser scanning microscope with a 100X (numerical aperture, 1.4) Oil immersion objective. The excitation wavelengths were 488 nm and 543 nm for GFP and PI, respectively. The emission filter for GFP was a 495/540 OlyMPFC1 filter, while PI was a 598/628 OlyMPFC2 filter. For the time-lapse series, images in the same field of view were taken at 0, 10, 30, and 60 min and created by ImageJ 1.44 ([Http://rsbweb.nih.gov/ij/download.html](http://rsbweb.nih.gov/ij/download.html)). Morphological observations of *S. mutans* treated with AMP were also examined by scanning electron microscopy (SEM). *S. mutans* were grown to log phase and diluted to 10⁵ CFU/ml in PBS. Bacteria suspension was mixed with GFP-PG1 (final concentration of 10 µg/ml) for 1 h at 37°C. After treatment, the bacteria were collected by filtration using 0.4 µm Millipore filters.

The deposits were fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 hour at room temperature and processed for SEM (Quanta FEG 250, FEI, Hillsboro, OR) observation. Untreated or bacteria treated with buffer only served as controls.

Evaluation of anti-biofilm activity

5 A well-characterized EPS-matrix producing oral pathogen, *S.mutans* UA159, was used to form biofilms on saliva-coated hydroxyapatite disc surfaces. Briefly, hydroxyapatite discs (1.25 cm in diameter, surface area of $2.7 \pm 0.2 \text{ cm}^2$, Clarkson, Chromatography Products, Inc., South Williamsport, PA) were coated with filter-sterilized, clarified human whole saliva (sHA) [Xiao J et al, 2012]. *S.mutans* was grown in UFTYE medium with 1% (w/v) glucose to mid-exponential
10 phase (37°C, 5% CO₂). Each sHA disc was inoculated with 10⁵ CFU of actively growing *S.mutans* cells per ml in UFTYE medium containing 1% (w/v) sucrose, and inoculated at 37°C and 5% CO₂ for 19 h. Before inoculum, the sHA discs were topically treated with GFP-PG1 solution (10 ug) for 30min. The inhibition effect of GFP-PG1 treatment on 3D biofilm architectures were observed via confocal imaging. Briefly, EPS was labeled using 2.5 µM Alexa
15 Fluor 647-labeled dextran conjugate (10 kDa; 647/668 nm; Molecular Probes Inc.), while the bacteria cells were stained with 2.5 µM SYTO9 (485/498 nm; Molecular Probes Inc.). The imaging was performed using Leica SP5 microscope with 20X (numerical aperture, 1.00) water immersion objective. The excitation wavelength was 780 nm, and the emission wavelength filter for SYTO 9 was a 495/540 OlyMPFEC1 filter, while the filter for Alexa Fluor 647 was a
20 HQ655/40M-2P filter. The confocal image series were generated by optical sectioning at each selected positions and the step size of z-series scanning was 2 µm. Amira 5.4.1 software (Visage Imaging, San Diego, CA, USA) was used to create 3D renderings of biofilm architecture [Xiao J et al. 2012, Koo H et al. 2010].

To examine the effects of the PG1 on biofilms formed with *S.mutans* for 19 h on sHA
25 discs, we examined the 3D architecture of the EPS-matrix and *in situ* cell viability using time-lapse confocal microscopy following biofilms incubation with 1) Control, 2) EPS-degrading enzymes only, 3) PG1 only, or 4) PG1 and EPS-degrading enzymes for up to 60 minutes. The EPS-degrading enzymes used here were dextranase and mutanase, which were capable of digesting the EPS derived from *S. mutans* by hydrolyzing α-1,6 glucosidic linkages and α-1,3
30 glucosidic linkages [Hayacibara et al. 2004]. Dextranase produced from *Penicillium sp.* was

commercially purchased from Sigma (St. Louis, MO) and mutanase produced from *Trichoderma harzianum* was kindly provided by Dr. William H. Bowen (Center for Oral Biology, University of Rochester Medical Center). Dextranase and mutanase were mixed at ratio of 5:1 before applying to biofilms [Mitsue F. Hayacibara et al. 2004]. Alexa Fluor 647-labeled dextran conjugate was used to label the EPS-matrix, while SYTO 9 and PI were used to label live cells and dead cells. Biofilms were examined using confocal fluorescence imaging at 0, 10, 30 and 60 min, and subjected to AMIRA/COMSTAT/ImageJ analysis. The total biomass of EPS matrix, live and dead cells in each series of confocal images was quantified using COMSTAT and ImageJ. The ratio of live to the total bacteria at each time point was calculated, and the survival rate of live cells (relative to live cells at 0 min) was plotted. The initial number of viable cells at time point 0 min was considered to be 100%. The percent-survival rate was determined by comparing to time point 0 min.

Microbiological assays

At selected time point (19 h), biofilms were removed, homogenized via sonication and subject to microbiological analyses as detailed previously [Xiao J et al. 2012, Koo H et al. 2010]; our sonication procedure does not kill bacteria cells while providing optimum dispersal and maximum recoverable counts. Aliquots of biofilm suspensions were serially diluted and plated on blood agar plates using an automated Eddy Jet Spiral Plater (IUL, SA, Barcelona, Spain). Meanwhile, propidium monoazide (PMA) combined with quantitative PCR (PMA-qPCR) was used for analysis of *S.mutans* cell viability as described. [Klein MI et al. 2012]. The combination of PMA and qPCR will quantify only the cells with intact membrane (i.e. viable cells) because the PMA cross-linked to DNA of dead cells and extracellular DNA modifies the DNA and inhibits the PCR amplification of the extracted DNA. Briefly, biofilm pellets were resuspended with 500 μ l TE (50 mM Tris, 10 mM EDTA, pH 8.0). Using a pipette, the biofilm suspensions were transferred to 1.5 ml microcentrifuge tubes; then mixed with PMA. 1.5 μ l PMA (20 mM in 20% dimethyl sulfoxide; Biotium, Hayward, CA) was added to the biofilm suspensions. The tubes were incubated in the dark for 5 min, at room temperature, with occasional mixing. Next, the samples were exposed to light for 3 min (600-W halogen light source). After photo-induced cross-linking, the biofilm suspensions were centrifuged (13,000 g/10 min/4°C) and the

supernatant was discarded. The pellet was resuspended with 100 µl TE, following by incubation with 10.9 µl lysozyme (100 mg/ml stock) and 5 µl mutanlysin (5U/µl stock) (37°C/30 min). Genomic DNA was then isolated using the MasterPure DNA purification kit (Epicenter Technologies, Madison, WI). Ten pictograms of genomic DNA per sample and negative controls (without DNA) were amplified by MyiQ real-time PCR detection system with iQ SYBR Green supermix (Bio-Rad Laboratories Inc., CA) and *S.mutans* specific primer (16S rRNA) [Klein MI et al 2010].

Statistical Analysis

Data are presented as the mean ± standard deviation (SD). All the assays were performed in duplicate in at least two distinct experiments. Pair-wise comparisons were made between test and control using Student's t-test. The chosen level of significance for all statistical tests in present study was $P < 0.05$.

The following examples are provided to illustrate certain embodiments of the invention. They are not intended to limit the invention in any way.

Example I

Creation and characterization of transplastomic lines

All fusion tags (CTB, PTD, protegrin, retrocyclin) were fused to the green fluorescent protein (smGFP) at N-terminus to evaluate their efficiency and specificity. Fusion constructs encoding these fusion proteins were cloned into chloroplast transformation vectors which were then used to transform plants of interest as described in US Patent application no. 13/101,389 which is incorporated herein by reference. To create plants expressing GFP fusion proteins, tobacco chloroplasts were transformed using biolistic particle delivery system. As seen in the Fig. 1B, each tag-fused GFP is driven by identical regulatory sequences - the *psbA* promoter and 5' UTR regulated by light and the transcribed mRNA is stabilized by 3' *psbA* UTR. The *psbA* gene is the most highly expressed chloroplast gene and therefore *psbA* regulatory sequences are used for transgene expression in our lab [7, 34]. To facilitate the integration of the expression cassette into chloroplast genome, two flanking sequences, isoleucyl-tRNA synthetase (*trnI*) and alanyl-tRNA synthetase (*trnA*) genes, flank the expression cassette, which are identical to the

native chloroplast genome sequence. The emerging shoots from selection medium were investigated for specific integration of the transgene cassette at the *trnI* and *trnA* spacer region and then transformation of all chloroplast genomes in each plant cell (absence of untransformed wild type chloroplast genomes) was confirmed by Southern blot analysis. Thus, stable
5 integration of all GFP expression cassettes and homoplasmy of chloroplast genome with transgenes were confirmed before extracting fusion proteins. In addition, by visualizing the green fluorescence under UV light, GFP expression of was phenotypically confirmed. Confirmed homoplasmic lines were then transferred and cultivated in an automated greenhouse to increase biomass.

10 To scale up the biomass of each GFP tagged plant leaf material, each homoplasmic line was grown in a temperature- and humidity-controlled greenhouse. Fully grown mature leaves were harvested in late evenings to maximize the accumulation of GFP fusion proteins driven by light-regulated regulatory sequences. To further increase the content of the fusion proteins on a weight basis, frozen leaves were freeze-dried at -40°C under vacuum. In addition to the
15 concentration effect of proteins, lyophilization increased shelf life of therapeutic proteins expressed in plants more than one year at room temperature [Daniell et al 2015; 2016]. Therefore, in this study, lyophilized and powdered plant cells expressing GFP-fused tag proteins were used for oral delivery to mice.

20 ***Expression and purification of GFP fused antimicrobial peptides from transplastomic tobacco.***

Tobacco leaves expressing GFP fused antimicrobial peptides RC101 and PG1 were harvested from greenhouse and subsequently lyophilized for protein extraction and purification. The average expression level of GFP-RC101 was found to be 8.8% of total protein in crude extracts while expression of GFP-PG1 was that of 3.8% of total protein based on densitometry.
25 The difference in expression levels was similar to what was reported previously (Lee et al 2011, Gupta et al, 2015).

Purification of GFP fused to different antimicrobial peptides (RC101 and PG1) was done in order to test the microbicidal activity against both planktonic and biofilm forming *S.mutans*. Lyophilized tobacco material expressing different GFP fusions was used for extractions and
30 subsequent downstream processing (See Figure 8) to obtain the finished purified product which was subsequently quantified to determine concentration of GFP fused peptides. Quantitation of

purified GFP-RC101 and GFP-PG1 was done by both western blot and Native GFP fluorescence method where purified GFP-RC101 show 94% average purity with an average yield of 1624 μ g of GFP (116 μ g of RC101 peptide) per gm of lyophilized leaf material (Fig 1A and 1B). In GFP-PG1 both methods (Fig 1C and 1D) show 17% average purity with an average yield of 58.8 μ g of GFP (4.2 μ g PG1 peptide) per gm of lyophilized leaf material. The difference in purity can be attributed to difference in the type of tags fused to GFP as seen in previous studies (Xiao et al 2015, Skosyrev et al 2003). The fold enrichment of purified GFP-RC101 and GFP-PG1 from plant extracts was 10.6 and 4.5 respectively. The western blots also show GFP standards at 27 kDa which corresponds to the monomer fragment along with a 54kDa GFP dimer with loadings ranging from 6-8 ng of GFP. In GFP-RC101 western blots, 29 kDa and 58 kDa fragments are clearly visible which correspond to the monomer and dimer forms of the fusion (Fig. 1A). This could be attributed to the ability of GFP to form dimers (Ohashi et al, 2007). Western blots of GFP-PG1 (Fig 1D) clearly show the 29 kDa monomer along with a 40 kDa fragment could be due to mobility shift caused by GFP-PG1 bound to other non-specific plant proteins which could have been co-purified as described previously (Morassuttia et al 2002). Native fluorescence of GFP-RC101 and GFP-PG1 (Fig 1B and 1D) show multimeric bands with some of them visible below the 27 kDa GFP standard size which could be because of GFP being fused to cationic peptides causing a electrophoretic mobility shift with each GFP fragment as described in previous studies (Lee et al, 2011).

Antibacterial Activity of AMPs

We first examined the antimicrobial activity of GFP-PG1 using dose-response time-kill studies as shown in Fig. 2 (A-E). GFP-PG1 displays potent antibacterial activity against *Streptococcus mutans*, a proven biofilm-forming and caries-causing pathogen, rapidly killing the bacterial cells within 1h at low concentrations (Fig. 2A). GFP-PG1 also killed the early oral colonizers *Streptococcus gordonii* and *Actinomyces naeslundii*, but showed limited antifungal activity against *Candida albicans* at the concentrations tested (Fig 2E). Time-lapse confocal imaging shows that *S. mutans* viability is affected as early as 10 minutes as shown in Fig 3A relative to the untreated controls (Fig. 3B). SEM imaging revealed disruption of *S. mutans* membrane surface, causing extrusion of the intracellular content as well as irregular cell morphology, while untreated bacteria showed intact and smooth surfaces without any visible cell

lysis or debris (Fig 3C). Having shown the antimicrobial efficacy of GFP-PG1 against *S. mutans*, we have examined the potential of this antimicrobial peptide to prevent biofilm formation or disrupt pre-formed biofilms.

Inhibition of Biofilm Initiation by AMPs

5 Preventing the formation of pathogenic oral biofilms is challenging because drugs need to exert therapeutic effects following topical applications. To determine whether GFP-PG1 can disrupt the initiation of the biofilm, we treated saliva coated apatitic (sHA) surface (tooth surrogate) with a single topical treatment of GFP-PG1 for 30 min, and then incubated with actively growing *S. mutans* cells in cariogenic (sucrose-rich) conditions. We observed substantial
10 impairment of biofilm formation by *S. mutans* with minimal accumulation of EPS-matrix on the GFP-PG1 treated sHA surface (Figs. 4B and 4C). The few adherent cell clusters were mostly non-viable compared to control (Fig. 4A), demonstrating potent effects of GFP-PG1 on biofilm initiation despite topical, short-term exposure.

Disruption of pre-formed biofilm by AMP with or without EPS-degrading enzymes

15 Disruption of formed biofilms on surfaces is challenging. Disruption of cariogenic biofilms is particularly difficult because drugs often fail to reach clusters of pathogenic bacteria (such as *S. mutans*) because of the surrounding exopolysaccharides (EPS)-rich matrix that enmeshes and protects them [Bowen and Koo, 2011]. EPS-degrading enzymes such as dextranase and mutanase could help digest the matrix of cariogenic biofilms, although they are
20 devoid of antibacterial effects. We first optimized the dextranase and/or mutanase required for EPS-matrix disruption without affecting the cell viability (data not shown). As shown in Figure 5, the combination of dextranase and mutanase can digest the EPS (in red) and ‘open spaces’ (see arrows) between the bacterial cell clusters (in green) and ‘uncover’ cells (see arrows). Thus, the combination of GFP-PG1 and EPS-degrading enzymes synergistically potentiate the overall
25 antibiofilm effects.

To explore this concept, *Streptococcus mutans* biofilms were pre-formed on sHA surface, and treated topically with GFP-PG1 and EPS-degrading enzymes (Dex/Mut) either alone or in combination. Time-lapsed confocal imaging and quantitative computational analyses were conducted to analyze EPS-matrix degradation and live/dead bacterial cells within biofilms (Fig

6A). The enzymes-peptide combination resulted in more than 60% degradation of the EPS-matrix, while increasing the bacterial killing when compared to either GFP-PG or Dex/Mut alone. These findings were further validated via standard culturing assays by determining colony forming units. The antibacterial activity of PG against *S. mutans* biofilms combined with
5 Dex/Mut was significantly enhanced than either one alone. Topical exposure of Dex/Mut alone showed no effects on biofilm cell viability, whereas GFP-PG-1 alone showed limited killing activity (Fig 6B). Together, the data demonstrate potential of this combined approach to synergistically enhance antimicrobial efficacy of GFP-PG-1 against established biofilms (Fig. 6C).

10

Uptake of GFP fused with different tags by human periodontal cells.

Purified GFP fusion proteins when incubated with human cultured cells, including HPDLS, MMS, SCC-1, GMSC, AGK and osteoblast cells (OBC) revealed interesting results. Although only one representative image of each cell line is presented, uptake studies were
15 performed in triplicate and at least 10-15 images were recorded under confocal microscopy. Without a fusion tag, GFP did not enter any tested human cell line. Both CTB-GFP and PTD-GFP effectively penetrated all tested cell types, although their localization patterns differed. Upon incubation with CTB-GFP, GFP signals localized primarily to the periphery of HPDLSC and MMSC, uniformly small cytoplasmic puncta in SSC-1, AGK, OBC and large cytoplasmic
20 foci in GMSC. PTD-GFP was observed as small cytoplasmic foci in MMSC, variably sized cytoplasmic puncta in HPDLSC, GMSC, AGK, OBC and both the cytoplasm and the periphery of SCC-1 cells. PG1-GFP is the most efficient tag in entering all tested human cells because GFP could be localized at tenfold lower concentrations than any other fusion proteins. PG1-GFP showed exclusively cytoplasmic localization in HPDLSC, SCC-1, GMSC and AGK cells and
25 localized to both the periphery and cytosol in MMSC, but it is only localized to the periphery of OBC. RC101-GFP was localized in SCC-1, GMSC, AGK and OBC, but its localization in HPDLSC was negligible and was undetectable in MMSC cells.

Discussion and Conclusions

The assembly of cariogenic oral biofilms is a prime example of how pathogenic bacteria accumulate on a surface (teeth), as an extracellular EPS matrix develops. Prevention of cariogenic biofilm formation requires disruption of bacterial accumulation on the tooth surface with a topical treatment. Chlorhexidine (CHX) is considered ‘gold standard’ for topical antimicrobial therapy (Flemmig and Beikler 2011; Marsh et al 2011; Caufield et al 2001). CHX effectively suppresses mutans streptococci levels in saliva, but it has adverse side effects including tooth staining and calculus formation, and is not recommended for daily preventive or therapeutic use (Autio-Gold 2008). As an alternative, several antimicrobial peptides (AMP) have been developed and tested against oral bacteria, and have shown potential effects against biofilms (albeit with reduced effects vs planktonic cells) (as reviewed by Silva et al., 2012). Unfortunately, most of these studies tested antibiofilm efficacy using continuous, prolonged biofilm exposure to AMPs (several hours) rather than topical treatment regimen as used clinically. Furthermore, synthetic AMPs are expensive to produce making them unaffordable for dental applications. Here, we show a plant-produced AMP, which demonstrates potent effects in controlling biofilm formation with a single, short-term topical treatment of a tooth-surrogate surface.

Developed cariogenic biofilms are characterized by bacteria embedded in EPS matrix, making biofilm treatment and removal extremely difficult (Paes Leme et al 2006; Koo et al 2013). EPS-rich matrix promotes microbial adhesion, cohesion and protection as well as hindering diffusion (Koo et al 2013; Flemming and Wingender 2010). EPS matrix creates spatial and microenvironmental heterogeneity in biofilms, modulating the growth and protection of pathogens against antimicrobials locally as well as a highly adhesive scaffold that ensures firm attachment of biofilms on tooth surfaces (Flemming and Wingender 2010; Peterson et al. 2015). CHX is far less effective against formed cariogenic biofilms (Hope and Wilson, 2004; Van Strydonck et al 2012; Xiao et al., 2012). The EPS are comprised primarily of a mixture of insoluble (with high content of α 1,3 linked glucose) and soluble (mostly α 1,6 linked glucose) glucans (Bowen and Koo 2011). Thus, the possibility of using EPS-matrix degrading dextranase or mutanase (from fungi) to disrupt biofilm and prevent dental caries has been explored and included in commercially available over-the-counter mouthwashes (e.g. Biotene PBF). However, topical applications of enzyme alone have generated moderate anti-biofilm/anti-caries effects

clinically (Hull 1980), possibly due to lack of antibacterial action and reduced enzymatic activity in the mouth (Balakrishnan et al 2000). Interestingly, a recent *in vitro* study has shown that a chimeric glucanase comprised of fused dextranase and mutanase is more effective in disrupting plaque-biofilms than either enzymes alone (Jiao et al 2014). However, an approach of combining antimicrobial agents with both EPS-matrix degrading enzymes into a single therapeutic system has not yet been developed, likely due to difficulties associated with cost and formulations. In this study we demonstrate that PG1 together with matrix-degrading enzymes act synergistically and effectively to disrupt cariogenic biofilms. This feasible and efficacious topical antibiofilm approach is capable of simultaneously degrading the biofilm matrix scaffold and killing embedded bacteria using antimicrobial peptides combined with EPS-digesting enzymes.

Retention of high level antimicrobial activity by protegrin along with GFP fusion opens the door for a number of clinical applications to enhance oral health, beyond disruption of biofilms. In addition to biofilm disruption, enhancing wound healing in the gum tissues is an important clinical need. We recently reported that both protegrin and retrocyclin can enter human mast cells and induce degranulation, an important step in the wound healing process (Gupta et al 2015). Therefore, antimicrobial peptides protegrin and retrocyclin play an important role in killing bacteria in biofilms and initiate wound healing through degranulation of mast cells. In addition, it is important to effectively deliver growth hormones or other proteins to enhance cell adhesion, stimulate osteogenesis, angiogenesis, bone regeneration, differentiation of osteoblasts or endothelial cells. Previously identified cell penetrating peptides have several limitations. CTB enters all cell types via the ubiquitous GM1 receptor and this requires pentameric form of CTB. PTD on the other hand does not enter immune cells (Xiao et al 2016).

In this study we tested ability of PG1-GFP or RC101-GFP to enter periodontal and gingival cells. PG1-GFP is the most efficient tag in entering periodontal or gingival human cells because GFP signal could be detected even at ten-fold lower concentrations than any other fusion proteins. Although there were some variations in intracellular localization, PG1-GFP effectively entered HPDLSC, SCC-1, GMSC, AGK, MMSC and OBC. In contrast RC101-GFP entered SCC-1, GMSC, AGK and OBC but its localization in HPDLSC and MMSC cells were poor or undetectable. Therefore, this study has identified a novel role for protegrin in delivering drugs to osteoblasts, periodontal ligament cells, gingival epithelial cells or fibroblasts to enhance oral health. It is feasible to release protein drugs synthesized in plant cells by mechanical grinding

and protein drugs bioencapsulated in lyophilized plant cells embedded in chewing gums provides an ideal mode of drug delivery for their slow and sustained release for longer duration. This overcomes a major limitation of current oral rinse formulations - short duration of contact of antimicrobials on the gum/dental surface.

- 5 Beyond topical applications, protein drugs fused with protegrin expressed in plant cells can be orally delivered to deeper layers of gum tissues in a non-invasive manner and increase patient compliance. Protein drugs bioencapsulated in plants can be stored for many years at room temperature without losing their efficacy (Su et al 2015; Daniell et al 2016). The high cost of current protein drugs is due to their production in prohibitively expensive fermenters,
 10 purification, cold transportation/ storage, short shelf life and sterile delivery methods. All these challenges could be eliminated using this novel drug delivery concept to enhance oral health. Recent FDA approval of plant cells for production of protein drugs (Walsh 2014) augurs well for clinical advancement of this novel concept.

References

- 15 Agrawal P, Verma D, Daniell H. 2011. Expression of *Trichoderma reesei* β -mannanase in tobacco chloroplasts and its utilization in lignocellulosic woody biomass hydrolysis. PLoS One. 6:e29302.
- Ajdić D, McShan WM, McLaughlin RE, Savić G, Chang J, Carson MB, Primeaux C, Tian R, Kenton S, Jia H, Lin S, Qian Y, Li S, Zhu H, Najar F, Lai H, White J, Roe BA, Ferretti JJ. 2002. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. Proc Natl Acad
 20 Sci U S A 99:14434-14439.
- Autio-Gold J. 2008. The role of chlorhexidine in caries prevention. Oper Dent. 33(6):710-6.
- Banas JA, Vickerman MM. 2003. Glucan-binding proteins of the oral streptococci. Crit Rev Oral Biol Med. 14(2):89-99.
- Balakrishnan M, Simmonds RS, Tagg JR. 2000. Dental caries is a preventable infectious disease.
 25 Aust Dent J. 45:235-45.
- Bowen WH. 1972. The effect of dextranase on caries activity in monkeys (*macaca irus*). Caries Res. 6:75-6.

- Bowen WH and Koo H. 2011. Biology of streptococcus mutans-Derived glucosyltransferases: Role in extracellular matrix formation of cariogenic biofilms. *Caries Res* 45:69-86.
- Burne RA. 1998. Oral streptococci... products of their environment. *J Dent Res.* 77(3):445-52.
- Caufield PW, Dasanayake AP, Li Y. 2001. The antimicrobial approach to caries management. *J Dent Educ* 65:1091-5.
- Chen J, Falla TJ, Liu H, Hurst MA, Fujii CA, Mosca DA, Embree JR, Louny DJ, Radel PA, Cheng Chang C, Gu L, Fiddes JC. 2000. Development of protegrins for the treatment and prevention of oral mucositis: structure-activity relationships of synthetic protegrin analogues. *Biopolymers.* 55:88-98.
- da Silva BR, de Freitas VA, Nascimento-Neto LG, Carneiro VA, Arruda FV, de Aguiar AS, Cavada BS, Teixeira EH. 2012. Antimicrobial peptide control of pathogenic microorganisms of the oral cavity: a review of the literature. *Peptides* 36: 315-321.
- Daniell H, Lin CS, Yu M, Chang WJ. 2016. Chloroplast genomes: diversity, evolution and applications in genetic engineering. *Genome Biology*, in press
- DeGray G, Rajasekaran K, Smith F, Sanford J, Daniell H. 2001. Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. *Plant Physiol.* 127(3):852-62.
- Dige I, Raarup MK, Nyengaard JR, Kilian M, Nyvad B. 2009. *Actinomyces naeslundii* in initial dental biofilm formation. *Microbiology* 155:2116-2126.
- Dye BA, Hsu KL, Afful J. 2015. Prevalence and Measurement of Dental Caries in Young Children. *Pediatr Dent* 37: 200-216.
- Flemmig TF, Beikler T. 2011. Control of oral biofilms. *Periodontol* 2000. 55:9-15.
- Flemming HC, Wingender J. 2010. The biofilm matrix. *Nat Rev Microbiol.* 8:623-33.
- Giles FJ, Miller CB, Hurd DD, Wingard JR, Fleming TR, Sonis ST, Bradford WZ, Pulliam JG, Anaissie EJ, Beveridge RA, Brunvand MM, Martin PJ; PROMPT-CT Trial Investigators. 2003. A phase III, randomized, double-blind, placebo-controlled, multinational trial of iseganan for the

prevention of oral mucositis in patients receiving stomatotoxic chemotherapy (PROMPT-CT trial). *Leuk Lymphoma*. 44:1165-72.

Guggenheim B, Regolati B, Schmid R, Mühlemann HR. 1980. Effects of the topical application of mutanase on rat caries. *Caries Res*. 14:128-35.

- 5 Guo L, McLean JS, Yang Y, Eckert R, Kaplan CW, Kyme P, Sheikh O, Varnum B, Lux R, Shi W, He X. 2015. Precision-guided antimicrobial peptide as a targeted modulator of human microbial ecology. *Proc Natl Acad Sci U S A* 112: 7569-7574.

- 10 Gupta, Kshitij, Akhil Kotian, Hariharan Subramanian, Henry Daniell, and Hydar Ali. "Activation of Human Mast Cells by Retrocyclin and Protegrin Highlight Their Immunomodulatory and Antimicrobial Properties." *Oncotarget* 6, no. 30 (October 6, 2015): 28573–87. doi:10.18632/oncotarget.5611.

Hajishengallis E, Parsaei M, Klein MI, Koo H. 2015. Advances in the microbial etiology and pathogenesis of early childhood caries. *Molecular Oral Microbiology* doi: 10.1111/omi.12152.

- 15 Hall-Stoodley L, Costerton JW, Stoodley P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2: 95-108.

Hayacibara MF, Koo H, Vacca-Smith AM, Kopec LK, Scott-Anne K, Cury JA, Bowen WH. 2004. The influence of mutanase and dextranase on the production and structure of glucans synthesized by streptococcal glucosyltransferases. *Carbohydrate Research* 339: 2127-2137.

- 20 Hope CK and Wilson M. 2004. Analysis of the effects of chlorhexidine on oral biofilm vitality and structure based on viability profiling and an indicator of membrane integrity. *Antimicrob Agents Chemother* 48:1461-8.

Hull PS. 1980. Chemical inhibition of plaque. *J Clin Periodontol*. 7:431–42.

Jin S, Daniell H. 2015. The Engineered Chloroplast Genome Just Got Smarter. *Trends Plant Sci* 20:622-640

- 25 Jiao YL, Wang SJ, Lv MS, Jiao BH, Li WJ, Fang YW, Liu S. 2014. Characterization of a marine-derived dextranase and its application to the prevention of dental caries. *J Ind Microbiol Biotechnol*. 41:17-26.

- Jones CG. 1997. Chlorhexidine: is it still the gold standard? *Periodontol* 2000 15: 55-62.
- Jin S, Kanagaraj A, Verma D, Lange T, Daniell H. 2011. Release of hormones from conjugates: chloroplast expression of β -glucosidase results in elevated phytohormone levels associated with significant increase in biomass and protection from aphids or whiteflies conferred by sucrose esters. *Plant Physiol.* 155:222-35.
- Kassebaum NJ, Bernabé E, Dahiya M, Bhandari B, Murray CJ, Marcenes W. 2015. Global burden of untreated caries: A systematic review and metaregression. *J Dent Res.* 94:650-658.
- Kohli N, Westerveld DR, Ayache AC, Verma A, Shil P, Prasad T, Zhu P, Chan SL, Li Q, Daniell H. 2014. Oral delivery of bioencapsulated proteins across blood-brain and blood-retinal barriers. *Mol Ther.* 22:535-46.
- Klein MI, Scott-Anne KM, Gregoire S, Rosalen PL, Koo H. Molecular approaches for viable bacterial population and transcriptional analyses in a rodent model of dental caries. *Molecular Oral Microbiology* 27: 350–361.
- Klein MI, DeBaz L, Agidi S, Lee H, Xie G, Lin AHM, Hamaker BR, Lemos JA, Koo H. 2010. Dynamics of *Streptococcus mutans* transcriptome in response to starch and sucrose during biofilm development. *PLoS One*: e1347840.
- Koo H, Falsetta ML, Klein MI. 2013. The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. *J Dent Res.* 92:1065-73.
- Koo H, Xiao J, Klein MI, Jeon JG. 2010. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. *J Bacteriol* 192:3024-3032.
- Kwon KC, Verma D, Singh ND, Herzog R, Daniell H. 2013. Oral delivery of human biopharmaceuticals, autoantigens and vaccine antigens bioencapsulated in plant cells. *Adv Drug Deliv Rev.* 65(6):782-99.
- Kwon KC, Nityanandam R, New JS, Daniell H. 2013. Oral delivery of bioencapsulated exendin-4 expressed in chloroplasts lowers blood glucose level in mice and stimulates insulin secretion in beta-TC6 cells. *Plant Biotechnol J.* 11:77-86.
- Odd FC. 1988. *Candida* and candidosis. 2nd ed. Bailliere Tindall, London, United Kingdom.

- Peterson BW, He Y, Ren Y, Zerdoum A, Libera MR, Sharma PK, van Winkelhoff AJ, Neut D, Stoodley P, van der Mei HC, Busscher HJ. 2015. Viscoelasticity of biofilms and their recalcitrance to mechanical and chemical challenges. *FEMS Microbiol Rev* 39: 234-245.
- Lakshmi PS, Verma D, Yang X, Lloyd B, Daniell H. 2013. Low cost tuberculosis vaccine antigens in capsules: expression in chloroplasts, bio-encapsulation, stability and functional evaluation in vitro. *PLoS One*. 8:e54708.
- Lai JR, Huck BR, Weisblum B, Gellman SH. 2002. Design of non-cysteine-containing antimicrobial beta-hairpins: structure-activity relationship studies with linear protegrin-1 analogues. *Biochemistry*. 41:12835-42.
- 10 Lee SB, Li B, Jin S, Daniell H. 2011. Expression and characterization of antimicrobial peptides Retrocyclin-101 and Protegrin-1 in chloroplasts to control viral and bacterial infections. *Plant Biotechnol J*. 9:100-15.
- Ma Z, Wei D, Yan P, Zhu X, Shan A, Bi Z. 2015. Characterization of cell selectivity, physiological stability and endotoxin neutralization capabilities of α -helix-based peptide amphiphiles. 15 *Biomaterials*. 52:517-30.
- Marsh PD, Moter A, Devine DA. 2011. Dental plaque biofilms: communities, conflict and control. *Periodontology 2000*. 55:16-35.
- Morassutti, Carla, Francesca De Amicis, Barbara Skerlavaj, Margherita Zanetti, and Stefano Marchetti. Production of a Recombinant Antimicrobial Peptide in Transgenic Plants Using a 20 Modified VMA Intein Expression System. *FEBS Letters* 519: 141–46.
- Ohashi, Tomoo, Stephane D. Galiacy, Gina Briscoe, and Harold P. Erickson. An Experimental Study of GFP-Based FRET, with Application to Intrinsically Unstructured Proteins. *Protein Science: A Publication of the Protein Society* 16, no. 7 (July 2007): 1429–38. doi:10.1110/ps.072845607.
- 25 Otsuka R, Imai S, Murata T, Nomura Y, Okamoto M, Tsumori H, Kakuta E, Hanada N, Momoi Y. 2015. Application of chimeric glucanase comprising mutanase and dextranase for prevention of dental biofilm formation. *Microbiol Immunol*. 59:28-36.

- Paes Leme AF, Koo H, Bellato CM, Bedi G, Cury JA. 2006. The role of sucrose in cariogenic dental biofilm formation-new insight. *J Dent Res.* 85:878-87.
- Sassi AB, Bunge KE, Hood BL, Conrads TP, Cole AM, Gupta P, Rohan LC. 2011. Preformulation and stability in biological fluids of the retrocyclin RC-101, a potential anti-HIV topical
5 microbicide. *AIDS Res Ther.* 8:27.
- Sassi AB, Cost MR, Cole AL, Cole AM, Patton DL, Gupta P, Rohan LC. 2011. Formulation development of retrocyclin 1 analog RC-101 as an anti-HIV vaginal microbicide product. *Antimicrob Agents Chemother.* 55:2282-9.
- Skosyrev, Vitaly S., Natalja V. Rudenko, Alexander V. Yakhnin, Vasily E. Zagranichny, Lubov
10 I. Popova, Mikhail V. Zakharov, Andrey Yu Gorokhovatsky, and Leonid M. Vinokurov. EGFP as a Fusion Partner for the Expression and Organic Extraction of Small Polypeptides. *Protein Expression and Purification* 27, no. 1 (January 2003): 55–62.
- Trotti A, Garden A, Warde P, Symonds P, Langer C, Redman R, Pajak TF, Fleming TR, Henke M, Bourhis J, Rosenthal DI, Junor E, Cmelak A, Sheehan F, Pulliam J, Devitt-Risse P, Fuchs H,
15 Chambers M, O'Sullivan B, Ang KK. 2004. A multinational, randomized phase III trial of iseganan HCl oral solution for reducing the severity of oral mucositis in patients receiving radiotherapy for head-and-neck malignancy. *Int J Radiat Oncol Biol Phys.* 58:674-81.
- Van Strydonck DA, Slot DE, Van der Velden U, Van der Weijden F. 2012. Effect of a chlorhexidine mouthrinse on plaque, gingival inflammation and staining in gingivitis patients: a
20 systematic review. *J Clin Periodontol.* 39:1042-55.
- Verma D, Kanagaraj A, Jin S, Kolattukudy PE, Daniell H. 2010. Chloroplast-derived enzyme cocktails hydrolyse lignocellulosic biomass and release fermentable sugars. *Plant Biotechnol J.* 8:332-50.
- Walsh G. 2014. Biopharmaceutical benchmarks 2014. *Nat Biotechnol.* 32:992-1000.
- 25 Wang W, Cole AM, Hong T, Waring AJ, Lehrer RI. 2003. Retrocyclin, an antiretroviral theta-defensin, is a lectin. *J Immunol.* 170(9):4708-16.

Xiao, Yuhong, Kwang-Chul Kwon, Brad E. Hoffman, Aditya Kamesh, Noah T. Jones, Roland W. Herzog, and Henry Daniell. Low Cost Delivery of Proteins Bioencapsulated in Plant Cells to Human Non-Immune or Immune Modulatory Cells. *Biomaterials* 80 (February 2016): 68–79. doi:10.1016/j.biomaterials.2015.11.051.

- 5 Xiao, J. et al. 2012. The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. *PLoS Pathog* 8, e1002623.

Yakhnin, A. V., L. M. Vinokurov, A. K. Surin, and Y. B. Alakhov. Green Fluorescent Protein Purification by Organic Extraction. *Protein Expression and Purification* 14, no. 3 (December 1998): 382–86. doi:10.1006/prep.1998.0981.

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Example II

Creation of chloroplast vectors expressing AMP, biofilm degrading enzymes and fusion proteins thereof

15

Effective treatment of biofilm-associated infections is problematic as antimicrobials often fail to reach clusters of microbes present within the surrounding extracellular matrix that enmeshes and protects them. Furthermore, development of novel therapies against biofilm-related oral diseases and maintenance of oral health needs to be cost-effective and readily accessible.

20

To ensure a continued supply of reagents, dextranase /mutanase and protegrin/retrocyclin are expressed independently and as fusion proteins in tobacco and other plant chloroplasts, such as lettuce. Proteins will be produced and used in low cost purification strategies. Tobacco plants produce a million seeds, and thus, it is feasible to scale up production easily. Each acre of tobacco will produce up to 40 metric tons of biomass, facilitating low cost large scale production of AMP, enzymes and fusion constructs encoding the same. In another approach, the proteins are produced in an edible plant such as lettuce.

25

Several dextranases (Dex) and mutanases (Mut) have been isolated from fungi and bacteria and characterized for their enzymatic activity. Optimal dextranase and mutanase enzymes should have enzymatic properties suitable for human oral environment. Based on short duration of oral treatments, strong binding/retention property to plaque-biofilms and catalytic activity to both types of EPS (dextrans and mutans) are highly desirable. The enzymes added in

commercial dextranase-containing mouthwashes (e.g. Biotene) are largely derived from fungi (*Penicillium* sp. and *Chaetomium erraticum*). However, fungal dextranases show higher temperature optima (50-60°C) than bacterial dextranases (35-40°C). Furthermore, bacterial dextranases are more stable and effective at oral temperature (~37°C) and are suitable for dental caries-prevention. Recently, a dextranase from *Arthrobacter* sp strain Arth410 showed superior dextran degradation properties at optimal temperatures (35-45°C) and pH values (pH 5-7) found in mouth and in cariogenic biofilms when compared to fungal dextranases. In addition, topical applications of bacterial dextranase are more effective in reducing dental caries *in vivo* than fungal dextranase. Likewise, a bacterial mutanase from *Paenibacillus* sp. strain RM1 shows that biofilm was effectively degraded by 6 hr incubation even after removal of the mutanase, preceded by first incubation with the biofilms for 3 min. Also, when compared to other microbial species, RM1 mutanase shows enhanced biofilm-degrading property. Notably, fungal enzymes require glycosylation, which precludes their expression in chloroplasts. In addition, immunogenicity of glycoproteins in human system may raise additional regulatory concerns. Therefore, the present invention involves use of bacterial dextranase and mutanase for expression in chloroplasts.

In order to increase the production of Arth410 dextranase and RM1 mutanase protein in chloroplasts, both sequences have been codon optimized for chloroplast expression. See Figures 9A and 9B.

Retrocyclin and Protegrin.

In order to maximize synthesis and reduce toxicity of AMPs, ten tandem repeats of PG1 or RC101, separated by protease cleavage sites as shown in Figure 10 are employed. For each copy of expressed gene, ten functional copies of PG1 or RC 101 will be made. For this purpose we have chosen the Tobacco Etch Virus (TEV) protease, which has high specificity and a short cleavage site of seven amino acids. Alternatively, furin cleavage sites can also be employed. This vector can also be engineered to include a nucleic acid encoding a biofilm degrading enzyme. The coding region can be expressed under the promoter utilized to express the AMP or can be ligated into the vector operably linked to a second promoter region. The biofilm degrading enzyme coding sequence may also contain TEV protease cleavage sites to facilitate release of the enzyme. This approach provides a safer and cleaner option than chemical cleavage methods. Most importantly, individual PG1 peptides in the fusion protein will not form

secondary structures before cleavage, thereby avoiding accumulation of functional peptides which can be lethal to the host production systems. Antimicrobial activity of the cleaved PG1/RC101, biofilm degrading enzymes or fusion proteins thereof can be used to degrade biofilms using the methods disclosed in Example I.

As mentioned above, the sequences encoding the AMP/biofilm degrading enzymes are optionally codon-optimized prior to insertion into chloroplast transformation vectors, such as pLD. Chloroplast transformation relies upon a double homologous recombination event. Therefore, chloroplast vectors comprise homologous regions to the chloroplast genome which flank the expression cassette encoding the heterologous proteins of interest, which facilitate insertion of the transgene cassettes into the intergenic spacer region of the chloroplast genome, without disrupting any functional genes. Although any intergenic spacer region could be used to insert transgenes, the most commonly used site of transgene integration is the transcriptionally active intergenic region between the *trnI-trnA* genes (in the *rrn* operon), located within the IR regions of the chloroplast genome (Figure 10). Because of similar protein synthetic machinery between *E. coli* and chloroplasts, efficiency of codon-optimization can also be assessed in *E. coli* and then plants can be created. Both systems could be used for expression of AMPs, biofilm degrading enzymes or fusion proteins thereof, as well as for purification and evaluation of AMPs or enzymatic activities.

Purification strategies

A hydrophobic interaction column (HIC; TOSOH Butyl Toyopearl 650m) can be used to purify PG1 fused with Green Florescent Protein (GFP). The GFP selectively binds to the HIC and facilitates Rc101/PG1 to >90% purity. Despite using the expensive HIC chromatography method, recovery is very poor (<20%). To address this problem and enhance yield, 10 tandem repeats of PG1 with an elastin like biopolymer (GVGV (SEQ ID NO: 11); Fig.10) are engineered into the vector. This biopolymer, has a unique thermal property of precipitating out of solution upon increasing temperature above its inverse transition temperature (T_t). GVGVP remains in soluble monomeric state below T_t and form insoluble aggregates above it. This phase transition from soluble to insoluble state is reversible by changing the temperature of the solution and this facilitates protein purification. Subsequently fused protein is re-solubilized by cooling below T_t and to remove any insoluble contaminants that have co-precipitated as shown in Figure

11. The process of heating (37°C) and cooling (4°C) is known as Inverse Transition Cycling (ITC) and performing 3-5 rounds of ITC results in highly purified proteins (>98% purity, Figure 11).

In an alternative approach, a signal peptide is fused with dextranase or mutanase for expression in *E. coli*, where the signal peptide will result in secretion of the enzymes into the extracellular media. In addition, secretory proteins should pass through two membrane systems of *E. coli*, during which they pass through the periplasmic environment where disulfide isomerases, foldases and chaperones are present. Therefore, correct folding and disulfide bond formation of secretory proteins are facilitated by the enzymes, resulting in enhancement of biological activity of proteins (ideal for AMPs). Another merit of this production strategy is the low level of proteolytic activity in the culture medium which serves to enhance the stability of the recombinant protein. The signal sequence of the secreted protein is cleaved during the export process, creating an authentic N-terminus to the native protein. There are several molecules useful for translocating proteins to extracellular media, such as TAT, SRP, or SecB-dependent pathways. However, rather than working independently, the different pathways closely interact with each other. Both SRP and SecB-dependent pathways can work together in targeting of a single protein. Also, under Sec-deficient conditions, translocation of Sec pathway substrates can be rescued by TAT systems.

Among numerous signal sequences, outer membrane protein A (OmpA) and Seq X (derived from lac Z) signal peptide demonstrate superior export functions and are capable of exporting fused protein into extracellular medium at up to 4g /L and 1g/L, respectively. Therefore, these signal sequences are used for efficient exporting of Arth 410 Dex and RM1 Mut to extracellular milieu. Accumulation of the dextranase and mutanase exported into media will be determined by protein quantitation and enzyme assays.

Successful expression of these proteins in *E. coli* has been achieved. See Western blot results shown in Figure 12. Chloroplast vectors harboring these sequences will be bombarded into tobacco or lettuce leaves to create plants capable of large scale production of extranase/mutanase/AMP proteins. After harvesting large scale biomass, leaves will be lyophilized and stored at room temperature. In another approach, clinically-proven anti-caries compounds such as (fluoride 250ppm) and a broad-spectrum bactericidal, chlorhexidine 0.12% can be included to assess whether these agents increase efficacy.

The AMP-enzyme combination effectively disrupts cariogenic biofilm formation and the onset of cavitation *in vivo*. Furthermore, AMP-enzyme fusion protein appears to be superior to current chemical modalities for antimicrobial therapy and caries prevention.

As mentioned previously, effective AMP-enzyme (independently or in combination) can be expressed in lettuce chloroplasts under the control of endogenous lettuce regulatory elements, for large scale GLP production and stability assessment. A key advantage is the lower production cost by elimination of prohibitively expensive purification processes. Freeze-dried leaf material expressing AMP/enzymes can be stored at ambient temperatures for several months or years while maintaining their integrity and functionality. See Figure 13. In addition to long-term storage, increase of protein drug concentration and decrease of microbial contamination are other advantages. Lettuce leaves, after lyophilization showed 20-25 fold increase in protein drug concentration when compared to fresh leaves, thereby reducing the amount of materials used for oral or topical delivery. Following lyophilization, the plant material can be incorporated into a chewing gum to deliver the biofilm degrading compositions contained therein.

The steps for producing the AMP/enzymes or fusions thereof are shown in Figure 12. The lettuce chloroplast vectors useful for expressing the proteins of the invention have been previously described in US Patent Application No. 12/059,376, which is incorporated herein by reference. Expression levels of up to 70% of total protein in case of therapeutic proteins like proinsulin in lettuce chloroplasts can be achieved using this system.

AMP-enzyme(s) expressed in the edible plants are preferably orally delivered (topically) when used for treatment of oral diseases and the prevention and inhibition of dental carie formation. For enhanced lysis of plant cells within the oral cavity, AMP-enzyme expressing plant cells are optionally mixed with plant cells expressing cell wall degrading enzymes, described in US Patent Application, 12/396,382, also incorporated herein by reference.

Chewing gum tablet preparation is shown in Figure 14. Using GFP as an example of the protein of interest, this data shows the amounts of GFP that can be incorporated in to a chewing gum tablet. GFP levels were assessed both via fluorescence and by western blot. The results are shown in Figure 15. The present inventors employed the chewing simulator shown in Figure 16 and artificial saliva to assess GFP release kinetics from the gum tablets comprising GFP. Figure 17 shows a graph illustrating the release kinetics over time from gum tablets comprising different amounts of GFP present in recombinant lettuce.

It is clear from these data that gum tablets comprising the AMP-enzyme fusion proteins of the invention will deliver the active material for a suitable time period to achieve bacterial kill and plaque or biofilm degradation. However, oral rinses, such as Listerine®, can also be employed to deliver the AMP-enzyme fusion proteins or combinations of the invention. Figure 18 demonstrates that crude extracts comprising the enzymes of the invention mixed with LISTERINE mouthwash are as effective as commercially produced and purified enzymes that are quite costly to prepare. The data reveal that the dual-enzyme at various combinations (both different ratio and amounts) markedly reduced the biomass of *S. mutans* biofilm, in a dose-dependent manner. Among different combinations, 25U Dex and 5U Mut (5:1, Dex:Mut ratio) was the most effective, resulting in more than 80% of the total biomass degradation within 120 minutes. Further experiments confirmed that 5:1 Dex/Mut activity ratio displayed the highest effectiveness for both EPS degradation and bacterial killing by LISTERINE mouthwash. Excitingly, the dual-enzyme pre-treatment dramatically enhanced the efficacy of LISTERINE mouthwash-mediated bacterial killing (> 3 log reduction vs vehicle pre-treatment and LISTERINE mouthwash). The inclusion of a third enzyme further enhanced the overall anti-biofilm activity. Furthermore, results from the mixed-species model indicated that the dual-enzyme combination was capable of not only enhancing the overall antibacterial activity, but also inducing targeted reduction of *S. mutans* dominance (while increasing the proportion of commensal/probiotic *S. oralis*) when LISTERINE mouthwash was used after enzymes pre-treatment. Accordingly, the enzyme+LISTERINE mouthwash strategy should selectively target the pathogen *S. mutans*, while increasing the proportion of commensal *S. oralis*, thereby preventing microecological imbalance within mixed-species biofilm.

AMPS have the ability to stimulate innate immunity and wound healing, in addition to antimicrobial activity. Harnessing this novel mast cell host defense feature of AMPs in addition to their antimicrobial properties expands their clinical applications. Biofilm-associated caries is the most challenging model for development of topical therapeutics. When developed, such topical drug delivery can be easily adapted to other biofilms, as matrix formation hinders drug efficacy in many other biofilm-associated diseases. Matrix is inherent in all biofilms thus the application goes beyond the biofilm in the mouth. The biofilm inhibiting compositions described herein can also be employed in coating stents, artificial joints, implants, valves and other medical devices inserted into the human body for the treatment of disease.

As discussed above, the AMP/enzymes, or leaves expressing the same can be incorporated into a chewing gum for effective topical application of the same for the treatment of oral disease. The compositions may also be incorporated into an oral rinse, such as LISTERINE mouthwash. As mentioned previously, other anti dental carrie agents such as fluoride or CHX
5 may included in such gums or oral rinses.

The references below in Table 2 describe a number of different mutanases from a variety of biological sources. Each of these references incorporated herein by reference.

	Reference	Year	Mutanase resource
1	Otsuka R, Imai S, Murata T, et al. (2014) Application of chimeric glucanase comprising mutanase and dextranase for prevention of dental biofilm formation. Microbiology and Immunology n/a-n/a	2014	Paenibacillus humicus NA1123
2	Wiater A, Pleszczyńska M, Rogalski J, Szajnecka L & Szczodrak J (2013) Purification and properties of an alpha-(1 → 3)-glucanase (EC 3.2.1.84) from Trichoderma harzianum and its use for reduction of artificial dental plaque accumulation. Acta Biochim Pol 60: 123-128.	2013	Trichoderma harzianum CCM F-340
3	Wiater A, Janczarek M, Choma A, Próchniak K, Komaniecka I & Szczodrak J (2013) Water-soluble (1 → 3),(1 → 4)-α-d-glucan from mango as a novel inducer of cariogenic biofilm-degrading enzyme. International Journal of Biological Macromolecules 58: 199-205.	2013	Trichoderma harzianum strain CCM F-340
4	Tsumori H, Shimamura A, Sakurai Y & Yamakami K (2012) Combination of Mutanase and Dextranase Effectively Suppressed Formation of Insoluble Glucan Biofilm by Cariogenic Streptococci. Interface Oral Health Science 2011,(Sasaki K, Suzuki O & Takahashi N, ed. eds.), p. pp. 215-217. Springer Japan.	2012	Paenibacillus humicus
5	Xiao J, Klein MI, Falsetta ML, et al. (2012) The Exopolysaccharide Matrix Modulates the Interaction between 3D Architecture and Virulence of a Mixed-Species Oral Biofilm. PLoS Pathog 8: e1002623.	2012	Trichoderma harzianum
6	Tsumori H, Shimamura A, Sakurai Y & Yamakami K (2011) Mutanase of Paenibacillus humicus from Fermented Food Has a Potential for Hydrolysis of Biofilms Synthesized by Streptococcus mutans. Journal of Health Science 57: 420-424.	2011	Paenibacillus humicus
7	Wiater A, Szczodrak J & Pleszczyńska M (2008) Mutanase induction in Trichoderma harzianum by cell wall of Laetiporus sulphureus and its application for mutan removal from oral biofilms. J Microbiol Biotechnol 18: 1335-1341.	2008	Trichoderma harzianum
8	Shimotsuura I, Kigawa H, Ohdera M, Kuramitsu HK & Nakashima S (2008) Biochemical and Molecular Characterization of a Novel Type of Mutanase from Paenibacillus sp. Strain RM1: Identification of Its Mutan-Binding Domain, Essential for Degradation of Streptococcus mutans Biofilms. Applied and Environmental Microbiology 74: 2759-2765	2008	Paenibacillus sp. strain RM1
9	Shimotsuura I, Kigawa H, Ohdera M, Kuramitsu HK & Nakashima S (2008) Biochemical and Molecular Characterization of a Novel Type of Mutanase from Paenibacillus sp. Strain RM1: Identification of Its Mutan-Binding Domain, Essential for Degradation of Streptococcus mutans Biofilms. Applied and Environmental Microbiology 74: 2759-2765.	2008	Paenibacillus sp. strain RM1
10	Wiater A, Szczodrak J, Pleszczyńska M, Próchniak K (2005) Production and use of mutanase from Trichoderma harzianum for effective degradation of streptococcal mutans. Braz. J. Microbiol. vol.36 no.2	2005	Trichoderma harzianum CCM F-340
11	Hayacibara MF, Koo H, Vacca Smith AM, Kopec LK, Scott-Anne K, Cury JA & Bowen WH (2004) The influence of mutanase and dextranase on the production and structure of glucans synthesized by streptococcal glucosyltransferases. Carbohydrate Research 339: 2127-2137	2004	Trichoderma harzianum
12	Kopec LK, Vacca Smith AM, Wunder D, Ng-Evans L & Bowen WH (2001) Properties of Streptococcus sanguinis glucans formed under various conditions. Caries Res 35: 67-74.	2001	Trichoderma harzianum
13	Kopec LK, Vacca-Smith AM & Bowen WH (1997) Structural aspects of glucans formed in solution and on the surface of hydroxyapatite. Glycobiology 7: 929-934.	1997	Trichoderma harzianum CCM F-341
14	Vacca-Smith AM, Venkitaraman AR, Quivey RG, Jr. & Bowen WH (1996) Interactions of streptococcal glucosyltransferases with alpha-amylase and starch on the surface of saliva-coated hydroxyapatite. Arch Oral Biol 41: 291-298.	1996	Trichoderma harzianum
15	Quivey RG, Jr. & Kriger PS (1993) Raffinose-induced mutanase production from Trichoderma harzianum. FEMS Microbiol Lett 112: 307-312.	1993	Trichoderma harzianum
16	Inoue M, Yakushiji T, Mizuno J, Yamamoto Y & Tanii S (1990) Inhibition of dental plaque formation by mouthwash containing an endo-alpha-1, 3 glucanase. Clin Prev Dent 12: 10-14.	1990	Pseudomonas sp. strain
17	Inoue M, Yakushiji T, Katsuki M, Kudo N & Koga T (1988) Reduction of the adherence of Streptococcus sobrinus insoluble α-d-glucan by endo-(1→3)-α-d-glucanase. Carbohydrate Research 182: 277-286.	1988	Pseudomonas sp.
18	Kelstrup J, Holm-Pedersen P & Poulsen S (1978) Reduction of the formation of dental plaque and gingivitis in humans by crude mutanase. European Journal of Oral Sciences 86: 93-102.	1978	Trichoderma harzianum
19	Kelstrup J, Holm-Pedersen P & Poulsen S (1978) Reduction of the formation of dental plaque and gingivitis in humans by crude mutanase. Scand J Dent Res 86: 93-102.	1978	Trichoderma harzianum
20	Guggenheim B, Regolati B & Mühlemann HR (1972) Caries and Plaque Inhibition by Mutanase in Rats. Caries Research 6: 289-297.	1972	Trichoderma harzianum OMZ 779

Additional biofilm degrading enzyme encoding sequences useful in the practice of the invention, include without limitation,

I) Paenibacillus humicus NA1123

See also <http://www.ncbi.nlm.nih.gov/nuccore/AB489092>

5 Genbank AB489092

Length:1,146

Mass (Da):119,007

Reference: Otsuka R, et al. Microbiol Immunol. 2015 Jan;59(1):28-36.

2. The protein sequence of mutanase from *Paenibacillus humicus* NA1123

10 >gi|257153265|dbj|BAI23187.1| putative mutanase [Paenibacillus humicus]

[illegible]

3. Sequence of mRNA from *Paenibacillus humicus* NA1123

30 >gi|257153264|dbj|AB489092.1| *Paenibacillus humicus* mut gene for putative mutanase, complete cds

1 aaaggaggat cgccaaccaa tcateccagc aaagaaggtg atggcagccc aagaattgaa

61 agcgctttga atttgaata tacggatttg gccgacctgc tgattcagtc gtattcaagc

121 gattatgccg cgaaccaatc gaacccgagg aggactataa tgcgtatccg cactaaatat

181 atgaactgga tgttggtgct cgtcctgac gccgccgget tcttcaggc tgccggcccc

241 atcgtcccg ccaccgtgc aggaggcgcg aatctgacgc tcggcaaac cgtcaccgcc
 301 agcggccagt cgcagacgta cagccccgac aatgtcaagg acagcaatca gggaacttac
 361 tgggaaagca cgaacaacgc ctcccgagc tggatccaag tcgacctgg cgccagcacg
 421 agcatcgacc agatcgtgct caagcttccg tccggatggg agactcgtac gcaaacgctc
 5 481 tcgatacagg gcagcgcgaa cggctcgacg ttcacgaaca tcgtcggatc ggccgggtat
 541 acattcaatc catccgtcgc cggcaacagc gtcacgatca acttcagcgc tgccagcgcc
 601 cgctacgtcc gcctgaattt cacggccaat acgggctggc cagcaggcca gctgtcggag
 661 ctgagatct acggagcgac ggcgccaacg cctactcca cgcctactcc aacaccaacg
 721 ccaacgcaa caccaacgcc aaccctaca gtaaccctg cgcctcggc cagccgact
 10 781 ccgactctc cggcaggcag caacatgcc gtagggaaat cgattacagc ctctccagc
 841 acgcagacct acgtagctgc aatgcaaat gacaacaata catccaccta ttgggaggga
 901 ggaagcaacc cgagcacgt gactctgat ttcggtcca accagagcat cacttcgctc
 961 gtctcaagc tgaatccggc ttcggaatgg gggactcgca cgcaaagat ccaagttctt
 1021 ggagcggatc agaacgcggc ctcttcagc aatctcgtct ctgccagtc ctatacgtt
 15 1081 aatccccgaa ccggcaatac ggtgacgatt ccggtctccg cgacggtaa gcgcctccag
 1141 ctgaacatta cggcgaactc cgcgccccct gccggccaga ttgccgagtt ccaagtgttc
 1201 ggcagccag cgcctaatec ggactgacc attaccggca tgtcctggac tccgtcttct
 1261 ccggtcgaga gggcgacat tacgtgaac gccgtcgtca agaacatcgg aactgcagct
 1321 gcaggcgcca cgacggtaa ttctacctg aacaacgaac tcgccggcac cgctccgta
 20 1381 ggcgcgcttg cggcaggagc ttctgcaaat gtatgatca atgcaggcgc caaagcagcc
 1441 gcaacgtatg cggtaagcgc caaagtcgac gagagcaacg ccgtatcga gcagaatgaa
 1501 ggcaacaaca gctactgaa cccgactaac ctctcgtag cgccggtgtc cagctccgac
 1561 ctctgcgcg tgacgtcatg gtcgccggc acgccgtgc agggagcggc ggtcgcatth
 1621 accgtcgcgc ttaaaaatca gggtagctg gcttccggc gcggagccca tcccgaacc
 25 1681 gtctgtctga aaaacgtgc cggagcgacg ctgcaaacct tcacgggcac ctacacaggt
 1741 tccctggcag caggcgcatc cgcaataac agcgtgggca gctggacggc agcgagcggc

1801 acctataccg tctcgacgac ggtagccgct gacggcaatg aaattccggc caagcaaagc
 1861 aacaatacga gcagcgcgag cctcacggtc tactcggcgc gcggcgccag catgccgtac
 1921 agccgttacg acacggagga tgcgggtgctc ggcgggcgag ctgtcctgag aacggcgccg
 1981 acgttcgacg agtcgctcat cgctccgaa gcatcgggac agaaatacgc cgcacttccg
 5 2041 tccaacggct ccagcctgca gtggaccgtc cgtcaaggcc agggcggtgc aggcgtcacg
 2101 atgcgcttca cgatgcccgga cagcagcgac ggcatgggcc agaacggctc gctcgacgtc
 2161 tatgtcaacg gaaccaaagc caaacgggtg tcgctgacct cttattacag ctggcagtat
 2221 ttctccggcg acatgccggc tgacgtccg ggcgggcgga ggccgctctt ccgcttcgac
 2281 gaagtcact tcaagctgga tacggcgtg aagccgggag acacgatccg cgtccagaag
 10 2341 ggcggtgaca gcctggagta cggcgtcgac ttcatcgaga tcgagccgat tccggcagcg
 2401 gttgcccgtc cggccaactc ggtgtccgtc accgaatacg gcgctgtcg caatgacggc
 2461 aaggatgate tcgccgctt caaggtgcc gtgaccgcag cggtagcggc cggaaaatcc
 2521 ctctacatcc cggaaggcac cttccacctg agcagcatgt gggagatcgg ctcggccacc
 2581 agcatgatcg acaacttcac gggtacgggt gccggcatct ggtatacga catccagttc
 15 2641 acgaatccca atgcatcggg cggcggcatc tccctgagaa tcaaaggaaa gcttgatttc
 2701 agcaacatct acatgaactc caacctgcgt tcccgttacg ggcagaacgc cgtctacaaa
 2761 ggctttatgg acaatttcgg cactaattcg atcatcatg acgtctgggt cgagcatttc
 2821 gaatcgggca tgtgggtcgg cgactacgcc catactctg cgatctatgc gagcgggctc
 2881 gtcgtgaaa acagccgcat ccgaacaat cttccgacg gcatcaactt ctcgcaggga
 20 2941 acgagcaact cgaccgtccg caacagcagc atccgcaaca acggcgatga cggcctcgcc
 3001 gtctggacga gcaacacgaa cggcgtccg gccggcgtga acaacacctt ctctacaac
 3061 acgatcgaga acaactggcg cgcggcgcc atgccttct tcggcggcag cggccacaag
 3121 gctgaccaca actacatcat cgactgtgtc ggcggtccg gcatccgat gaatacggtg
 3181 tcccaggct accacttcca gaacaacacc ggcatcacct tctcggatac gacgatcac
 25 3241 aacagcggca ccagccagga tetgtacaac ggcgagcgcg gagcgattga tctggaagct
 3301 tccaacgacg cgatcaaaaa cgtcaccttc accaacaatc acatcatcaa tgcacgagc

3361 gacggcggtc agatcggcta tggcgggcgc ttcgagaaca tcgtgttcaa caacatcacg
 3421 atcgacggca ccggccgcga cgggatatcg acatcccgt tctcgggacc tcattttggc
 3481 gcagccatct atacgtacac gggcaacggc tcggcgacgt tcaacaacct ggtgaccggg
 3541 aacatcgct atgcaggcgg caactacatc cagagcgggt tcaacctgac gatcaaatag
 5 3601 gctgcaaaaa aaaggaagct cctcggagct tcctttttt (SEQ ID NO: 13)

II) *Paenibacillus curdlanolyticus* MP-1

1. General information of of mutanase from *Paenibacillus curdlanolyticus* MP-1

<http://www.ncbi.nlm.nih.gov/nuccore/HQ640944>

10 Genbank HQ640944; **Length:**1,261; **Mass (Da):**131,631

Reference: Pleszczyńska M, et al. Protein Expr Purif. 2012 Nov;86(1):68-74.

2. The protein sequence of mutanase from *Paenibacillus curdlanolyticus* MP-1

>gi|315201261|gb|ADT91063.1| alpha-1,3-glucanase [Paenibacillus curdlanolyticus]

MRNKYVTWTLALTMLFSSFFLA VGPNKVVHAAGGTNLALGKNVTASGQSQTYSNNVKDSNQSTYWEST
 15 NNAFPQWIQVDLGATTSIDQIVLKL PAGWGTRTQTLAVQGSTDGSSFTNIVGSAGYVFNP AVANNAV TINF
 SAASTRYVRLNVTANTAWPAAQLSEFEIYGAGGTTTPPTTPAGTYEAESAALSGGAKVNTDHTGYTGTGF
 VDGWYWTQGATTTFTANVSAAGNYDVT LKYANASGSAKTL SVYVNGTKIRQTTLASLANWDTWGTKVET
 LSLNAGNNTIAYKYEASDSGNVNIDSIAVAPSTSTPVDPEPPITPPTGSNIAIGKAISASSNTQAFVAANANDN
 DTNTYWEGGAASSTLTLDLGANQNVTSIVLKLNPSSAWSTRTQTIQVLGHNQSTTTFSNLVSSQSYTFNPAT
 20 GNSVTIPVTATVKRLQLSITANS GSGAGQIAEFQVYGT PAPNPDLTITGMSWTPASPIETDAVTLNATVKNS
 GNADAPATTNVFYLNNELVGSSPVGAL AAGASSTVSLNVGTKAATYAVSAKVDESNSIIEQNDANNSYT
 NASSLVVAPVASSDLVGATTWTPSTPVAGNAIGFMVNLKNQGTIASASGAHGITVVVKNAAGAALQSFSG
 TYSGAIAAGASVNVTLPGTWTAVNGSYT VTTTVAVDANELTNKQGNVSTSNLVVYAQRGASMPYSRYD
 TEDATRGGGATLQTAPT FNQAQIASEASGQSYIALPSNGSSAQWTVRQGGGAGVTMRFTMPDSTDGMGL
 25 NGSLDVYVNGVKVKT VSLTSYYSWQYFSGDMPGDAPSAGRPLFRFDEVHVKLDTP LQPGDTIKIQKGNG
 DSLEYGIDFLEIEPVPTAI AKPANSLSVTEYGAVANDGQDDLA AFKATVTA AAVAAGKSVYIPAGTFNLSSM
 WEIGSANNMINNITITGAGY WHTNIQFTNPNAAGGGISLRISGQLDFSNVYMNSNLRSRYGQNAIYKGFMD
 NFGTNSKIHDVWVEHFECGMWVG DY AHTPAIYATGLV VENSRI RNNLADGINYSQGT SNSIVRNSSIRNNG
 DDGLAVWTSNTNGAPAGVNN TFSYNTIENNWRAGGIAFFGGGGHKADHNLIVDTVGGSGIRMNTVFPGY
 30 HFQNNTGITFSDNTLINTGTSQDLYNGERGAIDLEASND AIKNVTFTNIDIINTQRDAIQFGYGGGFENIVFNN
 ININGTGLDGVTTSRFAGPHKGAAIYTYTGNGSATFN NLTTSNVAYPGLNFIQQGFNLVIQ (SEQ ID NO: 14)

3. Sequence of mRNA from *Paenibacillus curdlanolyticus* MP-1

1 atgcgcaaca agtatgtcac atggacgctc gccctgacga tgctattttc gagctttctc

61 ctgcagtag gtccaacaa ggtcgttcac gcagcaggcg gaacgaattt agcgctcggc

121 aaaaacgtta cggcaagcgg ccaatcgcaa acgtatagtc ccaacaatgt aaaagacagc
 181 aatcaatcga cgtactggga aagcacgaac aatgcattcc cgcaatggat tcaagtagac
 241 ttaggcgcaa cgacgagcat tgaccaaate gtactgaagc tgcccgtgg atgggggtacg
 301 cgtacgcaaa cgtagctgt tcaaggaagc acggacgggt cctcggtcac gaatatcgtg
 5 361 ggctccgcag gctatgtatt taatctgct gttgccaata acgccgttac gattaactc
 421 tctgctgcaa gcacgcgtta tgtcgtctg aacgtaacag cgaacacggc ttggccagca
 481 gcgcagctgt ccgaattcga gatttatggc gctggcggca cgacgacgcc tccaacaacg
 541 ccagcaggca catatgaagc tgaatccgca gcattgtccg gcggtgcgaa agtgaacacg
 601 gatcataccg gctacacggg tacgggcttt gttgacggct actggacaca aggcgcgaca
 10 661 acgacgttca cggctaactg gtccgcagct ggcaactatg acgttacatt gaaatatgcc
 721 aacgcaagcg gcagtgccaa gacgctaagc gtttactca acggcacgaa gattcgccag
 781 acgacgtgg caagcctggc aaactgggac acttggggca cgaagggtga gacgtgagc
 841 ttgaatgccg gcaataatac gattgcatac aagtatgagg ctacgcgactc gggcaacgtg
 901 aatatcgact ccattgccgt ggcgccatcg acttcgacac cggtagatcc agaaccgccg
 15 961 atcacgccgc caacgggcag caatatcgca atcggcaaag cgatcagcgc atcttcaat
 1021 acgcaagcat tcgtagctgc caacgcgaac gataacgata cgaacacgta ctgggaaggc
 1081 ggagctgcat cgagcacgct gacgctggat cttggcgca accaaaatgt aacctgac
 1141 gtgctgaagc tgaatcctc ttcggcatgg agcacgcgta cgcaaacgat ccaagtgtt
 1201 ggccacaacc aaagcacgac gacgttcagc aatctggtat cttcgcaatc gtatacgtc
 20 1261 aatctgcaa cgggcaactc cgtgacgatt ccggttacgg caacagttaa gcgcttcag
 1321 ctgagcatta cggcgaactc gggttccggc gctggtcaaa ttgcggaatt ccaagtgtat
 1381 ggaacgccgg caccaaacc agacctgacg atcacaggca tgtctggac gctgcttcg
 1441 ccaattgaaa cggatgcagt tacgtgaat gcaacggta aaaacagcgg aatgcagac
 1501 gctctgcaa cgacggtaaa cttctactg aacaatgagc tcgtaggetc ctcgccagtt
 25 1561 ggcgcaactg ctgcaggcgc ttctcgacg gtttcgtga atgttggtac gaaaacggct
 1621 gcaacttatg cagttagcgc gaaagtcgat gagagcaatt cgattatcga gcaaaatgat

1681 gcgaacaaca gttatacgaa cgcatcctcg ctgctgctcg ctctgtgcg aagctctgac
 1741 ttggttgccg cgacgacgtg gacgcctagc acgccggttg ccggcaatgc aattggcttc
 1801 atggtaaate taaaaacca aggaacgatt gcatctgcaa gcggcgcgca tggcattaca
 1861 gttgtcgtga aaaatgccgc aggcgctgcg ctccaatcgt tcagcggcac ctacagcgga
 5 1921 gcaatcgag ctggcgcatc cgtaacgta accctgccag gtacgtggac ggctgtgaat
 1981 ggagctaca ccgtaacgac aacgggtgct gtcgatgcta acgagctgac gaacaacaa
 2041 gggaacaacg taagcacttc gaacctcgtt gtttatgcac aacgtggcg aagcatgcct
 2101 tacagccgtt atgacacgga agacgtaca cgtggcgccg gtgcaacgct gcaaaccgca
 2161 ccaacctca accaagcgca aatcgcttcg gaagcatccg gacaaagcta tatcgcgctg
 10 2221 ccttgaacg gctcctccg acaatggacg gtccgtcaag gacaaggcgg agctggcggtt
 2281 acgatgcgt tcacgatgcc ggattcgact gacggatgg gttgaacgg ttcgtcgac
 2341 gtttatgtca acggcgtaa agtaaaaacg gtatcgctca cgtcctacta cagctggcag
 2401 tatttctcgg gcgatatgcc tggcgatgcg ccgtccgctg gccgtccgtt gttccgctt
 2461 gacgaagtac actggaagct tgacacgcct ctcaaccag gcgacacgat caaatccaa
 15 2521 aaaggcaacg gagatagcct ggaatacggc attgacttcc tcgaaatga gccggttcca
 2581 acagcaatcg ctaaacctgc caactcgctt tccgttacgg agtatggcg ttagcaaac
 2641 gatggccaag acgacctgc cgattcaaa gcaacggta cggctgcagt tgctgctggc
 2701 aaatccgtt acattctgc tggcacgttc aatctgagca gcatgtggga aatcggatcg
 2761 gtaacaaca tgatcaacaa cattacgatt acaggcgag gctactggca tacgaacatt
 20 2821 caattcacga atccgaatgc agcaggcggc ggcatttcg tccgatttc cggacagctt
 2881 gatttcagca atgtttacat gaactccaac ctgcgttcg gttatgtca aatgcgatt
 2941 taaaagget tcatggacaa ctccggcaca aactcaaaa tccatgacgt atgggttgag
 3001 cacttcgagt gcggcatgtg ggtaggcgat tacgcgata cgccagcgat ctatgcaacg
 3061 ggtcttctg ttgaaaacag ccgatttcg aacaacctg cagacggcat caactactg
 25 3121 caaggcacga gcaattcgat cgtacgcaac agcagtatcc gcaataacgg tgatgacggt
 3181 ctggcggtt ggacgagtaa cacgaatggc gcgccagcag gcgtgaacaa cacgttctcg

3241 tacaacacga tcgaaaacaa ctggcgtgca ggccgtatcg cattcttcgg cggcggcggc
3301 cacaaggctg accacaacct gatcggtgat acggttggcg gctccggcat ccgcatgaac
3361 acggtattcc caggctacca ctccaaaac aacacgggta ttacgttctc cgacaacacg
3421 ctgatcaaca caggcacaag ccaagatttg tacaacggcg agcgcggtgc gatcgatctc
5 3481 gaagcatcga acgatgcaat caagaacgtc acgttcacga acatcgacat catcaacacc
3541 cagcgcgatg cgatacaatt cggctacggc ggccggttcg agaacatcgt atttaacaac
3601 attaacatta acggtacggg gcttgacggc gttacaacct cacggtttgc tggaccgcat
3661 aaagtgctg caatctacac gtacacgggc aatggctctg caacgttcaa taacctgacg
3721 acgagcaacg tggcatatcc aggcttgaat tcattcagc aaggctttaa tctggtgatc
10 3781 cagtag (SEQ ID NO: 15)

III) *Paenibacillus* sp. strain RM1.

1. General information of of mutanase

Genbank E16590; **Length:**1,291; **Mass (Da):**135kD

Reference: Shimotsuura I, et al. Appl Environ Microbiol. 2008 May;74(9):2759-65.2. The protein sequence of mutanase from *Paenibacillus* sp. strain RM1

↓

```

1  MRCKEVANSI VTAMLMASLL TAVGPFPGAS AAGGPNLTFG KPITASGQSQ
51  TYSFQNVKDG NQNTYWESTN NAFFQWICVD LGASTGIDQI VLKLPASWEA
101 RTQTLAVQGS LNGSTFTDIV GSANYVFSRS VGNNTVTINF TATSTRYVRL
151 YVTANTGWPA AQLSEFEIYG SGDQTPAPDT YQAESAAALSG GAKVNTDHAG
201 YIGTGFDVGY WTQGAFTTFS VNAPTAGNYD VTLRYGNATG SNKTVSLYVN
251 GAKIRQTTLP SLPNWDSSWS KTETLNLNAG SNTIAYKYDP GDSGNVNLQ
301 ITVEASTTTP TPTPTPTPTPT TPTPTPTPTPT TPTPTPTPTPT TPTPTPTPTPT
351 TPTPTGGNIAI GHSISASSHT QTYVAENAND NDVNTYWEGG GNPSTLTLOL
401 GANYNITSIV LRLNPSSIWA ARTQTIOVLG HDQNTTTFST LVSARYSFED
451 PASGNTVTIP VTATVRLQL NITENSGAPA GQVAEFQVFG TPAFNPDLTI
501 TGMSSWSPSP VETDAITLNA TVKNNGNASA AATTVNFXLM NELAGSAPVA
551 ALAAGASATV PLNVGAKTAA TYAVGAKVDE SNAVIELNES NNSYTNFASL
601 VVAFVSSSDL VGTVSWTPTST PIANNVSEFN VNLKNQGTIA SAGGSHGVTV
651 VLKNASGSTV QTFSGSYTGS LAPGASVNIT LPGTWTAAG SYTVTATVAA
701 DANELPIKQA NNANTASLTV YSARGASMPY SRYDTEDATL GCGATLKSAF
751 TFDQALTASE ATGQLYAALP SNGSYLOWTV RQCGGAGVT MRFTMPDSAD
801 GMGLNGSLEV YVNGTEVKTV SLTSYYSWQY FSGDMFGDAP SAGRPLFRFD
851 EVHWKLDTPL KPGDTIRIQK NNGDSLEYGV DFIEIEPVPA AISRPANSVS
901 VTDYGAVPND GQDQLTAFKA AVNAAVASDK ILYIEGTFH LNMWEIGSV
951 SNMIDHITIT GAGIWTNIIQ FTNANPASGG ISLRITGKLD FSNVYLMNSL
1001 BSKYQNAVY KGFMDNFGTN SVIROVWVEH FECGFVWGDY GHTFAIRASG
1051 LLIENSRIRN NLADGVNFAQ GTSNSTVRNS SLRNNQDDAL AVWTSTNTGA
1101 PRCVNNITFY NTIENNWRAG GIAFFGGSCH KADHNYIVDC VCGSGIRMNT
1151 VFPGYHFQNN TGIVFSOTTI VNCGTSKDLY NGERGAIDLE ASNDAIRNVT
1201 FENIDIINSQ RDAIQPGYGG GFTNIVFNNI NINGTGLOGV TTSRFSQPHL
1251 GAAIFTYTCN GSATFNNLAT SNIAYPNLYY IQSGPNLIIN N

```

Deduced amino acid sequence of mutanase RM1. The signal peptide region is underlined, and the linker region is boxed. The arrow indicates the cleavage site for the N-terminal domain of the protein. The DNA sequence was registered as GenBank accession number E16590. (SEQ ID NO: 16)

3. Sequence of mRNA from *Paenibacillus* sp. strain RM1

```

1  cccgggtacc agacctatcg ggaaaaacgc gagcggccct tcg'gcctta tgcgctacgg
10  61  acggtgctgg cgggcggttt gttttcatc atcattcccc tgatgatcta caggcatcg
121  tatatcccg tttgctcgt gccgggtccc ggacacgggt tgaaagacgt cgtctccgcc
181  cagaagtta tgttcaatta tcatagccgg cttaacgcca cccaccatt ctcgtcgtg
241  tgggtggagt ggctctcat ccgcaagccg atctggtatt acggagccgc ggaattggcg
301  cccggaaaaa tggcgagcat cgtgggcatg ggcaatccgg cggtgtgtg gacgggaacg
15  361  attcggtaa tcgggccttc tcgctcgccc tggaagaagc gggaccggag catgaccgtc

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421 gtcttcgttg gaategcctc gtcttatctt ccgtgggttt tcgtatccag actcaccttt
 481 atttatcaact ttttcgcttg cgttcggttt ctcggtcttt gcatcggtta ttggattcga
 541 aaaatggaat agcgtaagcc gggatatcgg attgcgacgc tcctttacgc aggcgcggtt
 601 ctggtgctgt tcattttgtt ttaccgatt ttgtcgggga ccgaaataga cgtttcttac
 5 661 gcggaccgcg ttctgaagtg gttcggcggg tggattttc acgggtaagc gagcgttgga
 721 agcaaggaag ggaaggaaga cgagcgtctc ctcccgaaa tccatccaat atcttgaat
 781 tgcatacatt ttctgaaga ttgcttcta tctgtctccc tccctgttc ttataatggg
 841 ggatcccaa cgaaaggagg gtttgaagc gctgtcagcs tgttgccga aagtctcgc
 901 atttgctgac ctacacttg aggaggagga attaatgcg ctgcaaattt gtcgcatggt
 10 961 cgcttggtac agccatgctg atggccagtt tgctgacggc tgtaggaccg ttcggccccg
 1021 ctccgcccgc gggaggaccg aatctgacgc cgggcaaacc cattacggcg agcggccaat
 1081 cccaaaccta cagccctcag aacgtaaaag acggcaatca aaatacgtat tgggaaagca
 1141 cgaacaacgc gttcccgcaa tggattcaag tggatttggg cgcaagcacg ggcatcgacc
 1201 aaattgtgct gaagctgccc gcaagctggg aagcgcgcac gcaaacgctg gccgttcaag
 15 1261 gcagettgaa cggttcgacg ttcacggaca ttgtcggctc cgccaattat gtattcagtc
 1321 cgtctgtcgg gaacaacacg gttacgatca actttaccgc gaccagcacg cgctacgtgc
 1381 gcttgatgt aacggccaac acgggctggc cggcggcgca gctgtccgaa ttcgaaattt
 1441 acggctccgg cgaccagacg ccggcgctg atacgtatca agccgaatcc gcggctctgt
 1501 ccggcggcgc gaaagtcaac acggaccatg ccggatatat cggcacgggc tttgttgacg
 20 1561 gttactggac gcaaggcgcg acgacgacct ttccggtcaa cgcgccgacg gcgggcaact
 1621 acgatgtaac gctgaggtag ggcaacgcaa ccggcagcaa caaacggta agcctctacg
 1681 tcaatggagc gaagattcgc cagaccacgc tgcccagcct gcctaactgg gattcatgga
 1741 gcagcaagac ggagacgctt aacctgaatg caggcagcaa caccattgcg tacaatacgc
 1801 acccggggcg ttcgggcaac gtcaatcttg accaaatcac ggtcgaagcg tcgactcaa
 25 1861 cgctactec tactccatcc cctactccta cacctacgcc aacgccgacg cctacgccta
 1921 cgctacacc cacactact ccgaccccg cgcctacgcc tacacctaca cctacaccta

1981 cgccgacgcc tctccgggc ggcaacatcg ccacggcaa atcgatttcc gcacctccc
 2041 acacgcagac gtacgttgcg gagaacgcga acgataacga tgtcaacacg tactgggaag
 2101 gcggcggcaa tccgagcacg ctgacgctcg atctcggagc gaactacaat attacgtcca
 2161 tcgtgctgaa gctgaacccg tctcgcata gggctgcgcg tacgcaaacg attcaagtgc
 5 2221 tcggacacga tcagaacacg acgaccttca gcaatctggt ctcggcgaaa tcgtactcgt
 2281 tcgatccggc ctccggcaat actgtgacca ttccggttac ggcgacggtg aaacgtttgc
 2341 agttgaacat tacgtcgaac tccggcgccc cggccggaca agtcgccgag ttccaggtgt
 2401 tcggcacgcc tgcgccgaat ccggacctga cgattaccgg catgtcctgg tcgccttctt
 2461 ctccggttga gaccgacgcc attacgctaa acgcaacggt gaagaacaac gggaatgcc
 10 2521 gcgcccggc gaccaccgtc aattctacc tgaacaacga gctggcgggt tccgcgccg
 2581 tagccgcgtt ggccgagcgc gttcggcaa cggcgccgtt gaatgtcggc gcgaaaaccg
 2641 ccgcgacata cgcggtcggc gccaaagtag acgagagcaa cgcggtcatc gagctgaacg
 2701 agtcgaacaa cagctacacg aatccggctt cactcgttgt ggccccggtt tccagtcgg
 2761 atctggtggg cacggttctg tggacgccga gcactccgat tgccaacaat gccgtttctt
 15 2821 ttaacgtaaa tcttaaaat caaggaacga ttgcttcgc cggcgggtct cacggcgtga
 2881 cggtcgtgct taaaatgct tccggttga ccgttcaac gttcagcggc tcctataccg
 2941 gcagctggc tccgggagcg tccgtcaaca tcaccttcc ggggacctgg acggcggcag
 3001 ccggcagcta cacggtaacg gccaccgttg cggcagacgc caacgaactt ccgatcaagc
 3061 aagccaacaa cgcgaacacc gcaagcctga ccgtatattc cggccgcggc gcgagcatgc
 20 3121 cgtacagccg gtatgacacc gaggacgcca cctcggcgg cggcgccacg ctgaagtccg
 3181 cgccgacatt cgatcaggcg cttacggcat cggaagccac cggccaactc tatgcggcgc
 3241 tgccctgaa cggctcctat cttcaatgga ccgtcagaca gggtcagggc ggcgcaggcg
 3301 tgacgatgag atttacgatg cccgactcgg cggacggcat gggattaaac ggttcgctag
 3361 acgtttacgt caacggcacc aaagtcaaaa ccgtatcgt gacctctac tacagctggc
 25 3421 agtatttctc gggcgatatg cccggagacg ctcccagcgc gggccgtccg ctcttccgt
 3481 ttgacgaagt gcactggaag ctggatactc cgtcaaac ccgagacacg attcgcattc

3541 agaagaacaa cggcgacagc ctggaatacg gtgtcgactt tattgaaatc gaaccgggtc
 3601 cggtcgcat ctcccgccg gccaaactcg ttccgtaac ggattacggc gctgtgccga
 3661 acgacggaca ggacgatctc accgccttta aagccgccgt aaacggggcg gtcgcatccg
 3721 acaagatctt gtacattccg gaaggaacgt tccacctcgg caacatgtgg gagatcggtt
 5 3781 ccgtcagcaa catgatcat cacattacga ttacgggagc cggtatctgg tatacgaaca
 3841 tccagtttac caacgccaat ccggcgccg gcggcatctc gctccggatt acgggcaagc
 3901 ttgatttcag caacgtgtac ctcaactcca attgcggtc gcggtatggt caaaatgcgg
 3961 ttacaaagg ctttatggac aacttcggga ccaattccgt catccgcgac gtctgggtcg
 4021 agcacttca atgcggcttc tgggtcgggg actacgggca tacgccggcg atccgcgcga
 10 4081 gcgggctgct gattgaaaac agccgaatcc gcaacaacct ggccgatggc gtcaactcg
 4141 cccaaggac cagcaatcg accgtacga acagcagcct gcgcaacaac ggcgacgacg
 4201 cccttgccgt atggacgagt aatacgaacg gcgcgccga aggcgtaaac aatacttct
 4261 cgtacaacac catcgaaaac aactggcgcg cgggaggcat cgccttctc ggaggaagcg
 4321 gacacaaggc cgaccacaac tacatcgctg actgcgtcgg cggttccggc atccggatga
 15 4381 acaccgtgtt ccccgatac cacttcaga acaataccgg cattgtgttc tcggacacga
 4441 ccatcgtaa ctgcggcacg agcaaagacc tataaacgg cgaacggcg gccatcgatc
 4501 tggaaagctc gaacgacgcc atccggaacg tgacgtttac caacatcat attatcaact
 4561 ctacgcgca tgcgatccag ttcggttac gcggcggtt caccaacatc gtgttcaaca
 4621 acatcaacat taacggaacc ggtcttgacg gcgtaaccac ctgcgggtc tcgggaccgc
 20 4681 atctgggcgc ggcgatctc acctataccg gcaacggctc cgccacgtc aacaatctga
 4741 ggaccagcaa tatcgcttac ccaatctgt attacatcca gagcgggtc aatctgatca
 4801 tcaataatta gatatctggg cccgtctcgc ggggaggaac tcttcggagc tcgaattcgt
 4861 aatcatggc atagctgtt cctgtgtgaa attgttatcc gtcacaatt ccacacaaca
 4921 tacgagccgg aagcataaag tgtaaagcct ggggtgccta atgagtgage taactcat
 25 4981 taattcggt gcgctcactg cccgcttcc agtcgggaaa ctgtcgtgcc agctgcatta
 5041 atgaatcggc caacgcgcgg ggagaggcsg ttkegtatt gggcgccctt (SEQ ID NO: 17)

IV) *Trichoderma harzianum* (CCM F-470)

1. General information of of mutanase from *Trichoderma harzianum*

Also see <http://www.uniprot.org/uniprot/Q8WZM7> Length:635

Mass (Da):67,726

5 Last modified:March 1, 2002 - v1

Checksum:iBB0D864E2F432C58

2. The protein sequence of mutanase from *Trichoderma harzianum*

<http://www.uniprot.org/uniprot/Q8WZM7.fasta>)

>tr|Q8WZM7|Q8WZM7_TRIHA Alpha-1,3-glucanase OS=Trichoderma harzianum GN=p3 PE=2 SV=1

10 MLGVFRRLRLGALAAAALSSLGSAAPANVAIRSLEERASSADRLVFCHFMIGIVGDRGSS
ADYDDDMQRAKAAGIDAFALNIGVDGYTDQQLGYAYDSADRNGMKVFISDFDNWWSPGNA
VGVGQKIAQYANRPAQLYVDNRPFASSFAGDGLDVNALRSAAGSNVYFVPNFHHPGQSSPS
NIDGALNWMawDNDGNNKAPKPGQTVTVADGDNAYKNWLGGKPYLAPVSTWVFNHFGPEV
SYSKNWVFPSGPLIYNRWQQVLQQGFPRVEIVTWNDYGESHYVGPLKSKQFHDGNSKWVN
15 DMPHDGFLDLSKPFIAAYKNRDTDISKYVQNEQLVYWYRRNLKALDCDATDTTSNRPANN
GSGNYFEGRPDGWQTMDDTVYVAALLKTAGSVTVTSGGTTQTFQANAGANLFIQIPASIGQ
QKFALTRNGQTVFSGTSLMDITNVCSCGIYNFNPNYVGTIPAGFDDPLQADGLFSLTIGLH
VTTCQAKPSLGTNPPVTSGPVSSLPASSTTRASSPPVSSSTRVSSPPVSSPPVSRTSSAP
PPPGNSTPPSGQVCVAGTVADGESGNYIGLCQFSCNYGYCPPGPCKCTAFGAPISPPASN
20 GRNGCPLPGEGDGYLGCLCSFSCNHNYCPPTACQYC (SEQ ID NO: 18)

3. Sequence of mRNA (*Trichoderma harzianum*

<http://www.ebi.ac.uk/ena/data/view/AJ243799&display=fasta>)

25 >ENA|AJ243799|AJ243799.1 Trichoderma harzianum mRNA for alpha-1,3-glucanase (p3 gene)
ATGTTGGGCGTTTTCCGCCGCCTCAGGCTCGGCGCCCTTGCCGCCGCAGCTCTGTCTTCT
CTCGGCAGTGCCGCTCCCGCCAATGTTGCTATTCGGTCTCTCGAGGAACGTGCTTCTTCT
GCTGACCGTCTCGTATTCTGTCATTTTCATGATTGGGATCGTGGGTGACCGTGGCAGCTCG

GCAGATTATGATGACGATATGCAACGTGCCAAAGCCGCTGGCATTGACGCCTTCGCCCTG
AACATCGGCGTTGACGGCTATACCGACCAGCAGCTCGGCTATGCCTATGACTCTGCCGAT
CGTAATGGCATGAAAGTCTTCATTTCAATTTGATTTCAACTGGTGGAGCCCCGGCAATGCA
GTTGGTGTGGCCAGAAGATTGCGCAGTATGCCAACCGCCCTGCCCAGCTGTATGTGCAC
5 AACCGGCCATTTCGCTCTTCCTTCGCCGGTGACGGTCTGGATGTAAATGCGTTGCGCTCT
GCTGCAGGCTCCAACGTTTACTTTGTGCCCAACTTCCACCCTGGTCAATCTTCCCCCTCC
AACATTGATGGCGCCCTTAAGTGGATGGCCTGGGATAATGATGGAAACAACAAGGCACCC
AAGCCGGGCCAGACTGTCACAGTGGCAGACGGTGACAACGCTTATAAGAATTGGTTGGGT
GGCAAGCCTTACCTGGCGCCTGTCTCAACTTGGGTTTTCAACCATTTCGGGCCCCGAAGTT
10 TCATATTCCAAGAACTGGGTTTTCCCAAGTGGGCCTCTGATCTATAACCGGTGGCAACAA
GTCTTGACAGCAAGGGTTCCCAAGGGTTGAGATCGTTACCTGGAATGACTACGGGGAATCT
CACTACGTCGGTCCCCTGAAGTCTAAGCAATTTTCATGATGGGAACTCCAAATGGGTCAAT
GATATGCCCCACGATGGATTCTCTGGATCTTTTGAAGCCGTTTCATAGCCGCATATAAAAAC
AGGGATACCGACATCTCCAAGTATGTTCAAAATGAGCAGCTTGTTTACTGGTACCGCCGC
15 AACTTAAAGGCACTGGACTGTGACGCCACCGACACAACCTCTAACCGCCCCGGCTAACAAT
GGAAGCGGCAATTACTTTGAGGGACGCCCCGATGGTTGGCAAACCTATGGATGATACGGTT
TACGTGGCGGCACTTCTCAAGACTGCCGGTAGCGTCACGGTCACGTCTGGTGGCACCCT
CAAACGTTCCAGGCCAACGCCGGAGCCAATCTCTTCCAAATCCCGGCCAGCATCGGCCAG
CAAAAGTTTGCTCTGACTCGTAACGGTCAGACCGTCTTTAGCGGAACCTCATTGATGGAT
20 ATCACCAACGTTTGCTCTTGCGGTATCTACAACCTTCAACCCATATGTTGGCACCATTCT
GCCGGCTTTGACGACCCTCTTCAGGCTGACGGTCTTTTCTCTTTGACCATCGGATTGCAC
GTCACAACTTGTGAGGCCAAGCCATCTCTTGGAACCTAACCTCCTGTCACCTCCGGCCCT
GTGTCCTCGCTTCCAGCTTCTCTCCACCACCCGCGCATCCTCGCCGCCTCCTGTTTCTTCA
ACTCGTGTCTCTTCTCCCCCTGTCTCTTCCCCTCCAGTTTCTCGCACCTCTTCTGCCCCCT
25 CCCCCTCCGGGCAACAGCACGCCGCCATCGGGTCAGGTTTGC GTTGCCGGCACCGTTGCC
GACGGCGAGTCTGGCAACTACATCGGCCTGTGCCAATTCAGCTGCAACTACGGTTACTGC

CCACCAGGACCGTGTAAGTGCACCGCCTTTGGTGCTCCCATCTCGCCACCGGCATCCAAC
 GGCCGCAACGGCTGCCCTCTGCCGGGAGAAGGCGATGGTTATCTGGGCCTGTGCAGTTTC
 AGTTGTAACCATAATTACTGCCCCGCCAACGGCATGTCAATACTGCTAGGAGGGATCAATC
 TCAGTATGAGTATATGGAGGCTGCTGAAGGACCAATTAGCTGTTCTTATCGGCAGACGAA
 5 ACCCATAGAGTAAGAAGTTAAATAAAATGCAATTAATGTGTTTTCAAAAAAAAAAAAAAAAAA
 A (SEQ ID NO: 19)

(There is a polyA tail since *Trichoderma harzianum* is fungi)

V) *Trichoderma harzianum*

1. General information of of mutanase from *Trichoderma harzianum*

10 Also see <http://www.uniprot.org/uniprot/Q8WZM7>;

2. The protein sequence of mutanase from *Trichoderma harzianum*

See: <http://www.uniprot.org/uniprot/Q8WZM7.fasta>)

>tr|Q8WZM7|Q8WZM7_TRIHA Alpha-1,3-glucanase OS=Trichoderma harzianum GN=p3 PE=2 SV=1
 MLGVFRRRLRLGALAAAALSSLGSAAPANVAIRSLEERASSADRLVFCHFMIGIVGDRGSS
 15 ADYDDDMQRAKAAGIDAFALNIGVDGYTDQQLGYAYDSADRNGMKVFISFDNWWSPGNA
 VGVGQKIAQYANRPAQLYVDNRPFASSFAGDGLDVNALRSAAGSNVYFVPNFHPGQSSPS
 NIDGALNMAWDNDGNNKAPKPGQTVTVADGDNAYKNWLGGKPYLAPVSTWVFNHFGPEV
 SYSKNWVFPSGPLIYNRWQQVLQQGFPRVEIVTWNDYGESHYVGPLKSKQFHDGNSKWVN
 DMPHDGFLDLSKPFIAAYKNRDTDISKYVQNEQLVYWYRRNLKALDCDATDTTSNRPAN
 20 GSGNYFEGRPDQWQTMDDTVYVAALLKTAGSVTVTSGGTTQTFQANAGANLQIPASIGQ
 QKFALTRNGQTVFSGTSLMDITNVCSGIIYFNPNYVGTIPAGFDDPLQADGLFSLTIGLH
 VTTCQAKPSLGTNPPVTS GPVSSLPASSTTRASSPPVSSSTRVSSPPVSSPPVSRTSSAP
 PPPGNSTPPSGQVCVAGTVADGESGNYIGLCQFSCNYGYCPPGPCKCTAFGAPISPPASN
 GRNGCPLPGEGDGYLGLCSFSCNHNYCPPTACQYC (SEQ ID NO: 20)

25

**3. Sequence of mRNA (*Trichoderma harzianum* Further information can be found at
<http://www.ebi.ac.uk/ena/data/view/AJ243799&display=fasta>)**

>ENA|AJ243799|AJ243799.1 *Trichoderma harzianum* mRNA for alpha-1,3-glucanase (p3 gene)

ATGTTGGGCGTTTTCCGCCGCCTCAGGCTCGGCGCCCTTGCCGCCGCAGCTCTGTCTTCT
CTCGGCAGTGCCGCTCCCGCCAATGTTGCTATTCGGTCTCTCGAGGAACGTGCTTCTTCT
5 GCTGACCGTCTCGTATTCTGTCAATTCATGATTGGGATCGTGGGTGACCGTGGCAGCTCG
GCAGATTATGATGACGATATGCAACGTGCCAAAGCCGCTGGCATTGACGCCTTCGCCCTG
AACATCGGCGTTGACGGCTATACCGACCAGCAGCTCGGCTATGCCTATGACTCTGCCGAT
CGTAATGGCATGAAAGTCTTCATTTCAATTTGATTTCAACTGGTGGAGCCCCGGCAATGCA
GTTGGTGTGGCCAGAAGATTGCGCAGTATGCCAACCGCCCTGCCAGCTGTATGTGCAC
10 AACCGGCCATTCGCCTCTTCCTTCGCCGGTGACGGTCTGGATGTAAATGCGTTGCGCTCT
GCTGCAGGCTCCAACGTTTACTTTGTGCCCAACTTCCACCCTGGTCAATCTTCCCCCTCC
AACATTGATGGCGCCCTTAAGTGGATGGCCTGGGATAATGATGGAAACAACAAGGCACCC
AAGCCGGGCCAGACTGTACAGTGGCAGACGGTGACAACGCTTATAAGAATTGGTTGGGT
GGCAAGCCTTACCTGGCGCCTGTCTCAACTTGGGTTTTCAACCATTTCCGGGCCCCGAAGTT
15 TCATATTCCAAGAACTGGGTTTTCCCAAGTGGGCCTCTGATCTATAACCGGTGGCAACAA
GTCTTGCAGCAAGGGTTCCCAAGGGTTGAGATCGTTACCTGGAATGACTACGGGGAATCT
CACTACGTCGGTCCCCTGAAGTCTAAGCAATTCATGATGGGAACTCCAAATGGGTCAAT
GATATGCCCCACGATGGATTCTTGGATCTTTCGAAGCCGTTTCATAGCCGCATATAAAAAC
AGGGATACCGACATCTCCAAGTATGTTCAAAATGAGCAGCTTGTTTACTGGTACCGCCGC
20 AACTTAAAGGCACTGGACTGTGACGCCACCGACACAACCTCTAACCGCCCCGGCTAACAAT
GGAAGCGGCAATTACTTTGAGGGACGCCCCGATGGTTGGCAAACCTATGGATGATACGGTT
TACGTGGCGGCACTTCTCAAGACTGCCGGTAGCGTCACGGTCACGTCTGGTGGCACCCT
CAAACGTTCCAGGCCAACGCCGGAGCCAATCTCTTCCAAATCCCGGCCAGCATCGGCCAG
CAAAAGTTTGCTCTGACTCGTAACGGTCAGACCGTCTTTAGCGGAACCTCATTGATGGAT
25 ATCACCAACGTTTGCTCTTGCGGTATCTACAACCTTCAACCCATATGTTGGCACCATTCT
GCCGGCTTTGACGACCCTCTTCAGGCTGACGGTCTTTTCTCTTTGACCATCGGATTGCAC
GTCACAACCTTGTCAGGCCAAGCCATCTCTTGGAACCTAACCTCCTGTCACCTCCGGCCCT

GTGTCCTCGCTTCCAGCTTCTCCACCACCCGCGCATCCTCGCCGCCTCCTGTTTCTTCA
 ACTCGTGTCTCTTCTCCCCCTGTCTCTTCCCCTCCAGTTTCTCGCACCTCTTCTGCCCCT
 CCCCCTCCGGGCAACAGCACGCCGCCATCGGGTCAGGTTTGCGTTGCCGGCACCGTTGCC
 GACGGCGAGTCTGGCAACTACATCGGCCTGTGCCAATTCAGCTGCAACTACGGTTACTGC
 5 CCACCAGGACCGTGTAAGTGCACCGCCTTTGGTGCTCCCATCTCGCCACCGGCATCCAAC
 GGCCGCAACGGCTGCCCTCTGCCGGGAGAAGGCGATGGTTATCTGGGCCTGTGCAGTTTC
 AGTTGTAACCATAATTACTGCCCGCCAACGGCATGTCAATACTGCTAGGAGGGATCAATC
 TCAGTATGAGTATATGGAGGCTGCTGAAGGACCAATTAGCTGTTCTTATCGGCAGACGAA
 ACCCATAGAGTAAGAAGTTAAATAAAAATGCAATTAATGTGTTTTCAAAAAAAAAAAAAAAAAA

10 A (SEQ ID NO: 21)

(There is a polyA tail since *Trichoderma harzianum* is fungi)

Dextranase (Dex) gene from *Penicillium minioluteum*

GenBank: L41562.1

(<http://www.ncbi.nlm.nih.gov/nuccore/L41562.1>)

15 The mature protein has 574 amino acids with MW at 67KD. The optimum reaction condition is pH 5.5 and 40°C. The pH range is 3-6.

Amino acid sequence

MATMLKLLALTLAISEAIGAVMHPPGNSHPGTHMGTTNNTHCG

20 ADFCTWWHDSGEINTQTPVQPGNVRQSHKYSVQVSLAGTNNFHDSFVYESIPRNGNGR
 IYAPTDPPNSNTLDSSVDDGISIEPSIGLNMAWSQFEYSHDVDVKILATDGSSLGSPS
 DVVIRPVSIY AISQSDDGGIVIRVPADANGRKFSVEFKTDLYTFLSDGNEYVTSGGS
 VVGVEPTNALVIFASPFLPSGMIPHMTDPDNTQTMTPGPINNGDWGAKSILYFPPGVYW
 MNQDQSGNSGKLGSNHRLNSNTYWVYLAPGAYVKGAIEYFTKQNFYATGHGILSGEN
 25 YVYQANAGDNYIAVKSDSTSLRMWWHNNLGGGQTWYCVGPTINAPPFNTMDFNGNSGI
 SSQISDYKQVGAFFFQTDGPEIYPNSVVHDFVWHVNDDAIKIYYSYGASVSRATIWKCH
 NDPIIQMGWTSRDISGVTIDTLNVIHTRYIKSETVVP SAIIGASPFYASGMSPDSRKS
 ISMTVSNVVCEGLCPSLFRITPLQNYKNFVKNVAFPDGLQNSIGTGESIIPAASGL
 TMGLNISNWTVGGQKVTMENFQANSLGQFNIDGSYWGEWQIS (SEQ ID NO: 22)

30

DNA sequence

1 ggcataagtaa tcccacagc cgagtatgat ggagcttctt cggataatga tagcggcacc

61 agaccttgc ttagctggag agctaaaaca ttaaacgcca cagaccaac actctcatta

121 gttgcgatag atgatgctcg gagctgttga aactcagaaa ttcttctat gcggggtctc
 181 caagatcgat cctgggggat gtgaatacta cgggtggacct aattgacgcc ttgacagggtg
 241 atgttaagcg aaccaaggaa gaataatctg gggctagatg aagatgttga gctgtaaggt
 301 acggtacgtt cctattggct ttatcggagc ttctccgggt tactcagctt ttccgggagc
 5 361 atgatcattt ttgtattgtc caatagtaag cagaaactga gagccaccac aaactcaaaa
 421 cctcggtagc gaagtttccc ggaaccagtc aggatttca gaaactgtgc tcgtgttgcg
 481 gggaaatccgc attctacgtc gtctggagca aggaaatgtt cgtgctggat tgaggaggat
 541 aggtaggttg gagaatctct tcagctaacc aatctataag catgtcccg taacctttag
 601 agtttccat tcaacgtaat ttccaagata gccagagcgt cttgaatta ctatgtagaa
 10 661 atcctaaaa ttccctgtga aaatgcaagt caacgagatg cgtgccctca atgtctctcg
 721 gcgtacccc gaaatgatg cataaggcca agaattgcac ccgtaactt ttcttcaga
 781 atatcctaag atttccatca aacacagtcg aataggtaaa tgctcgcgag agactttctg
 841 ccttactct acgtctact catagaagtt caacggctca attccggggt aatctagagt
 901 ttggacctca agggagatgt tgcaacaaat tgtactagaa cgatgcgctt gctttccaat
 15 961 acagtagttg acttcatata gcttcaaca aaagggatgg ggatgaaggc tctatagcga
 1021 gaagtctata agaaatgtc ctcatacctg tatctctcag tcgttcgaga acaatcccgg
 1081 aaactatctt atcttgcgag aaagaagaca atatcctaaa cttatggcca caatgctaaa
 1141 gctacttgcg ttgaccttg caattagcga gtccgccatt ggagcagtc tgcacccacc
 1201 tggcaattct catcccggta ccatatggg cactacgaat aataccatt gcggcgccga
 20 1261 ttctgtacc tgggtgcatg attcagggga gatcaatacg cagacacctg tccaaccagg
 1321 gaacgtgcgc caatctcaca agtattccgt gcaagtgcgc ctactgtgta caaacaattt
 1381 tcatgactcc ttgtatatg aatcgatccc ccggaacgga aatggtcga tctatgtcc
 1441 caccgatcca ccaacagca acacactaga ttcaagtgtg gatgatggaa tctcgattga
 1501 gcctagtatc ggccttaata tggcatggc ccaattcgag tacagccacg atgtagatgt
 25 1561 aaagatctg gccactgatg gctcatcggt gggctcgcca agtgatgtt ttattgccc
 1621 cgtctcaatc tctatgcga ttctcagtc tgacgatggt gggattgtca tccgggtccc
 1681 agccgatcgc aacggccgca aatttctagt tgagtcaaa actgacctgt acacattcct
 1741 ctctgatggc aacgagtacg tcacatcggg aggcagcgtc gtcggcggtg agcctaccaa
 1801 cgcacttgt atcttcgcaa gtccgttct tcttctggc atgattcctc atatgacacc
 30 1861 cgacaacacg cagaccatga cgccaggtcc tatcaataac ggcgactggg gcgccaagtc
 1921 aattctttac ttcccaccag gtgtatactg gatgaacaa gatcaatcgg gcaactcggg
 1981 gaagtttaga tctaatcata tacgtctaaa ctggaacact tactgggtct accttgcccc
 2041 cgggtcgtac gtgaagggtg ctatagagta tttaaccaag cagaacttct atgcaactgg
 2101 tcatggtatc ctatcgggtg aaaactatgt ttaccaagcc aatgccggcg acaactacat
 35 2161 tgcagtcaag agcgattcaa ccagcctccg gatgtggtgg cacaataacc ttgggggtgg
 2221 tcaaacatgg tactgcgttg gcccgacgat caatgcgcca ccattcaata ctatggattt
 2281 caatggaaat tctggcatct caagtcaaat tagcgactat aagcagggtg gagccttctt
 2341 ctccagacg gatggaccag aaatatatcc caatagtgc gtgcacgacg tcttctggca
 2401 cgtcaatgat gatgcaatca aaatctacta ttccggagca tctgtatgc gggcaacgat
 40 2461 ctggaatgt cacaatgacc caatcatcca gatgggatgg acgtctcggg atatcagtgg
 2521 agtgacaatc gacacattaa atgtattca cccccgtac atcaaatcgg agacggtggt

2581 gccttcggct atcattgggg cctctccatt ctatgcaagt gggatgagtc ctgattcaag
 2641 aaagtccata tccatgacgg ttcaaactgt tgttgcgag ggtctttgcc cgtccctatt
 2701 ccgcatcaca ccccttcaga actacaaaaa ttttgtgtc aaaaatgtgg ctttcccaga
 2761 cgggctacag acgaatagta ttggcacagg agaaagcatt attccagccg catctggtct
 5 2821 aacgatggga ctgaatatct ccaactggac tgttgggtga caaaaagtga ctatggagaa
 2881 ctttcaagcc aatagcctgg ggcagttcaa tattgacggc agctattggg gggagtggca
 2941 gattagctga attccagctc tcggagcgcg tgagtgttc taccgctcc ttacccttg
 3001 tcgagagata aaggcataag ttagctcatg tgaaggcgt ttcagttcat tctctctttt
 3061 tggagcttat ttctgttcg accaattgtg acaccaactt gccttcaaa agacgtggac
 10 3121 gatattgtga cggtaatacag tcaaatgaac gtcaacattc atttaataag gacatttcca
 3181 ggtttcctta ctctgtcat tatgcctaac tcgggttgat gtctgtcag gatggaaaat
 3241 ctctgtgtgt acttcagtg aaatgggcag ggctaagccc taaacctaa cgcatacaat
 3301 ttgtaggcac ctacctatgt aagttcacac ccagtcgact tataagtcta gatatttatg
 3361 ctatgcaggc tctggaatga ttacattcc atgctataca tagttatttg caagaatttg
 15 3421 cagacgagat aaaaatcaat ggacgaataa tcacgcatta ctccacaggc tcatgccacg
 3481 gagcaagggt tccccgaat ctaggccaga ccgggatgat attcaaccga ttctttttgc
 3541 agtaactatc tccgtacgag ctgcacgagc taaacggatt atataaaggt gctaactgag
 3601 cattggatcc gtcagttata tgaatgca (SEQ ID NO: 23)

20 **2. Dextranase (Dex) gene from *Penicillium aculeatum* (*Talaromyces aculeatus* strain z01)**
 GenBank: KF999646.1 on the world wide web at ncbi.nlm.nih.gov/nuccore/KF999646.1

The optimum pH is around 5. The pH range is 3-6.

Amino acid sequence

25 MATMLKLLTLALAISESAIGAVLHPPGSSHPSTRDITNNTNTHCG
 ADFCTWWHDSGEINTQTPVQPGNVRQSHKYSVQVSLAGANNFQDSFVYESIPRNGNGR
 IYAPTDPPNSNTLDSSVDDGISIEHSIGLNMWWSQFEYSQDVIKILAADGSSLGSPS
 DVVIRPVSISYAISQSDDGIVIRVPADANGRKFSVEFKNDPYTFLSDGNEYVTSGGS
 VVGVEPTNALVIFASPLPSGMIPHMTDPNTQTMTPGPINNGDWGSKSILYFPPGVYW
 30 MNQDQSGNSGKLGSNHIRLNSNTYWVYFAPGAYVKGAIEYFTKQNFYATGHGVLSEN
 YVYQANAGENYVAVKSDSTSLRMWWHNNLGGGQTWYCVGPTINAPPFNTMDFNGNSGI
 SSQISDYKQVGAFFFQTDGPEIYPNSVVHDFVFWHVNDDAIKIYYSGASVSRATIWKCH
 NDPIIQMGWTSRDISGVTIDTLNVIHTRYIKSETVVPSAIGASPFYASGMSPDSSKS
 ISMTVSNVVCEGLCPSLFRITPLQNYKNFVKNVAFPDGLQTNISGTGESIIPAASGL
 35 TMGLDISNWSVGGQKVTMKNFQANSLGQFDIDGSYWGEWQIN (SEQ ID NO: 24)

DNA sequence

1 atggccacaa tgctaaagct acttacgttg gcccttgcaa ttacgagtc tgccattgga
 61 gcagctctgc acccacctgg cagttctcat cccagtaccc gtacggacac tacgaataat
 121 acccattgcg gtgccgactt ctgtacctgg tggcatgatt caggcgagat caacacacag
 40 181 acacctgtcc aaccggggaa cgtgcgcaa ttcacaagt attccgtaca agtgagccta
 241 gctggtgcga acaacttca ggactccttt gtatatgaat cgtacccctg gaacggaaat
 301 ggtcgcacat atgctccac cgtaccaccc aacagcaaca cactagattc aagtgttgat

361 gatggaatct cgattgaaca tagtattggc ctcaatatgg catgggccca attcgagtac
 421 agccaggatg tcgatataaa gatcctggcc gctgatggct catcgttggg ctaccaagt
 481 gatgtgttta ttcgccccgt ctcaatctcc tatgcaattt ctcaatccga cgatggcgga
 541 attgtcattc ggggtccagc cgatgcgaac ggccgcaaat tttagtcga gttcaaaaat
 5 601 gacccgtaca cgttctctc tgacggcaac gactacgtca catcgggagg cagcgtgtc
 661 ggcgttgagc ctaccaacgc acttgtgac ttcgaagcc cgttcttcc gtcaggcatg
 721 attcctcata tgacaccgga caacacgcag accatgacac caggacctat caataacggc
 781 gactggggct ccaagtcaat tctttattc ccaccggcg tatactggat gaaccaagat
 841 caatcaggca actcggggaa attaggatc aatcatatac gcctgaactc gaacacctac
 10 901 tgggtctact ttgccccagg tgcgtacgtg aagggtgcta tagagtattt caccaagcag
 961 aactctatg caactggta tgggtccta tcgggtgaaa actatgttta ccaagccaat
 1021 gctggcgaaa actacgttc ggtcaagagc gattcgacta gcctccggat gtggtggcac
 1081 aataacctgg gaggtggaca aacatgttac tgcgttggc ctacgatcaa tgcgccgcca
 1141 ttaacacaa tggatttcaa tggaaattc ggtatctcaa gtcaaatag cgactataag
 15 1201 caggtgggag ctttctctt tcagacggat ggaccagaaa ttatcccaa tagtgtctg
 1261 cagcagctc tctggcatg caatgatg gcaatcaaaa tctactatc cggagcatc
 1321 gtctcgagg caacgatc gaaatgtac aacgatcaa tcatccagat gggatggacg
 1381 tctcgggata tcagtgagt gacaatgac acattgaatg tcatccacac ccgtacatc
 1441 aagtcggaga cgggtgtgcc ttggctatc attggggctt ctccattcta tgcaagtggg
 20 1501 atgagtcctg attcaagcaa gtctatatcc atgacggtt caaacgtgt ctgcgaggga
 1561 ctttggccgt ctctgttccg aatcacacct ttacagaact acaagaattt tttgtcaaa
 1621 aatgtggctt tccagatgg gctacagacg aatagtattg gcacgggaga aagcattatt
 1681 ccagccgat ctggtctaac gatgggactg gatattcca actggtctg tgggtgacg
 1741 aagtgacta tgcagaactt tcaagccaat agtctggggc aattcgacat tgacggcagc
 25 1801 tattgggggg agtggcagat taactagctg aataatattg cagcttccag ggcgcatgag
 1861 tgctgtacc cgtccttta ccctgtc (SEQ ID NO: 25)

3. *Penicillium funiculosum* dexA gene for dextranase

GenBank: AJ272066.1 (<http://www.ncbi.nlm.nih.gov/nuccore/7801166>)

30 The optimum pH is around 5.5. The optimum temperature is 60°C. The pH range is 5-7.5
 (<http://www.sciencedirect.com/science/article/pii/S0032959298001277>)

Amino acid sequence

MATMLKLLALTLAISESAIGAVMHPPGVSHPGTHTGTTNNTHCG
 ADFCTWWHDSGEINTQTPVQPGNVRQSHKYSVQVSLAGTNNFHDSFVYESIPRNGNGR
 35 IYAPTDPNSNTLDSSVDDGISIEPSIGLNMAWSQFEYSQDVDIKILATDGSSLGSPS
 DVVIRPVSISYAIQSNDGGIVIRVPADANGRKFSVEFKNDLYTFLSDGNEYVTSGGS
 VVGVEPTNALVIFASPFPLPSGMIPHKPHNTQTMTPGPINNGDWGAKSILYFPPGVYW
 MNQDQSGNSGKLGSNHRLNSNTYWVYLAPGAYVKGAIEYFTKQNFYATGHGVLSEN
 YVYQANAGDNYVAVKSDSTSLRMWWHNNLGGGQTWYCVGPTINAPPFNTMDFNGNSGI
 40 SQISDYKQVGAFFFQTDGPEIYPNSVVHDFVFWHVNDDAIKIYSGASVSRATIWKCH
 NDPIIQMGWTSRDISGVTIDTLNVIHTRYIKSETVVPASIIIGASPFYASGMSPDSSKS
 ISMTVSNVVCEGLCPSLFRITPLQNYKNFVVKNVAFPDGLQTNISGTGESIIPAASGL
 TMGLNISSWTVGGQKVTMENFQANS LGQFNIDGSYWGEWQISRIS SQSA
 (SEQ ID NO: 26)

DNA sequence

1 atggccacaa tgctaaagct acttgcgttg acccttgcaa ttacgagtc cgccattgga
 61 gcagtcatgc acccacctgg cgtttctcat cccggtaccc atacgggcac tacgaataat
 121 acccattgcg gcgccgactt ctgtacctgg tggcatgatt caggggagat caacacgcag
 5 181 acacctgtcc aaccagggaa cgtgcgcaa tctcacaagt attccgtgca agtgagtcta
 241 gctggtacaa acaactttca tgactccttt gtatatgaat cgatcccccg gaacggaaat
 301 ggtcgcatct atgctccac cgatccatcc aacagcaaca cattagattc aagcgtggat
 361 gatggaatct cgattgagcc tagtatcggc ctcaatatgg catggtccca attcgagtac
 421 agccaggatg tcgatataaa gatcctggca actgatggct catcgttggg ctaccaagt
 10 481 gatgttgta ttcgccccgt ctcaatctcc tatgcgattt ctacgtcaa cgatggcggg
 541 attgtatcc gggtcccage cgatgcgaac ggccgcaaatt ttcagtcca attcaaaaat
 601 gacctgtaca ctttctctc tgatggcaac gagtacgtca catcgggagg tagcgtcgtc
 661 ggcgttgagc ctaccaacgc acttgtgac ttcgaagtc cgtttctcc ttctggcatg
 721 attctcata tgaacccca caacacgcag accatgacgc caggctctat caataacggc
 15 781 gactggggcg ccaagtcaat tctttacttc ccaccagggt tatactggat gaaccaagat
 841 caatcgggca actcgggtta attaggatct aatcatatac gtctaaactc gaacattac
 901 tgggtctacc ttgcccccg tgcgtacgtg aagggtgcta tagagtattt caccaagcaa
 961 aacttctatg caactgtgca tgggtccta tcaggtgaaa actatgttta ccaagccaat
 1021 gctggcgaca actatgtgc agtcaagagc gattcgacca gcctccggat gtggtggcac
 20 1081 aataaccttg ggggtggtca aacatggtac tgcgttggcc cgacgatcaa tgcgccacca
 1141 ttcaacacta tggatttcaa tggaaattct ggcatctcaa gtcaaattag cgactataag
 1201 caggtgggag ccttcttctt ccagacggat ggaccagaaa tctatcccaa tagtgcgtg
 1261 cagcagctct tctggcacgt caatgatgat gcaatcaaaa tctactattc gggagcatct
 1321 gtatcgcggg caacgatctg gaaatgtcac aatgacccaa tcatccagat gggatggaca
 25 1381 tctcgggata tcagtgaggat gacaatcgac acattaaatg ttattcacac ccgctacac
 1441 aaatcggaga cgggtggtgcc ttccgctatc attggggcct ctccattcta tgcaagtggg
 1501 atgagtcceg attcaagcaa gtccatatac atgacggttt caaacgttgt ttgcgaggg
 1561 ctttggccgt cctgttccg catcacaccc ctacagaact acaaaaattt tgtgtcaaa
 1621 aatgtggctt tccagatgg gctacagaca aatagtattg gcacaggaga aagcattatt
 30 1681 ccagccgcat ctggtctaac gatgggacta aatatctcca gctggactgt tgggtggaca
 1741 aaagtgacaa tggagaactt tcaagccaat agcctggggc agttcaatat tgacggcagc
 1801 tattgggggg agtggcagat tagtgaatt tccagctctc agagcgcgtg agtgcttcta
 1861 cccgctcctt taccttctc gaaggatcaa ggcatlaagt agctcatgtg aaggcgattt
 1921 cagttcatc tctcttttt ggagctcatt tcttttga ccaattgtga caccaaattg
 35 1981 ccatgtgtac tgtaattgtt caaatgaacg ttaaccttcg atttaatatg gacatttcca
 2041 ggtttcccta ctctgtcat tatgcctaac tcgggttgat gtctgtcag gatgaaaatc
 2101 tctgtgtcat glacttcgag tgaatgggc agggctaacc cctaagccct aacgccaat
 2161 cgacttataa gtctagatgt ttatgctatg caggctctgg aatgatttac attccatgct
 2221 ataca (SEQ ID NO: 27)

40

45

Example III

A Dual-Targeting Antibiofilm Approach for EPS Matrix Degradation and Enhanced Bacterial Killing

As noted above, biofilms are difficult to treat using conventional antimicrobial
5 monotherapy as they comprise structured microbial communities embedded in an extracellular
matrix associated with bacterial adhesion-cohesion and drug tolerance. In the present example,
we further investigated a multi-targeted approach combining exopolysaccharides (EPS) matrix-
degrading glucanohydrolases with clinically used essential oils-based antimicrobials to enhance
antibiofilm efficacy. Our data showed that dextranase synergized with mutanase to breakdown
10 EPS glucan-matrix in pre-formed oral biofilms, while markedly potentiating bacterial killing by
antimicrobials (3-log increase vs antimicrobial alone). Further analyses revealed that EPS-
degrading/antimicrobial dual-approach (EDA) breaks down the matrix scaffold causing ‘physical
collapse’ of the bacterial clusters and inducing cellular dispersion, exposing the bacterial cells for
rapid antimicrobial killing. Interestingly, a single pre-treatment of apatitic surface using the EDA
15 approach described prevented biofilm accumulation by inhibiting EPS synthesis and bacterial
binding in situ. Unexpectedly, we found that EDA-approach can also selectively target the EPS-
producing oral pathogen *Streptococcus mutans*, disrupting its colonization and overgrowth in a
mixed-species ecological biofilm model. We observed an intriguing mechanism whereby
localized EPS degradation and exposure of the embedded pathogen resulted in enhanced killing
20 (vs. other species), promoting the dominance of commensal bacteria. Together, these results
demonstrate an approach that can enhance antibiofilm efficacy and precision by dismantling the
EPS matrix and its protective microenvironment thereby potentiating the killing of bacterial cells
within.

The Materials and Methods below are provided to facilitate the practice of Example III.

25 Bacterial strains and growing conditions

S. mutans UA159 serotype c (ATCC 700610), a proven cariogenic dental pathogen and
also the primary producer of water-insoluble EPS matrix (26) was used for single-species biofilm
model. *Actinomyces naeshundii* (ATCC® 12104™), and *Streptococcus oralis* (ATCC® 35037™)

were selected commensals to generate mixed-species biological biofilm with *S. mutans*, because these three species are all detected in high abundance in the supragingival plaque of human(37). *S. oralis* is one of the earliest pioneer colonizers on the saliva-coated tooth surface (37). They were known to produce soluble glucans from sucrose and to be acid-tolerant(38). *A. naeslundii* also colonizes on saliva pellicle during the early stages of plaque formation. Strains were stored at -80°C in tryptic soy broth (TSB) containing 25% glycerol. All strains were grown in ultrafiltered (10kDa molecular-weight cut-off membrane; Prep/Scale, Millipore, MA) buffered tryptone-yeast extract broth (UFTYE medium; 2.5% tryptone and 1.5% yeast extract, pH 7.0) supplemented with 1% (w/v) glucose at 37°C and 5% CO₂ to mid-exponential phase before use(25).

EPS-degrading enzymes and antimicrobials

EPS-degrading enzymes are glucanohydrolases that can cleave the glucosidic linkages of polysaccharides. Dextranase[α -(1→6) glucanase; EC 3.2.1.11] which can catalyze the hydrolysis of glucoside bonds in (1→6)- α -D-glucans of different origins was purchased from Sigma (St. Louis, MO). Mutanase[α -(1→3) glucanase; EC 3.2.1.59] that hydrolyzes glucoside bonds in (1→3)- α -D-glucans was a kind gift from Johnson & Johnson (New Brunswick, NJ). The glucanohydrolase activity was determined as described by Kopec et al. (39) with some modifications. Briefly, purified *S. mutans* GtfD glucans[α -(1→6)-linked glucans] or GtfB glucans[α -(1→3)-linked glucans] were incubated for 1h at 37°C with dextranase or mutanase in 0.1M sodium acetate buffer (pH=5.5). The amount of reducing sugar released from the glucans by each glucanohydrolase was determined colorimetrically using Somogyi-Nelson Method (40). One unit (U) of activity of dextranase was defined as the amount of enzyme which will liberate 1.0 μ mol of reducing sugar (measured as maltose) per minute from α -(1→6)-linked glucans at pH 5.5 at 37°C. One unit (U) of mutanase was defined as the quantity of enzyme which will liberate 1.0 μ mol of reducing sugar (measured as glucose) per minute from α -(1→3)-linked glucans at pH 5.5 at 37°C. An alcohol-free essential oils (EOs)-based solution comprising the antimicrobial combination of menthol, thymol, eucalyptol, and methyl salicylate was used for Example III. LISTERINE ZERO[®] comprising these EOs, was kindly provided by Johnson & Johnson, was used as model antimicrobial agent.

Single- and mixed-species biofilm model

Single- and mixed-species biofilms were formed on vertically-suspended saliva-coated hydroxyapatite (sHA), a model to mimic the biological dental surface, as detailed previously(25). Briefly, Hydroxyapatite discs (1.25 cm in diameter, surface area of 2.7 ± 0.2 cm², Clarkson, Chromatography Products, Inc., South Williamsport, PA) were coated with filter-sterilized human whole saliva. For single-species biofilm, each sHA disk was inoculated with 10^5 CFU/mL actively growing *S. mutans* in UFTYE medium containing 1% (w/v) sucrose, and was grown for 19h (37°C and 5% CO₂)(25). The mixed-species biofilm model was designed to mimic the biofilm formation based on the “ecological plaque-biofilm” concept(41), as described by Xiao et al. (24). The bacterial cultures of *S. mutans*, *S. oralis* and *A. naeslundii* were diluted to provide an inoculum with a defined microbial population of *S. mutans* (10^2 CFU/mL), *S. oralis* (10^7 CFU/mL), and *A. naeslundii* (10^6 CFU/mL) in UFTYE medium with 0.1% (w/v) sucrose. The organisms were grown for the first 29h to form the initial biofilm community, with a medium change at 19 h. At 29, biofilms were transferred to UFTYE medium containing 1% (w/v) sucrose to induce environmental changes to simulate a cariogenic challenge. Biofilms were analyzed at 43h (early cariogenic biofilm) or 67h (mature cariogenic biofilm). The culture medium was replaced twice daily (8 am and 6 pm) with UFTYE medium with 1% (w/v) sucrose for experimental period longer than 43h.

Screening of combinatory effect of dextranase and mutanase on EPS degradation

For screening of combinatory effect on EPS degradation by dextranase and mutanase at different activity ratio, 96-well plate was coated with filter-sterilized human whole saliva and inoculated with 10^5 CFU/mL actively growing *S. mutans* in UFTYE medium containing 1% (w/v) sucrose. Biofilms were grown for 19h (37°C and 5% CO₂) before the combinatory effect of dextranase and mutanase to disrupt *S. mutans* biofilms were determined by checkerboard microdilution assay as described previously with some modification (42). In brief, biofilms in each well were incubated with 0.1M sodium acetate buffer (pH=5.5) with serially diluted test glucanohydrolases in combination for 120min at 37°C. The final concentrations of dextranase and mutanase ranged from 0-17.50U/mL. After incubation, each well was briefly washed with 0.89% sodium chloride to remove solubilized/detached biomass and residual biofilms were stained with 0.2% crystal violet for visualization and quantification. Relative reduction rate was

calculated using that of the vehicle control group as control(0% reduction). Coefficient of Drug Interaction(CDI) was used to evaluate the synergism/antagonism between the two glucanohydrolases with the equation $CDI = \frac{R_{Dex+Mut}}{R_{Dex} \times R_{Mut}}$ (R_{Dex} and R_{Mut} , relative reduction with dextranase or mutanase alone; $R_{Dex+Mut}$, relative reduction of the Dex/Mut combination).

- 5 CDI<0.7 indicates a significantly synergistic effect; CDI=1 indicates an additive effect; CDI>1 indicates an antagonistic effect (43).

Treatment regimen of EPS-Degrading/Antimicrobial (EDA) approach

Antibiofilm efficacy of EDA approach was tested using both preformed biofilms (efficacy of biofilm disruption) and in the process of biofilm formation (efficacy of biofilm prevention). For biofilm disruption, pre-formed biofilms on sHA (19h for single-species and 43h/67h for mixed-species biofilms) were treated with: 1) vehicle control (0.1M sodium acetate buffer, pH=5.5); 2) EPS-degrading enzyme(s) at the optimum concentration for 120 min, after which the biofilms were challenged with antimicrobial (EOs) at full strength for 1min, and harvested for further analysis. For biofilm prevention, sHA discs were topically treated with EPS-degrading enzymes (at the optimum concentration) or vehicle control (0.1M sodium acetate buffer, pH=5.5) for 60 min before inoculum and were incubated to allow single- or mixed-species bacterial colonization and biofilm formation. At the end of the experimental period (19h for single-species biofilm and 43h for mixed-species biofilms), biofilms were exposed to EOs at full strength for 1min and analyzed upon harvest.

Quantitative biofilm analysis

Biofilms were subjected to biochemical and microbiological analysis, as detailed elsewhere (22, 25). Briefly, biofilms on HA surfaces were harvested, homogenized by sonication and plated on blood agar after serial dilution using an automated Eddy Jet Spiral Plater (IUL, SA, Barcelona, Spain) to determine the CFU on each hydroxyapatite disk (CFU per biofilm). For mixed-species biofilms, the three species were differentiated by observation of colony morphology on blood agar, and proportion of different species was calculated(total CFU per biofilm was regarded as 100%). An aliquot of biofilm suspension was centrifuged (5,500g, 10min, 4°C), and the pellet was washed with water and dried in oven (105°C, 24h) before measuring water-insoluble dry weight. Water-soluble and water-insoluble polysaccharides in

biofilm matrix were extracted and colorimetrically quantified using phenol-sulfuric acid method as detailed previously (44, 45). At least 3 independent biofilm experiments were performed for each of the assays.

The effect of EDA approach on the biofilm 3D architecture was assayed by super-resolution fluorescence confocal microscopy at different time-point using our well-established protocols (24). Briefly, EPS were dynamically labelled via incorporation of Alexa Fluor 647 dextran conjugate (Final concentration, 1 μ M; molecular weight, 10 kDa; absorbance/fluorescence emission maxima of 647/668 nm; Molecular Probes, Eugene, OR) which served as primers for glucan synthesis during biofilm formation while bacterial cells were stained with 2.5 μ M SYTO 9 green-fluorescent nucleic acid stain (485/498 nm; Molecular Probes). Imaging was performed using a single-photon laser scanning microscope (LSM800, Zeiss, Germany) equipped with a 20 $\times$ (1.0 numerical aperture) water immersion objective. Each biofilm was scanned at 5 randomly selected areas, and confocal image series were generated by optical sectioning at each of these positions. Computational analysis of confocal images using Comstat2 (www.comstat.dk) was conducted to determine the biovolumes of bacteria and EPS in order to complement our biochemical and microbiological analysis (46). Amira 5.4.1 software (Visage Imaging, San Diego, CA, USA) was used to create 3D renderings of biofilm architecture.

4-dimensional time-lapse confocal live imaging and analysis

The dynamic impact of EDA on biofilm EPS structure, mechanical stability and the killing efficacy were assessed using 4-dimensional(x, y, z, t) time-lapse confocal live imaging as described by Xiao et al. (24) with modifications. EPS was labeled using 1mM Alexa Fluor 647 dextran conjugate supplemented in the culture medium. At the end of biofilm formation(19h for single-species biofilm and 67h for mixed-species biofilms), the sHA disk was transferred to a Petri dish (diameter 35mm) containing 5 μ M SYTO9 (for staining live cells; Molecular Probes) and 30 μ M propidium iodide (for staining dead, or membrane compromised cells; Molecular Probes) in 4mL of 0.1M sodium acetate buffer(pH5.5), which enabled continuous labeling and real-time visualization of live and dead bacterial cells over the entire experimental period. After initial staining for 30min at 37°C, multi-channel images were acquired using LSM800 single-

photon high-resolution confocal microscope (Zeiss) equipped with a 20× water immersion objective. Immediately after completion of the first scan, EPS-degrading enzymes or the vehicle buffer were carefully added to the solution and mixed well. For 4D time-lapse series, images of the same field of view were acquired every 10 minutes after adding EPS-degrading enzymes. At 5 120 min, antimicrobial (EOs) was added to the same buffer solution and mixed to yield a final concentration of 25% of the full strength. This concentration was optimized in our preliminary experiment for best killing efficacy with minimal effects on imaging. Imaging of the same field of view was initiated 1min after the antimicrobial challenge. The total biomass of EPS in each series of confocal images was algorithmically analyzed using COMSTAT2 (46). Computational 10 analysis of the dynamics of biofilm structural stability(single particle tracking) was performed using the open-source platform TrackMate (47, 48). The movement of each microcolony was quantified as cumulative displacement, a measure of how far it moved over the period of observation. Fiji(a distribution of ImageJ) (49) and Amira (Visage Imaging) were used for image processing and creating time-lapse renderings of biofilm architecture.

15 **Effect of EPS-degrading enzymes on glucan synthesis by purified GtfB**

GtfB was obtained from *Streptococcus milleri* KSB8 and purified to near homogeneity by hydroxyapatite column chromatography as detailed by Venkitaraman et al. and Koo et al. (50, 51). Gtf activity was measured by the incorporation of ¹⁴C-glucose from radiolabeled sucrose (PerkinElmer, MA, USA) into glucans (50). One unit(U) of GtfB activity is defined as the 20 amount of enzyme that incorporates 1 μ mol of glucose over a 4-h reaction. Glucan synthesis in the presence of EPS-degrading enzymes was performed as detailed by Hayacibara et al. (21) with some modifications. Briefly, GtfB (10U) was mixed with dextranase or mutanase (ranging from 0-50U), and incubated with ([¹⁴C]glucosyl)-sucrose substrate (0.2 μ Ci/mL; 200.0 mM sucrose, 40 μ M dextran T-10, 0.02% sodium azide in Adsorption Buffer: 50mM KCl, 0.35mM 25 K₂HPO₄, 0.65mM KH₂PO₄, 1mM CaCl₂, 0.1mM MgCl₂· 6H₂O, pH 6.5) for 4h at 37°C to allow glucan synthesis. For the combinatory effect of dextranase and mutanase, 1U of mutanase with various amount of dextranase(0-10U) was used in the assay. Insoluble glucans were collected after centrifugation (13,400g, 4°C, 10min) and washed three times with water. Soluble glucans

were precipitated with ethanol (final concentration: 70%) for 18h at -20°C. The amount of radiolabeled insoluble and soluble glucans were quantified by scintillation counting (50).

Fluorescence *in situ* hybridization (FISH) of mixed-species biofilm

Mixed-species biofilm morphogenesis and spatial distribution of polymicrobial population in the ecological biofilm model was assessed using FISH in order to understand the mechanism of enhanced precision and antibiofilm efficacy induced by EDA, as described elsewhere with modifications (52–54). Briefly, the mixed-species biofilms formed on sHA were gently washed twice with phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde (in PBS, pH 7.4) for 4h at 4 °C. After washing with PBS, sample was transferred to 50% ethanol (in PBS, pH 7.4) and stored at -20 °C. For hybridization, we first permeabilized bacterial cells by treatment with lysozyme (Sigma; 400,000U/mL in 20mM Tris-HCl pH 7.5, 5mM EDTA) for 14min at 37°C. Biofilms were incubated for 4h at 46°C in the hybridization solution (25% formamide, 0.9M NaCl, 0.01% SDS, 20mM Tris-HCl, pH 7.5) containing FISH oligonucleotide probes (SMU587, 5'- ACTCCAGACTTTCCTGAC-3' (SEQ ID NO: 28) with Alexa Fluor 488 for *S. mutans*; MIT588, 5' -ACAGCCTTTAACTTCAGACTTATCTAA-3' (SEQ ID NO: 29) with Cy3 for *S. oralis*; ACT476, 5'-ATCCAGCTACCGTCAACC-3' (SEQ ID NO: 30) with Alexa Fluor 594 for *A. naeslundii*, each probe at a final concentration of 1 μ M), and were washed against washing solution (0.2M NaCl, 20mM Tris-HCl pH 7.5, 5 mM EDTA, 0.01% SDS) at 37 °C for 15 min. High-resolution images were acquired using LSM800 single-photon confocal microscope (Zeiss) equipped with a 20× water immersion objective. Each field of view was imaged using sequential excitation with 640-, 561- and 488-nm laser lines, and the whole fluorescence spectrum emitted was detected as lambda stacks (55). Linear unmixing algorithm was applied on the lambda stack with Zeiss ZEN software using reference spectra of all fluorophores in the specimen. Unmixed images were assembled and false colored using Fiji (49).

Assessment of bacterial initial adherence

The cariogenic pathogen *S. mutans* produce glucan-binding proteins which greatly promote its colonization on tooth surface in the presence of Gtf-synthesized glucans (56) while other commensals rely on adhesin-receptor interaction that mediates preferential adhere to salivary pellicle (57). In order to investigate the influences of surface-adsorbed EPS-degrading

enzymes on polymicrobial binding affinities to mechanistically understand their impact on early microbial colonization, we assayed bacterial adherence using radioisotope tracing spectroscopy (21, 58). Briefly, *S. mutans*, *S. oralis* and *A. naeslundii* were grown in UFTYE medium supplemented with 10 μ Ci 3 H-thymidine (PerkinElmer) and 1% (w/v) glucose at 37°C, 5% CO₂ to mid-exponential phase to radiolabel bacteria and the cell density (cells/mL) was measured using Petroff-Hausser counting chamber. Saliva-coated Hydroxyapatite beads (Bio-rad, CA, USA; 80 μ m particle size) were pretreated with either vehicle or glucanohydrolases at the optimum combination before GtfB immobilization, and glucans were synthesized *in situ* in the presence of sucrose substrate (200.0 mM sucrose, 40 μ M dextran T-10, 0.02% sodium azide in Adsorption Buffer, pH6.5) for 4h. The glucan-coated surfaces were then incubated with 1.0×10^9 cells/mL isotope-labeled bacteria in Adsorption Buffer for 1h at 37°C. Hydroxyapatite beads with salivary pellicle only was used as the control. Unbound bacteria were removed by washing the beads with Adsorption Buffer 3 times. Affinity of bacterial adhesion was quantified by scintillation counting. The actual number of adherent cells on the sHA beads was calculated using calibration curves of measured radioactivity (counts per minutes, CPM) versus number of radiolabeled bacteria.

Results

EPS-degrading enzymes synergistically enhance antimicrobial killing at the optimum activity ratio.

EPS produced by streptococcal Gtfs are key components in cariogenic biofilm matrix, and contain a mixture of glycosidic linkages, comprised predominantly of α -(1 $\rightarrow$ 3)-, α -(1 $\rightarrow$ 6)- and α -(1 $\rightarrow$ 4)-linked glucans with high structural polymorphism(21). Therefore, targeting the EPS-rich matrix is challenging, and may require breakdown of more than one specific chemical bond. Glucanohydrolase, such as dextranase [α -(1 $\rightarrow$ 6) glucanohydrolase] and mutanase [α -(1 $\rightarrow$ 3) glucanohydrolase], were shown to disrupt the glucan synthesis by Gtfs and digest preformed EPS matrix(16, 21, 22). For optimal efficacy, we first screened the EPS-degrading activity of various combinations of dextranase and mutanase using a high-throughput biofilm model (Figure 19D). Neither dextranase nor mutanase alone could efficiently degrade the biofilm, even at the highest concentration tested (17.5U/mL, <25% of reduction rate). However, the dextranase and mutanase

in combination exhibited synergistic effect on EPS degradation, with the highest reduction rate(>60%) and the highest synergy(Coefficient of Drug Interaction, CDI=0.635, indicating significant synergy) achieved with 8.75U/mL dextranase and 1.75U/mL mutanase (5:1 activity ratio).

To test our multi-targeting concept, we developed a combinatory therapy combining glucanohydrolases with clinically-used essential oils (EOs)-based formulation, as a model antimicrobial agent. Antibiofilm effect was assessed using an established saliva-coated hydroxyapatite (sHA) oral biofilm model (Figure 19A). Biofilms were topically treated with EPS-degrading enzymes and then immediately exposed to antimicrobials (Figure 19B).

Consistent with glucanohydrolases optimization, the most potent killing activity was observed using 8.75U/mL dextranase and 1.75U/mL mutanase (1000-fold more effective versus antimicrobial alone) (Figure 19C). Because the glucan matrix also harbors significant proportion of α -(1→4) glycosidic linkages, we also investigated whether efficacy could be further improved by including glucoamylase [α -(1→4) glucanase]. We observed only a moderate increase in the killing efficacy (~ 0.5 log CFU) when compared to the optimized dual-enzyme combination (Figure 20F), indicating that dual-enzyme approach provide sufficient high EPS-degrading activity for testing the proof-of-concept proposed in this study. Altogether, the data suggest that dextranase and mutanase can synergistically degrade the protective EPS-matrix, and significantly enhance killing of biofilm cells.

EPS-degrading enzymes dismantle biofilm matrix *in situ* and facilitate antimicrobial targeting within biofilms.

EPS-matrix has been recognized as an important factor for biofilm drug recalcitrance (4). However, how the structural organization of the matrix and biofilm 3D architecture hinder antimicrobial efficacy remains poorly understood. Using high-resolution time-lapse confocal microscopy, we observed the spatiotemporal morphological changes that biofilms undergo following EPS-degradation. As shown in Figure 20A, the vehicle-treated biofilm display clusters of densely-packed bacterial (in green) cells, termed microcolonies (1–3) embedded by abundant amount of EPS matrix (in red), typically found in cariogenic biofilms. In sharp contrast, biofilm treated by dextranase in combination with mutanase were essentially devoid of EPS, while showing dispersion of bacterial cells (white arrows; Fig 20A-d). Neither dextranase nor

mutanase alone could completely degrade the matrix of preformed biofilm. Consistent with the confocal imaging data, the dual-enzyme treatment resulted in significantly less biomass (Figure 20C) with minimal exopolysaccharide content (Figure 20D) compared to either glucanohydrolase alone, indicating that targeting a single glycosidic bond was insufficient to fully dismantle the biofilm matrix.

Next, we investigated how EPS-degradation could modulate the antimicrobial killing profile at single microcolony level. Time-lapse killing assay was performed using real-time bacterial live/dead staining and EPS imaging (Figure 20 B and Figure 21E). The biofilm without dual-enzyme treatment (vehicle-treated) contained many live cells (in green) after brief (1 min) and prolonged (5min) exposure to topical antimicrobial agent (EOs) (Figure 20B, top). We found that bacterial cells close to the surface (white arrow) were mostly dead (in magenta) by antimicrobial exposure, whereas cells residing inside the microcolony remained mostly vital (in green). However, following EPS degradation by glucanohydrolases, the antimicrobial agent efficiently killed bacteria both inside and outside of the disrupted microcolony (Figure 20 B, bottom). These findings were further validated by microbiological analysis, showing that glucanohydrolases in combination synergistically potentiated antimicrobial killing of antimicrobial (~3-log more effective killing versus EOs alone, $p < 0.001$) while the enzymes alone did not have antibacterial activity (versus vehicle-treated control, $p > 0.05$) (Figure 20E) Thus, glucanohydrolases appear to ‘disable the physical barrier’ provided by the EPS matrix, thus exposing the bacterial cells for enhanced antibacterial activity.

To further understand the dynamics of enzymatic EPS-matrix dismantling and its associated protective mechanism of recalcitrance, we examined the spatiotemporal degradation of EPS *in situ* using time-resolved super-resolution confocal imaging (Figure 21A and 21B). Visualization of the ultrastructural features of the matrix within a single microcolony reveals a complex network of cross-linked EPS (Figure 21A, in red). Intriguingly, the dual-enzyme combination efficiently degraded the EPS located both outside and inside of the microcolony (Figure 21B, white arrows), resulting in a more effective and homogeneous bacterial killing following 1 min exposure to EOS (Figure 21D, live and dead bacteria in green and magenta, respectively). In contrast, the vehicle-treated microcolony showed a center core of bacteria (Figure 21C, dashed circle) comprised of mostly live cells (in green) while the outer layers harbored a mix of live and dead cells after antimicrobial exposure (live bacteria shown in the

inserted dashed boxes). The data indicate that dextranase and mutanase combination, in addition to degrading the surrounding EPS, is capable of reaching the interior of the bacterial microcolony and digest the EPS-scaffold within to enhance antimicrobial efficiency.

5 **Mechanical stability and integrity of the biofilm scaffold is damaged by EPS-degradation.**

The presence of exopolysaccharide matrix provides essential scaffold for mechanical stability and modulates the resistance of biofilms to mechanical clearance from the substratum (23), posing another challenge for the development of biofilm-disruptive strategies. Here, we examined whether disassembling the matrix-scaffold could impact the physical integrity and

10 stability of the biofilm architecture. Using time-lapsed imaging, we tracked the dynamic changes of the biofilm structure during the EPS degradation. We observed an ‘implosion-like’ collapse of the physical structure as a result of EPS removal where entire microcolony essentially crumbled down to the bottom of the sHA surface accompanied with cellular dispersion (Figure 22A). To further measure this dynamic process, computational analysis were performed to generate time-

15 resolved EPS degradation (red squares) and microcolony spatial displacement (green circle) curves. As shown in Figure 22B, the EPS was removed over time with higher breakdown rate during the first 30min (~70% reduction), at which point the EPS degradation slowed down as the time elapsed. Notably, the ‘physical collapse’ was detected during the later-phase of EPS

20 degradation (after 70 min), which manifested as detectable displacement of microcolonies, indicating that complete dismantling of the EPS scaffold may be required to fully compromise the biofilm mechanical stability and 3D integrity. Altogether, the data show that EPS-degrading/antimicrobial dual-approach (EDA) can potentially disrupt biofilms mediated by EPS-producing and cariogenic *S. mutans*.

25 **EDA locally degrade EPS for enhanced targeting of EPS-producing pathogen in mixed-species biofilms.**

The production of EPS in the presence of sucrose favors the establishment of *S. mutans* modulating the transition from commensal-rich community to pathological biofilms (22). Here, we hypothesized that EDA approach could target the EPS-producing pathogens in an mixed-species model that mimics the ecological development of cariogenic biofilms (22, 24). In the

ecological biofilm model, *S. mutans* is co-cultured with the early colonizers *S. oralis* and *A. naeslundii* to form an initial polymicrobial community on sHA (Figure 23A and 23B). Then, sucrose is introduced to induce ecological shifts from a commensal-rich community (with high levels of *S. oralis*, few *A. naeslundii* cells and *S. mutans* as the least abundant species) to a

5 biofilm characterized by EPS-rich matrix and acidic microenvironments (22, 25) that favors *S. mutans* growth (Figure 23A and 23B). Hence, we examined whether EDA approach can disrupt the establishment of pathogen-dominant biofilm under cariogenic conditions (treatment regimen shown in Figure 23A). To show this concept, we tested EDA approach in two different time

10 points, representing different ecological stages of the mixed-species biofilm (Figure 23B). At 43 h (the early stage), when *S. mutans* was not dominant with low EPS production, we observed similar killing by antimicrobial (EOs) with and without EPS-degradation (Figure 23C). At 67 h (the late stage), when *S. mutans* became dominant with high abundance of EPS accumulated, EDA had higher overall killing efficacy compared to antimicrobial (EOs) alone (Figure 23D, top). Intriguingly, the inclusion of EPS-degrading enzymes resulted in more selective bacterial

15 killing (Figure 23D, top), with significantly higher efficacy against *S. mutans* (~2.0-log reduction versus antimicrobial) than on *S. oralis* (~0.5-log reduction), resulting in commensal's dominance in the biofilm (Figure 23D, bottom).

Given the efficiency in disrupting pathogenic dominance, we further characterized the spatial organization of EPS-matrix, the pathogens and commensals in the mature mixed-species

20 biofilm with fluorescence *in situ* hybridization (FISH). Representative confocal images showed the bacterial components were organized into a highly structured and distinctive spatial organization (Figure 23E). The biofilm structure was characterized by the presence of EPS-embedded *S. mutans* cell cluster that were surrounded by *S. oralis* cells forming a peripheral layer (Figure 23E b, cross-section superimposed view). We use the phrase “core-corona” to

25 describe this consortium structure generated from the radially oriented *S. oralis* community (depicted in yellow) which forms a halo-like structure surrounding a densely packed *S. mutans* microcolony (depicted in green) enmeshed by EPS (depicted in red) (Figure 23E b-f). Such spatial and protected organization appears to make it difficult to target *S. mutans* within the established mixed biofilms using antimicrobials alone. Conversely, time-lapse confocal

30 microscopy revealed that the inclusion of dual-enzyme combination can locally degrade the EPS, exposing the embedded bacterial for enhanced killing by antimicrobial (Figure 23 F). Taken

together, EDA approach could target the EPS-producing pathogens in an mixed-species by eliminating their structural and virulent traits, thus enhancing antibiofilm precision and efficacy.

EPS-degrading enzymes prevent biofilm formation by inhibiting glucan synthesis *in situ*

5 Previous studies have shown that EPS formed on hydroxyapatite and microbial surfaces promote bacterial adhesion and cohesion. Specifically, EPS glucans synthesized from dietary sucrose by streptococcal Gtfs are of central importance in adhesive interactions providing scaffolding material for biofilm initiation (26). Our data indicate that EPS-degrading enzymes could also prevent biofilm formation given their potent efficacy on pre-formed biofilms. To
10 address this question, EPS-degrading enzymes were topically applied to the experimental salivary pellicle surface (sHA) before biofilm formation (Figure 24A). We confirmed glucanohydrolase activity of dextranase/mutanase pretreated sHA surface (Table 3), which were capable of degrading the surface-formed EPS (Figure 22C). Representative 3D renderings of the *S. mutans* biofilm formed on sHA were shown in Figure 24B. The biofilm formation on dual-
15 enzyme pretreated sHA was highly defective and unable to form an EPS matrix or microcolonies, whereas those formed on single enzyme pretreated sHA showed formation of altered microcolony structure (white arrow, Figure 24B) with detectable EPS (albeit reduced). The microbiological data showed that the dual-enzyme combination had higher effect than either dextranase or mutanase alone in inhibiting the biomass accumulation (Figure 24C) and reducing
20 the total CFU in biofilms (Figure 24D). We also exposed the biofilms formed on pre-treated sHA surfaces to antimicrobials to examine whether the susceptibility to killing would be enhanced. A 100-fold more effective killing (resulting in >4-log CFU reduction in total) was observed in biofilms on enzyme-pretreated sHA surface (versus vehicle-pretreated ones) (Figure 23E).

Table 3

Dex/Mut Activityy on sHA beads (U/g)

Dex 6.72 ± 0.12

Mut 0.88 ± 0.01

To further understand the EPS degradation *in situ*, we performed glucan synthesis assay in the presence of dextranase and/or mutanase to determine whether biosynthesis and composition of Gtf-derived glucans would be affected. In general, both glucanohydrolases interfered with the amount and proportion of soluble/insoluble glucan synthesized (Figure 24F).

5 The presence of dextranase affected the ability of GtfB to synthesize insoluble glucans but reached the plateau of inhibitory activity (up to 60% less insoluble glucan reduction versus vehicle control) (Figure 24F, left), consistent with previous observations (21). Mutanase completely inhibited insoluble glucan synthesis at low concentration but the soluble glucan production was enhanced (Figure 24 F, middle). However, the dual-enzyme combination
10 completely disrupted both insoluble and soluble glucans synthesis, confirming a synergistic action between dextranase and mutanase (Figure 24F, right). Taken together, these data indicated the EPS-degrading enzymes could inhibit glucan biosynthesis by bacteria-derived Gtf, thus preventing biofilm formation *in situ* via a glucan-dependent mechanism.

EDA approach selectively prevents early colonization *S. mutans* in mixed-species biofilms.

15 The surface-formed EPS were shown to provide binding sites for glucan-binding cariogenic pathogens (e.g. *S. mutans*) in the presence of sucrose and enhance their colonization, disrupting the commensal-rich community (22). Using a mixed-species ecological model (as described earlier), we first explored the impact of EDA approach on dynamics of microbial population change using the treatment regimen as illustrated in Figure 25A. At 43h (early
20 cariogenic biofilm, 14h after introduction of sucrose), dramatic changes of microbial population were noted. Early mixed biofilms formed on sHA pretreated with EPS-degrading enzymes showed a 2-log reduction of *S. mutans* population (CFU) in comparison to the vehicle control, while neither *S. oralis* or *A. naeslundii* population was affected (Figure 25B), suggesting selective inhibitory effect on bacterial colonization. FISH using three species-specific probes
25 (representative images shown in Figure 25C) showed large numbers of *S. oralis* (depicted in yellow) associated with *A. naeslundii* (depicted in cyan) and *S. mutans* (depicted in green) cells (Figure 24C left). In contrast, commensals, particularly *S. oralis*, dominated the biofilms formed on enzyme-treated sHA (Figure 25B right graph and 25C right). Notably, topical treatment with antimicrobial completely eliminated *S. mutans* in the enzyme-pretreated group, while high
30 numbers of *S. mutans* cells were detected in vehicle-pretreated group (Figure 25D).

Since *S. mutans* could express glucan-binding proteins that facilitated avid adhesion to surface-formed EPS(27), we reasoned that glucanohydrolases could selectively disrupt the colonization of the EPS-producing pathogen. To further understand the selective *S. mutans* disruption, bacterial adherence to glucan-coated sHA surface was assessed by ³H-thymidine radioisotope tracing spectroscopy. We found that the commensals (*S. oralis* and *A. naeslundii*) bound more effectively to the salivary pellicle than the pathogen (*S. mutans*), while glucans produced on pellicle significantly suppressed binding of the commensals and enhanced that of *S. mutans* (Figure 25E). Intriguingly, glucanohydrolase pretreatment of pellicle before glucan formation showed divergent effects on the bacterial binding affinity, favoring commensal adherence with detriment to that of *S. mutans*. This finding indicated the selective inhibition on bacterial colonization was related to the disrupted adhesion of pathogens by glucan-dependent mechanism.

Discussion

Biofilm drug recalcitrance imposes great challenges for existing antimicrobial monotherapies and indicates urgent need for multi-targeted or combinatorial approaches(2). Here, we demonstrate that readily available EPS-degrading enzymes combined with a clinically used antimicrobial agent can synergize and potentiate antibiofilm efficacy by targeting the protective matrix and amplifying localized killing under topical treatment regimen. Importantly, this strategy can spatially target EPS-producing pathogens within mixed-species biofilms by disrupting colonization and exposing them *in situ* for enhanced antimicrobial elimination. Our work indicates that matrix disruption accompanied by antimicrobial action produces optimal biofilm control while also targeting EPS-producing pathogens in the context of mixed-species community, thereby providing a potent yet selective antibiofilm approach.

The importance of the extracellular matrix in biofilm antimicrobial recalcitrance has been increasingly recognized (28). However, the mechanisms by which the matrix enhances bacterial resistance against antimicrobial remain poorly understood. Detailing the spatiotemporal degradation of the EPS-matrix and its impact on potentiating bacterial killing and causing biofilm disassembly could provide further insights on the role of EPS-matrix in creating a ‘protective physical scaffold’ for the residing bacteria. Using time-resolved biofilm imaging with highly specific EPS-degrading enzymes, we found how matrix dismantling can optimize biofilm

prevention and disruption and help overcome antimicrobial resistance. First, enhancement of antimicrobial efficacy by glucanohydrolases was closely associated with effectiveness of EPS-degradation. Our data revealed that simultaneous degradation of α -(1 $\rightarrow$ 3) and α -(1 $\rightarrow$ 6) glycosyl linkages of the glucan matrix was required to effectively digest the matrix and disorganize the microcolony structure. Concurrently with structural dismantling, we observed significant potentiation of antibacterial efficacy by promoting access and killing of bacterial cells in the interior of the EPS-degraded microcolony. Furthermore, sHA surfaces pre-treated with glucanohydrolases can reduce EPS production *in situ* preventing establishment of EPS-embedded bacterial clusters and facilitating killing of the resultant defective biofilm. Thus, despite lack of intrinsic antibacterial activity, glucanohydrolases can be potent adjuvants for various types of antimicrobials. Although the combination of dextranase and mutanase was effective in EPS matrix degradation, additional glucosyl linkages such as α -(1 $\rightarrow$ 4) as well as other biopolymers have been also found in the matrix of cariogenic biofilms(29–31). For example, the presence of extracellular DNA and lipoteichoic acid in the dental biofilm matrix is also important for the biofilm structure and topical treatment with DNase has been shown to help disperse early biofilms. We also found that inclusion of glucoamylase which targets α -(1 $\rightarrow$ 4) bonds can enhance the overall digestion of the EPS matrix and further enhance antimicrobial efficacy. Therefore, we predict that the matrix-degrading performance can be maximized by targeting the various extracellular polymeric substance, which may further potentiate efficacy in eliminating matrix-induced antimicrobial recalcitrance.

The resistance to mechanical clearance represents another important mechanism by which the biofilms persistently attach to abiotic and biotic surfaces, making physical removal challenging (13, 32). Our data highlight the ability of EPS-degrading enzymes to weaken the mechanical stability and cause structural collapse of the biofilm architecture. The “physical collapse” observed strongly indicate that the glucanohydrolases are capable of dismantling the core structural-scaffold structure of the biofilm. Notably, the enzymes can also induce dispersal of the bacterial cells by degrading the adhesive polysaccharide that encases and stabilizes the bacterial cells. In the clinical setting, the dispersed cells from biofilms will return to an planktonic state and become more sensitive to killing by antimicrobial agents and host defenses(2). However, it is noteworthy that biofilm dispersal also contributes to the communicable transmission and mediates the intra-host spread/persistence of the pathogen(33),

posing risk of recolonization or bacteraemia. Thus, reinforcing the concept that EPS degradation approaches should be co-administered with antimicrobials to synergistically kill the bacteria and prevent bacterial recolonization *in situ*. These findings suggest the potential use of EDA as an antibiofilm therapeutic approach with both dispersing and prophylactic efficacy.

5 Bacterial species within a mixed-species biofilm display extensive and complex competition affected by the multifactorial microenvironment(34). Previous studies on matrix-targeting strategies were performed primarily using mono-species biofilm model. Here, we assess the efficacy of this approach for the first time using a mixed-species biofilm model. Unexpectedly, we found that EDA approach can selectively target the EPS-producing oral
10 pathogen *S. mutans*, preventing their colonization and overgrowth in a mixed-species ecological biofilm model, thus providing a competitive advantage to the commensals. We found that glucanohydrolases can target *S. mutans* colonization by disrupting their glucan-dependent binding mechanisms, which provides an advantage for adherence and accumulation of the pathogenic bacteria. Unlike the adherence of commensals (such as *S. oralis* and *A. naeslundii*)
15 which largely depend on interactions of saliva-derived adhesin and surface-anchored receptors(35), *S. mutans* greatly promote their adhesion by glucan-dependent mechanism such as surface-adsorbed Gtf and the glucan-binding proteins(Gbps) (36). Using single-cell based analysis, we found that glucanohydrolases selectively interfered with the colonization of the EPS-producing pathogens by disrupting glucan production *in situ*. We found that glucan-coated
20 sHA surface favored *S. mutans* cells adhesion and further accumulation. Conversely, EPS-degradation of the glucan-coated surface reestablished colonization by commensals likely by exposing the pellicle receptors for adhesin-mediated binding by the commensal bacteria.

Because of the heterogeneous architecture and diverse multi-species distribution, certain species are able to develop protective microenvironment against killing (2). In our model, *S.*
25 *mutans* can create an EPS-microcolony complex which confers higher resistance to the antimicrobial challenge than the commensals. Our data show that EDA approach can spatially target EPS-producing pathogens by locally degrading the protective matrix and facilitating bacterial killing *in situ*. Such synergy provides clear advantages compared to conventional antimicrobial monotherapy whereby the agent is incapable of eradicating the matrix of the pre-
30 formed biofilms, leaving behind pathogens enmeshed in the matrix. Collectively, our combined

approach has a community-level impact on the mixed-species biofilms, which from the ecological point of view, prevent the colonization and overgrowth of pathogenic bacteria.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such
5 embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

In the claims:

1. A composition comprising one or more essential oils and at least one biofilm degrading enzyme which act synergistically to degrade biofilm structures and inhibit biofilm deposition, in a biologically acceptable carrier for delivery of said composition.
- 5 2. The composition as claimed in claim 1 where the essential oils comprise one or more of menthol, thymol, eucalyptol and methyl salicylate and the at least one biofilm degrading enzyme is selected from mutanase, dextranase and glucoamylase.
3. The composition as claimed in claim 2 where the essential oils are menthol, thymol, eucalyptol and methyl salicylate, said biofilm degrading enzymes are mutanase and dextranase.
- 10 4. The composition as claimed in claim 2, wherein said biofilm degrading enzymes are produced in a plant plastid.
5. The composition of claim 3 further comprising glucoamylase.
6. The composition of claim 1 wherein said biofilm degrading enzymes are dextranase to mutanase ratio in said composition is 5:1.
- 15 7. A composition comprising an effective amount of one or more biofilm degrading enzymes selected from mutanase, dextranase and glucoamylase and one or more essential oils selected from the group consisting of menthol, thymol, eucalyptol, methyl salicylate, and combinations of two or more thereof in a carrier suitable for oral delivery, wherein said carrier is selected from the group consisting of mouthwash, mouth rinse, mouth spray, toothpaste, chewing gum, tooth
20 gel, sub-gingival gel, mousse, foam, chewable tablet, dentifrice, lozenge, and dissolvable strip.
8. The composition of claim 7, wherein said carrier is mouthwash, mouth rinse, or mouth spray.
9. The composition of claim 7, wherein the carrier is a chewing gum, chewable tablet, lozenge, or dissolvable strip.
10. The composition of claim 7, wherein the carrier is a chewing gum.
- 25 11. The composition of claim 7, wherein said essential oils comprise a combination of menthol, thymol, eucalyptol and methyl salicylate.

12. A method of degrading and/or removing unwanted biofilm from the oral cavity, comprising administering an effective amount of one or more biofilm degrading enzymes selected from the group consisting of mutanase, dextranase, glucoamylase, and combinations of two or more thereof, and one or more essential oils selected from the group consisting of menthol, thymol, eucalyptol, methyl salicylate, and combinations of two or more thereof, to a surface of the oral cavity having said biofilm, said enzymes degrading said film and said essential oil(s) exerting a bactericidal effect, thereby synergistically reducing or eliminating said unwanted biofilm.
13. The method of claim 11, wherein the one or more essential oils comprises at least two essential oils selected from menthol, thymol, eucalyptol and methylsalicylate.
14. The method of claim 13, wherein said essential oils comprise a combination of of menthol, thymol, eucalyptol, and methyl salicylate.
15. The method of any one of claim 12, claim 13, or claim 14, which is effective to selectively kill pathogenic *S. mutans* without adversely affecting commensal bacteria, *A. naeslundii* and *S. oralis*.
16. The method of claim 12 wherein said administering step comprises first administering the biofilm degrading enzymes followed by administering the essential oils.
17. A method for inhibiting biofilm deposition on the surface in the oral cavity comprising contacting a surface of the oral cavity susceptible to biofilm deposition with an effective amount of one or more biofilm degrading enzymes selected from the group consisting of mutanase, dextranase, glucoamylase, and combinations of two or more thereof, and one or more essential oils selected from the group consisting of menthol, thymol, eucalyptol, methyl salicylate, and combinations of two or more thereof, to a surface of the oral cavity having said biofilm said enzymes degrading said biofilm and said essential oils exerting a bactericidal effect, thereby synergistically inhibiting deposition of said unwanted biofilm..
18. The method of claim 17, wherein the antimicrobial oral rinse comprises at least two essential oils selected from menthol, thymol, eucalyptol and methylsalicylate.
19. The method of claim 18, wherein said essential oils comprise a combination of of menthol, thymol, eucalyptol, and methyl salicylate.

20. The method of any one of claim 17, claim 18, or claim 19, which is effective to selectively kill pathogenic *S. mutans* without adversely affecting commensal bacteria, *A. naeslundii* and *S. oralis*.

21. The method of claim 17 wherein said administering step comprises first administering the
5 biofilm degrading enzymes followed by administering the essential oils.

22. The composition of claim 4, further comprising a plant remnant.

23. The composition of claim 20, wherein said plant is a tobacco or lettuce plant.

24. The composition of claim 1, wherein said enzymes are recombinant and are expressed as fusion proteins in bacteria and purified therefrom.

10 25. The composition of claim 1, wherein said enzymes are purified from non recombinant enzyme expressing bacteria.

FIG. 1A

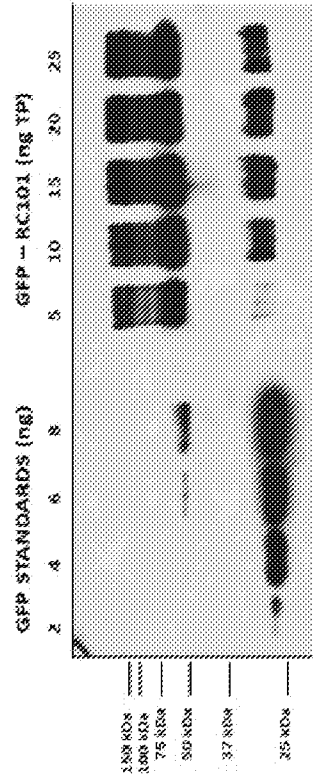


FIG. 1B

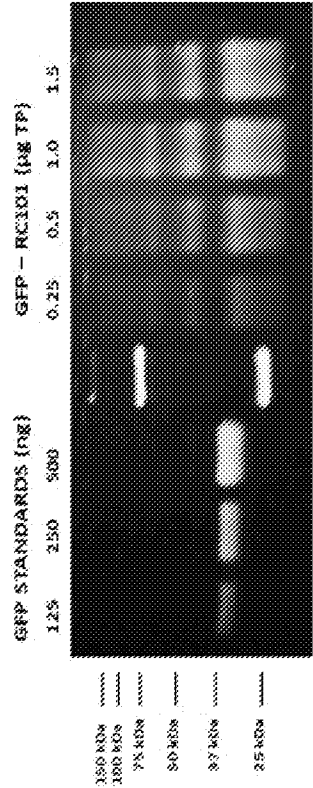


FIG. 1C

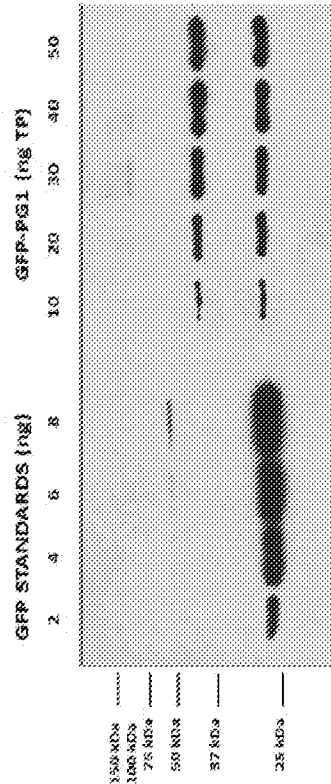
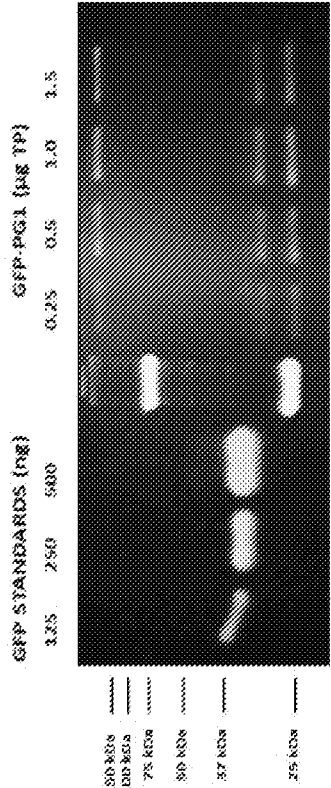
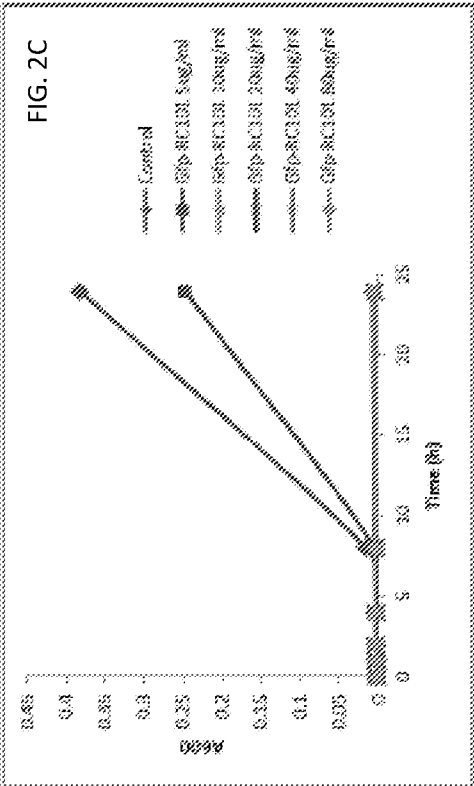
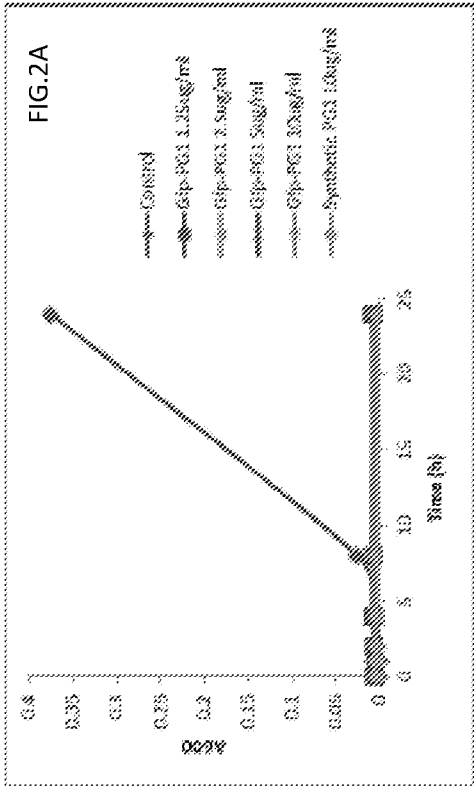
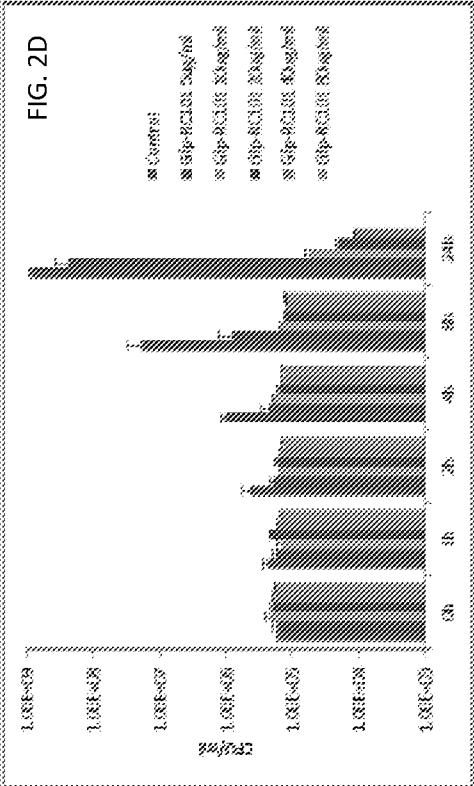
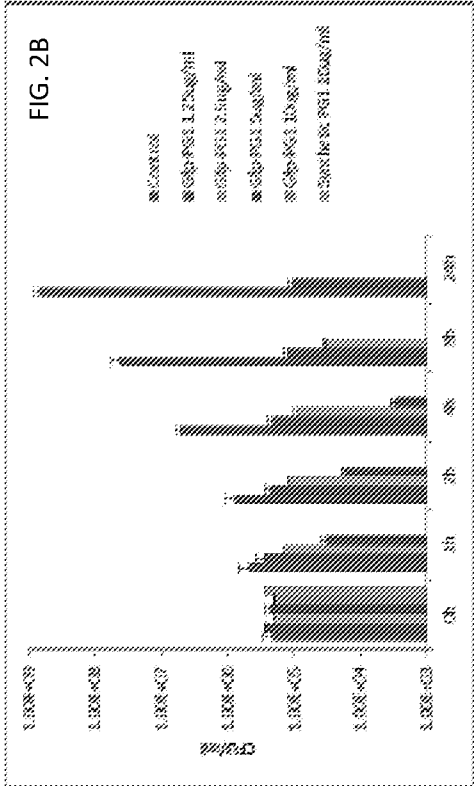


FIG. 1D



FUSION	AVERAGE EXPRESSION LEVEL (ng/g)	AVERAGE PURITY (%)	PURIFIED GFP YIELD (ng/gm lyophilized material)	PURIFIED PEPTIDE YIELD (µg/gm lyophilized material)	FOLD ENRICHMENT
GFP-RC101	8.30%	94%	1624	116	10.6
GFP-PG1	3.80%	17%	58.8	4.2	4.3



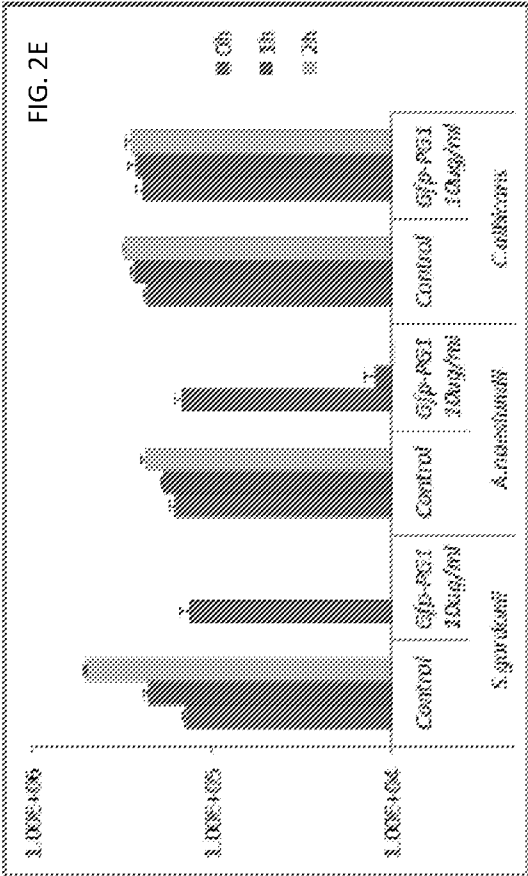


FIG. 3A

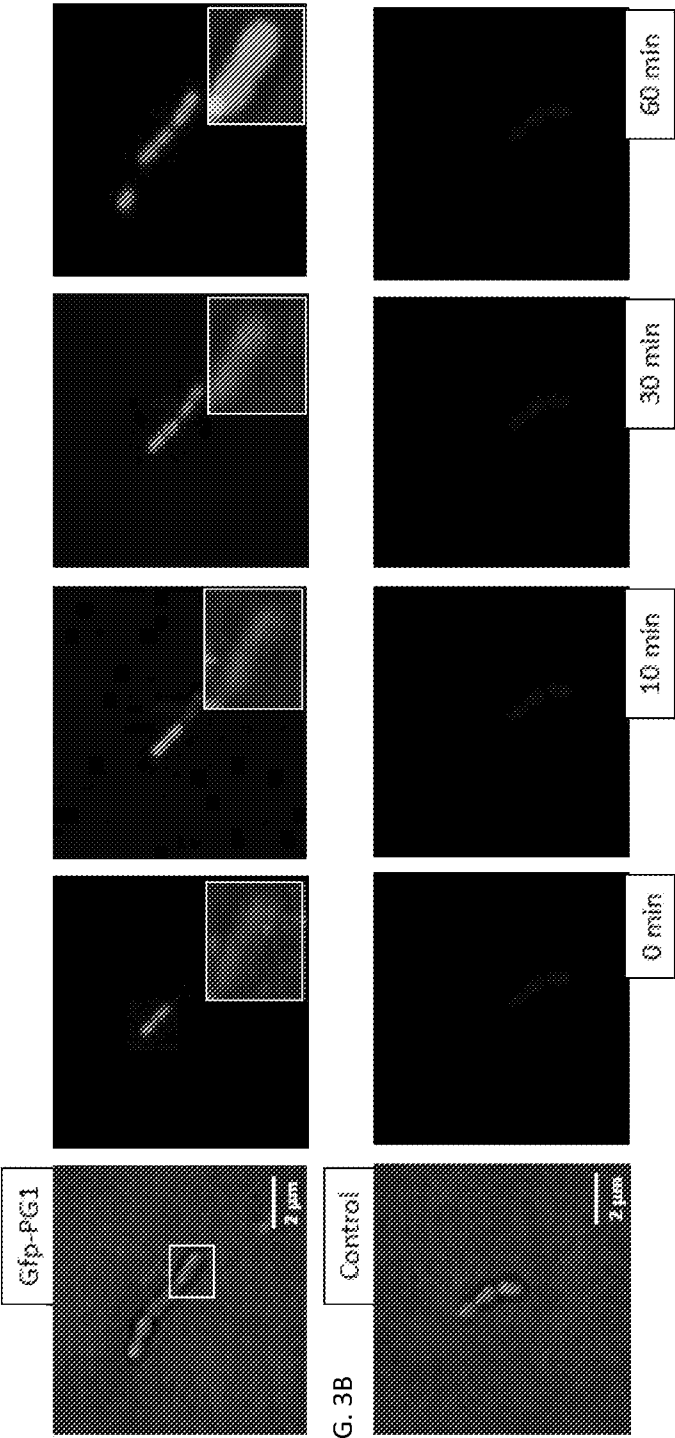


FIG. 3B

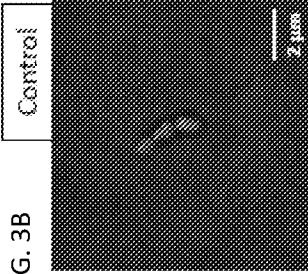


FIG. 3C

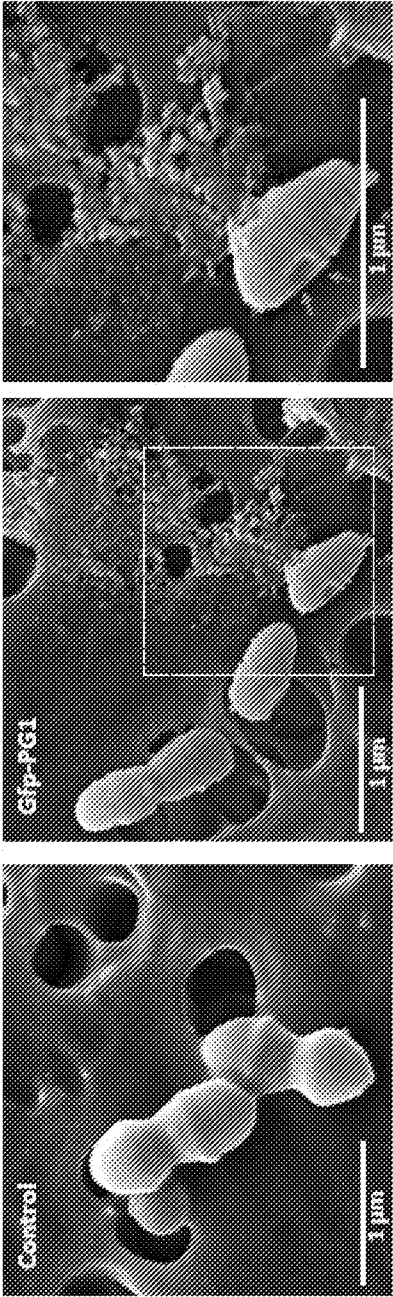


FIG. 4A

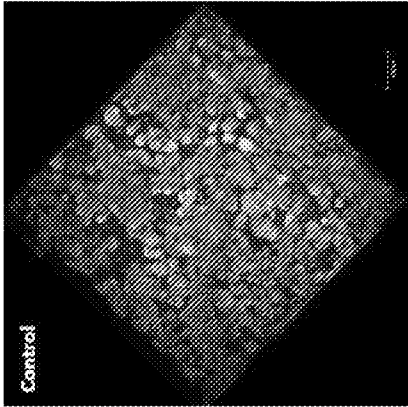


FIG. 4B

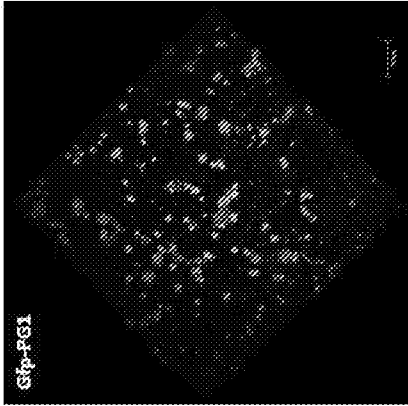
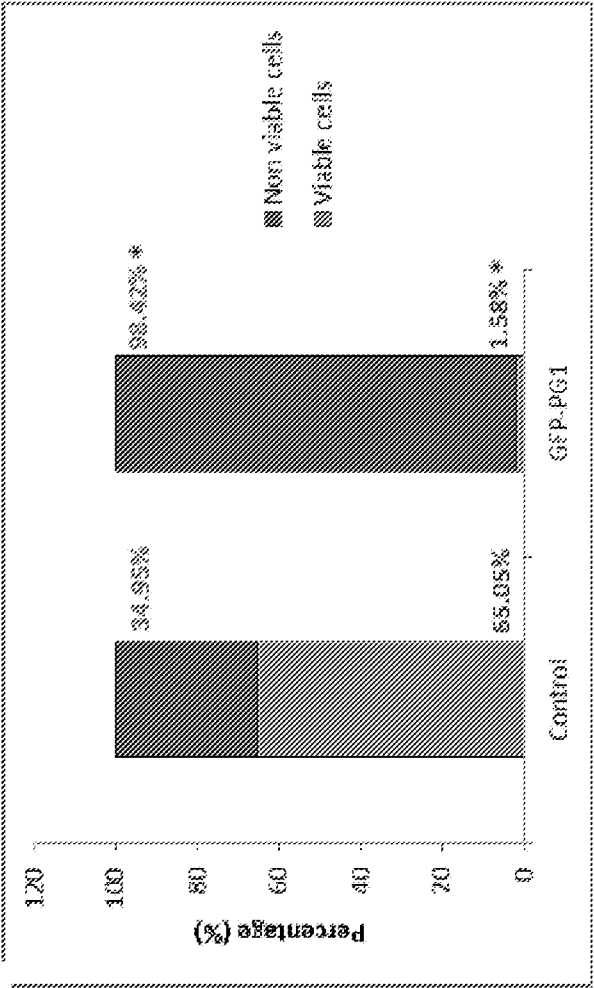


FIG. 4C



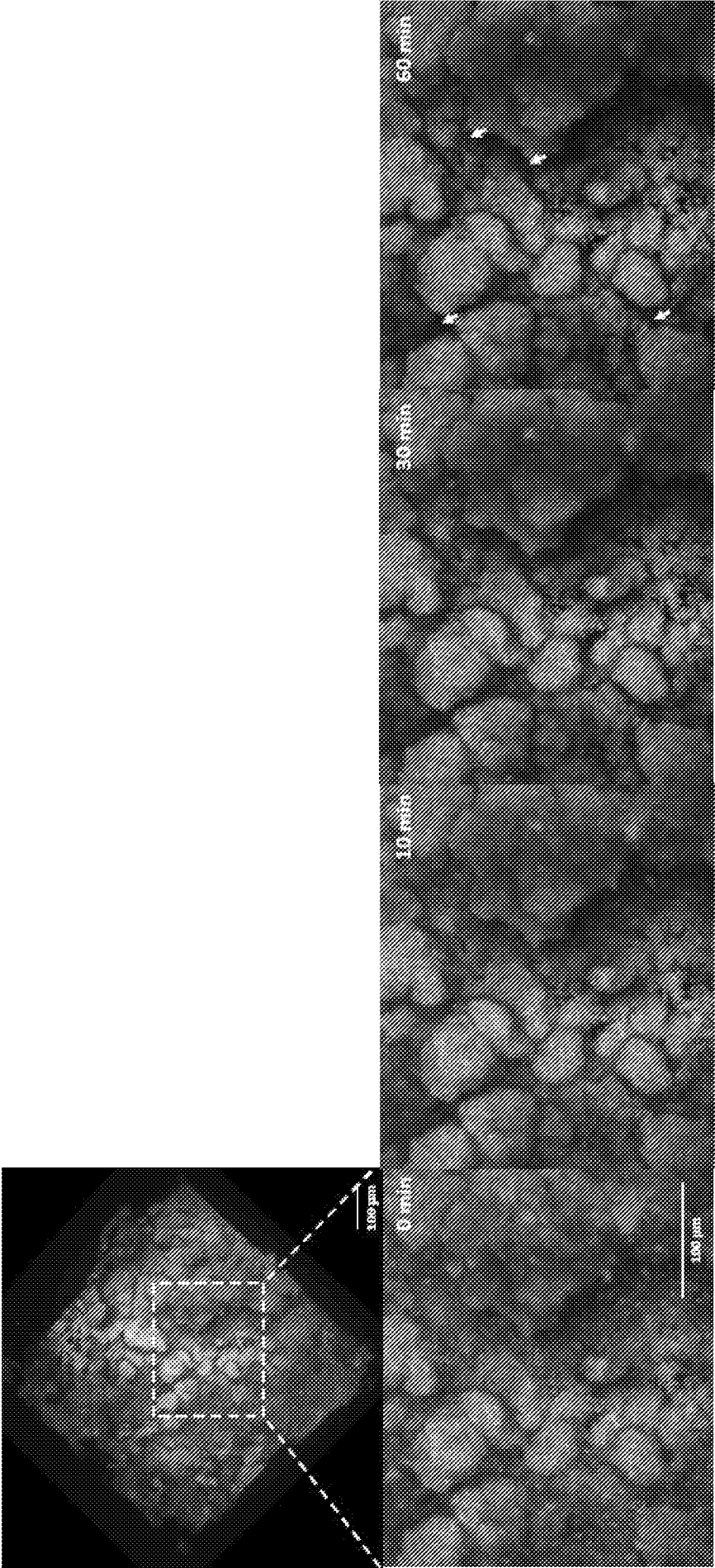


FIGURE 5

FIG. 6C

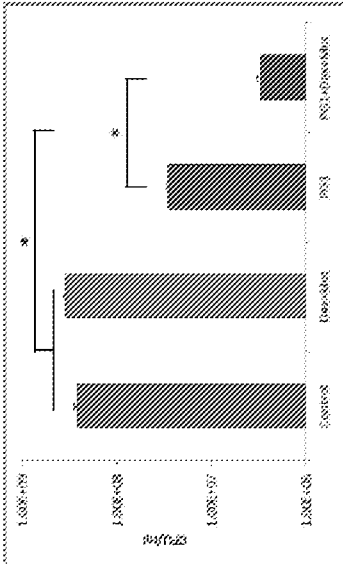


FIG. 6B

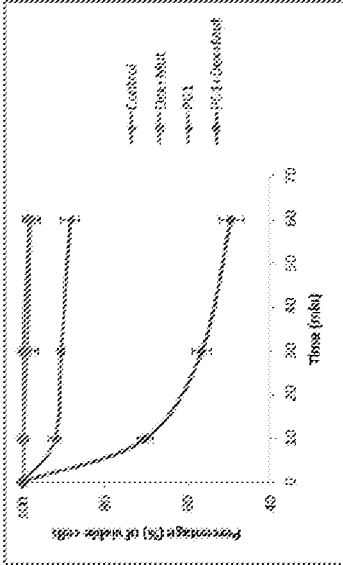


FIG. 6A

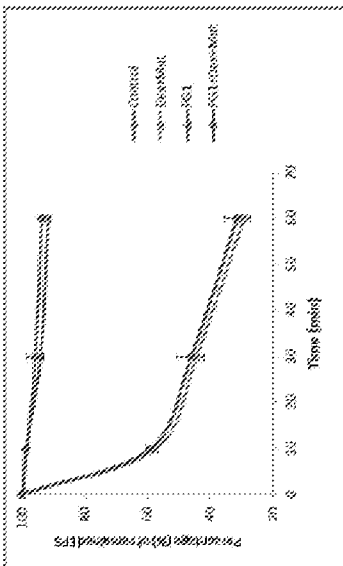


FIGURE 7

Uptake of GFP fused with different tags by human periodontal cell lines

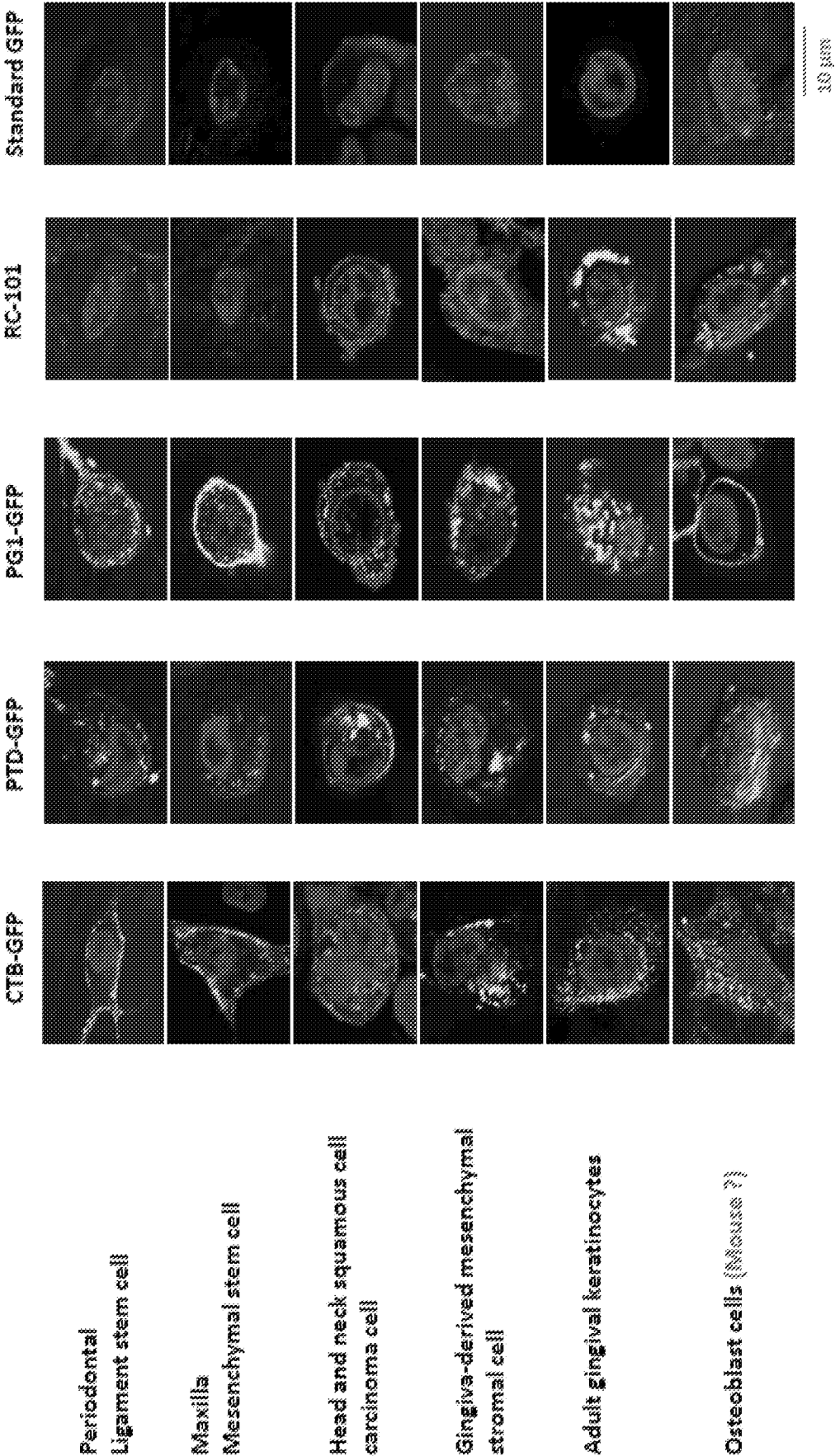


FIGURE 8

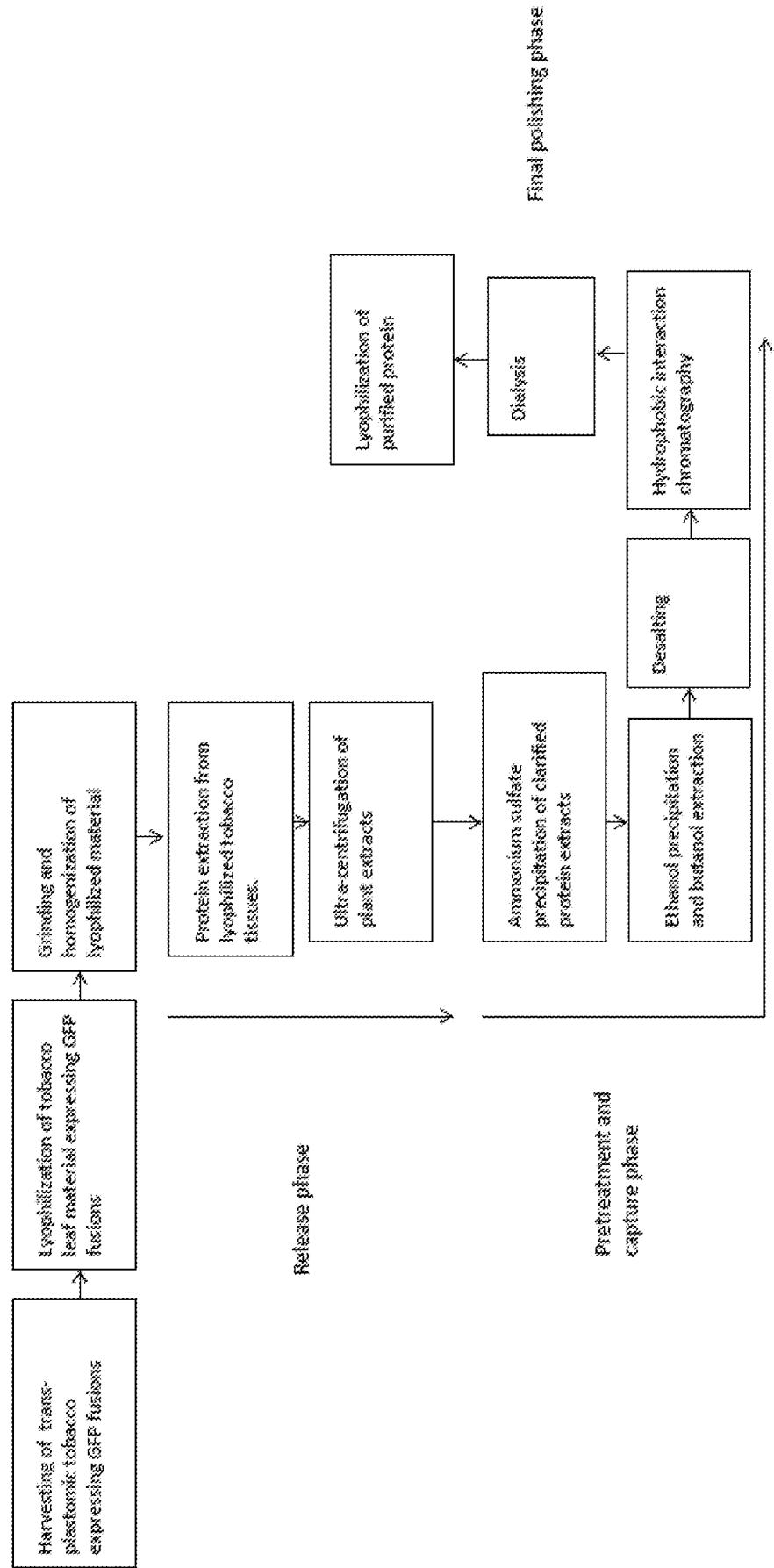


FIG. 9A
Construction of codon-optimized mutanase sequence from *Paenibacillus* sp. Strain RM1 into chloroplast transformation vector

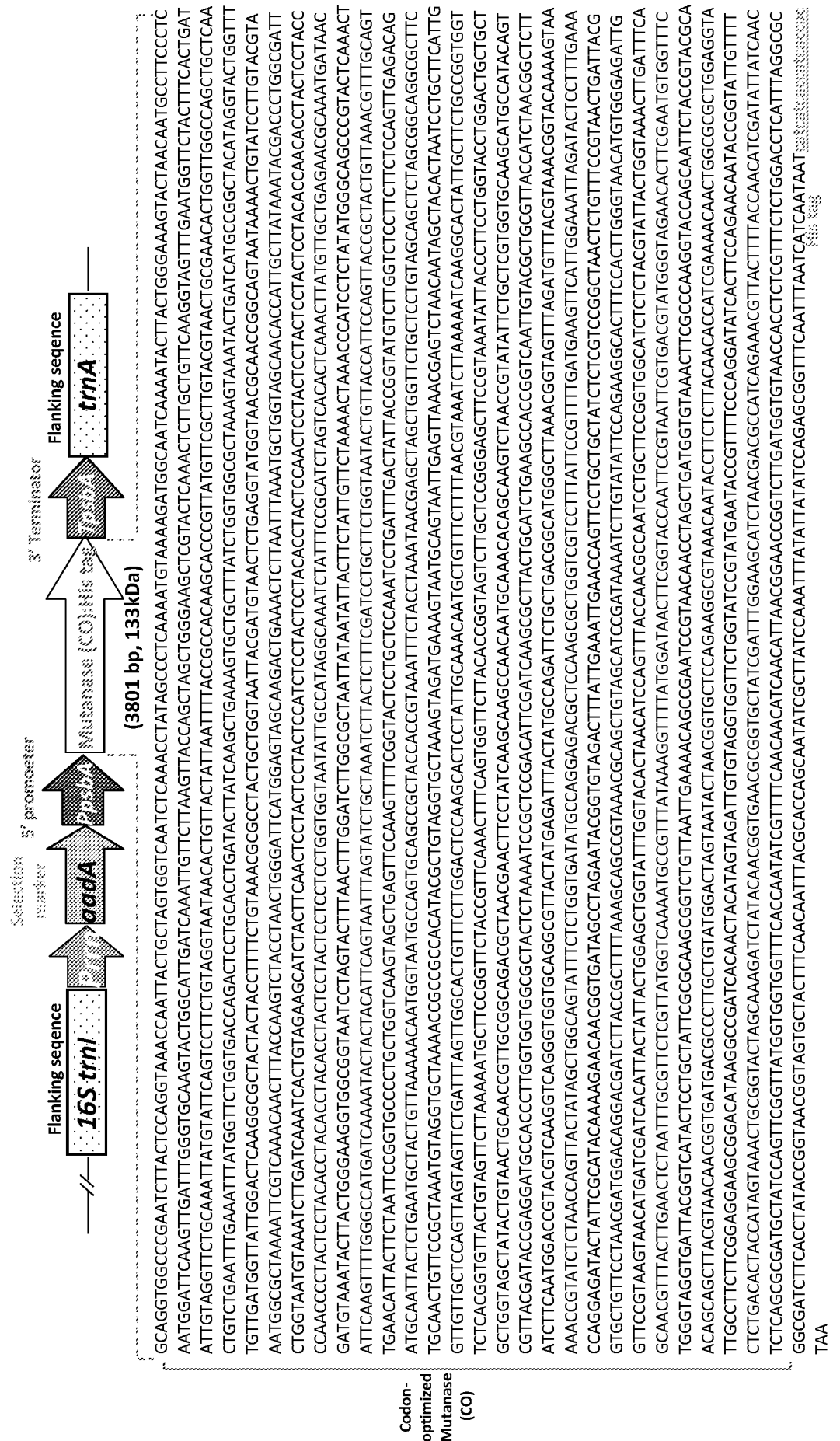
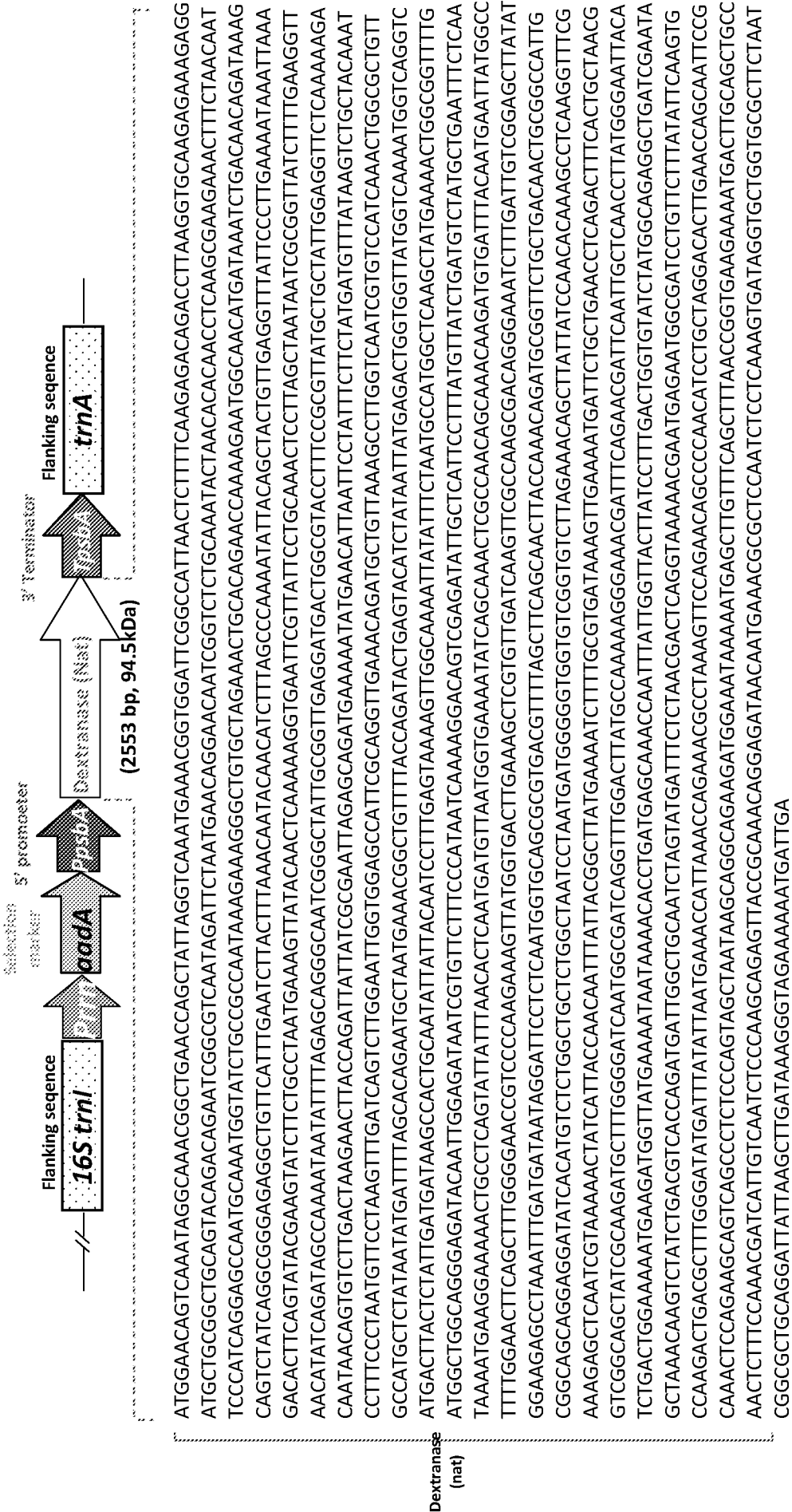


FIG. 9B. Construction of Dextranase sequence from Streptococcus mutans into chloroplast transformation vector



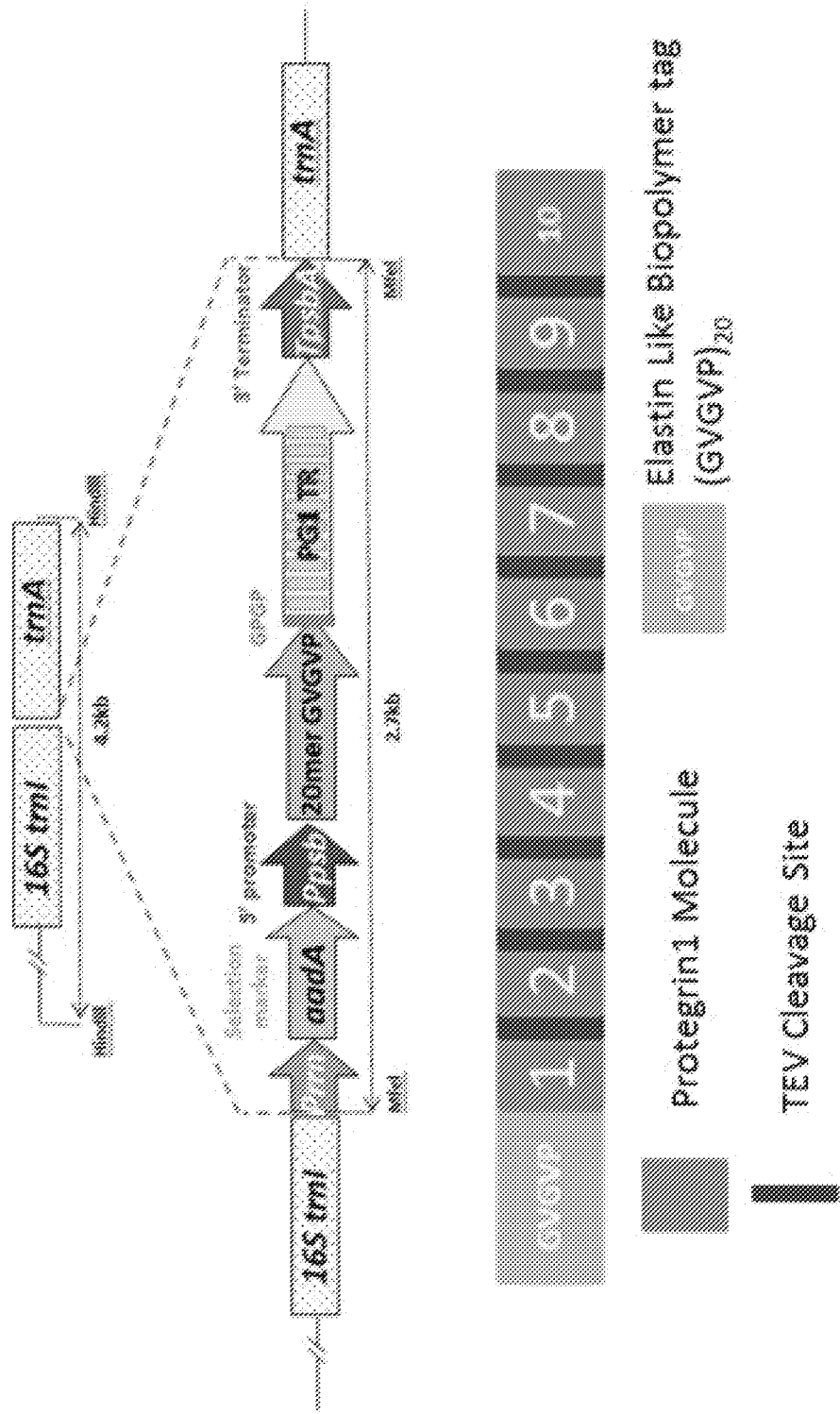


Figure 10

Figure 11

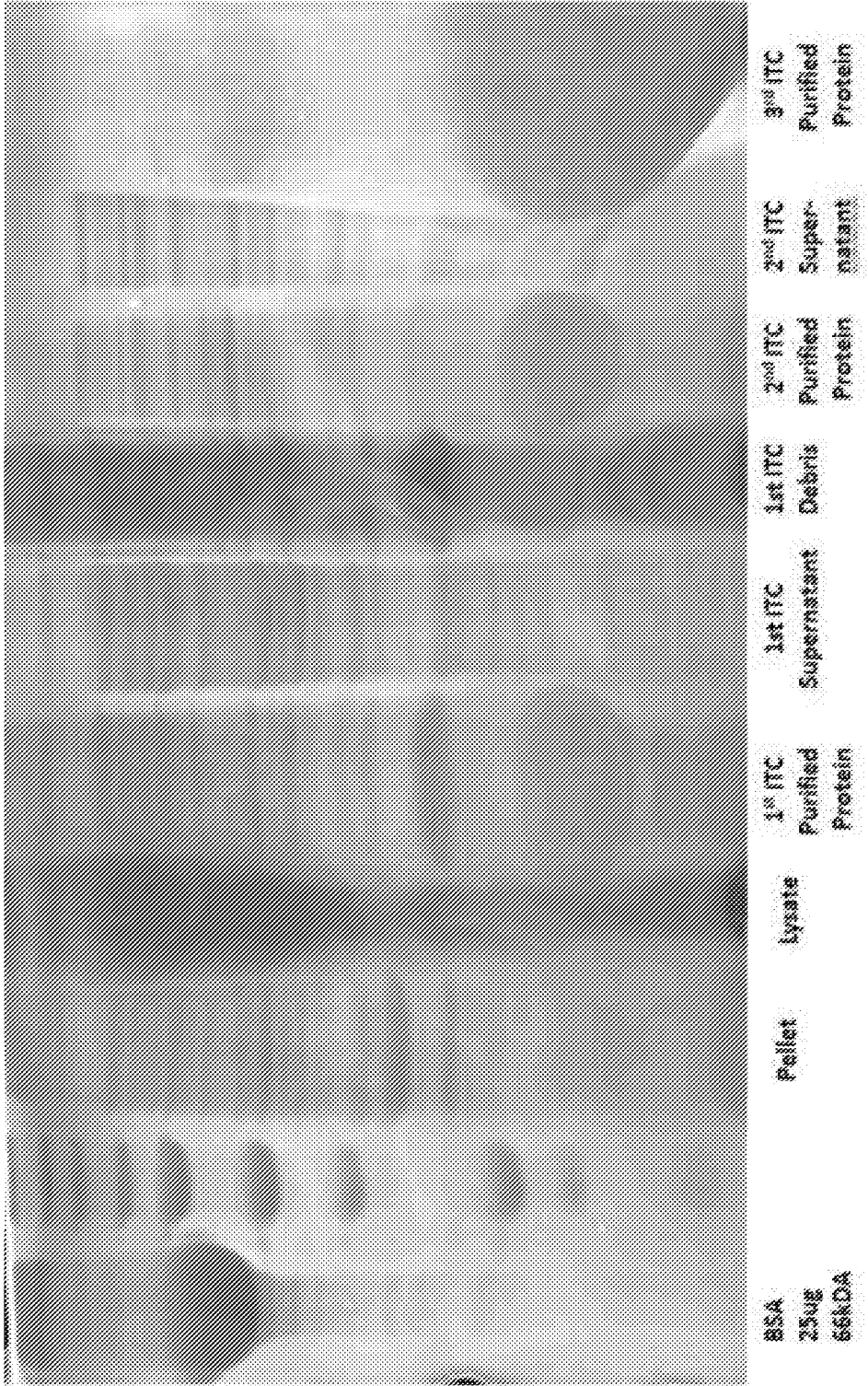
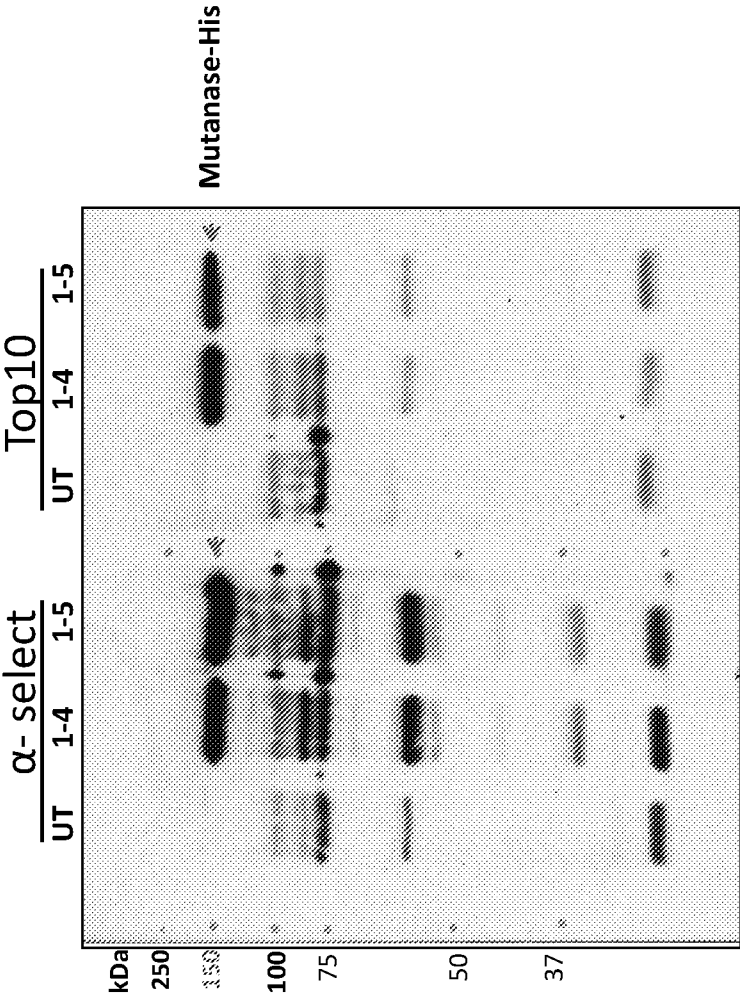


FIG. 12A



- Two different E.coli strains (α -select and Top10) were tested for expression of Mutanase
- Each lane was loaded with 0.3 ug of total E.coli protein
- Immunoprobed with anti-His antibody

Activity test of Dextranase expressed in E.coli
FIG. 12B

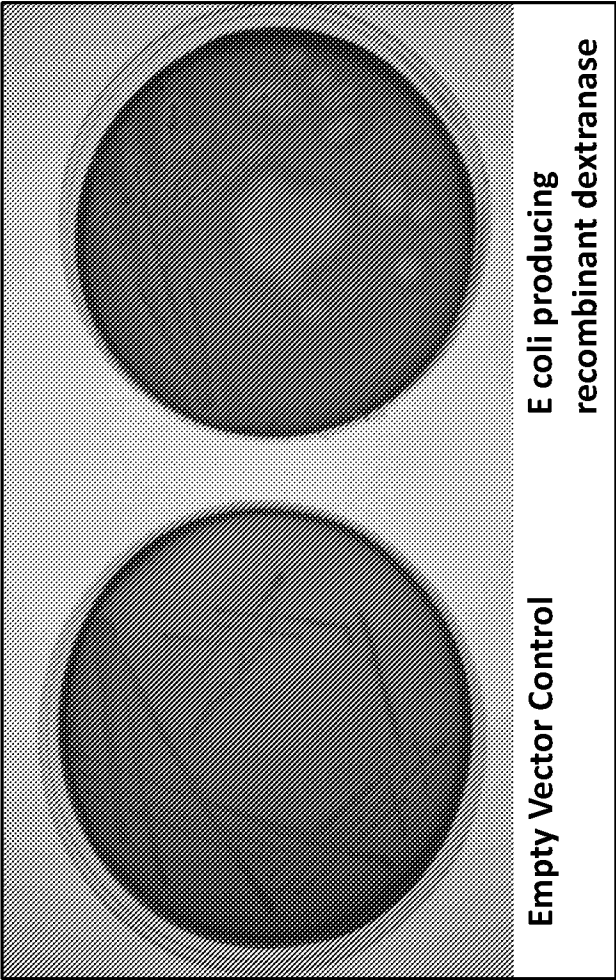


FIG. 12C

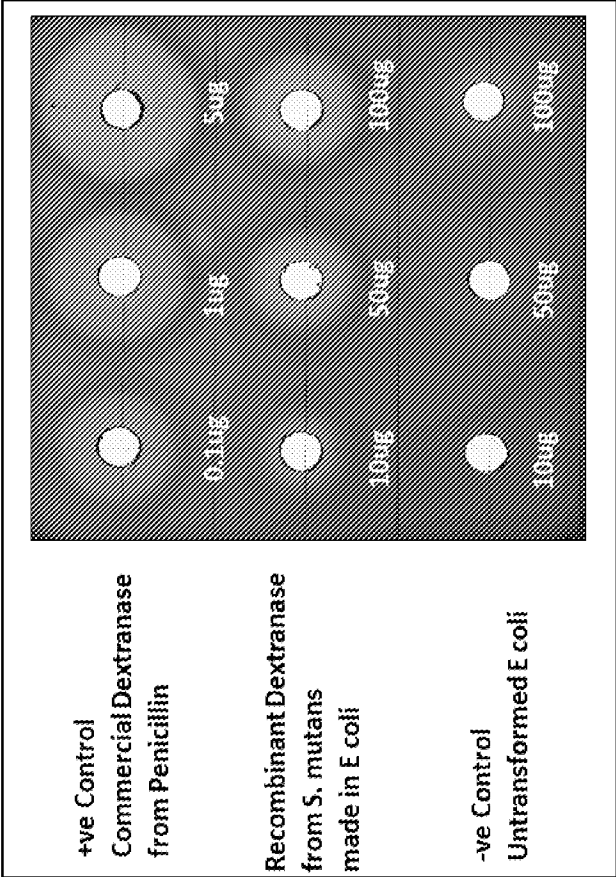


FIGURE 13

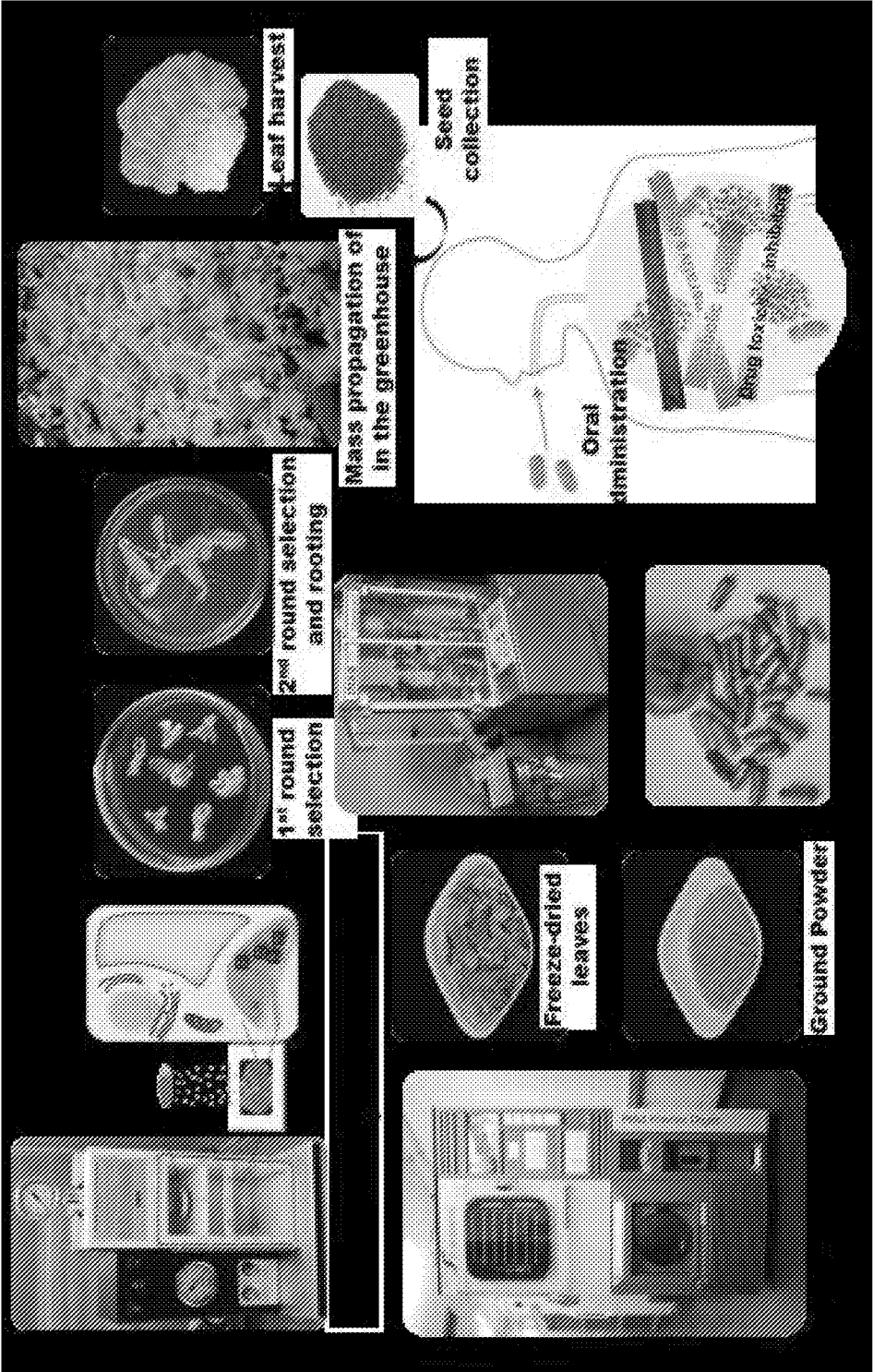
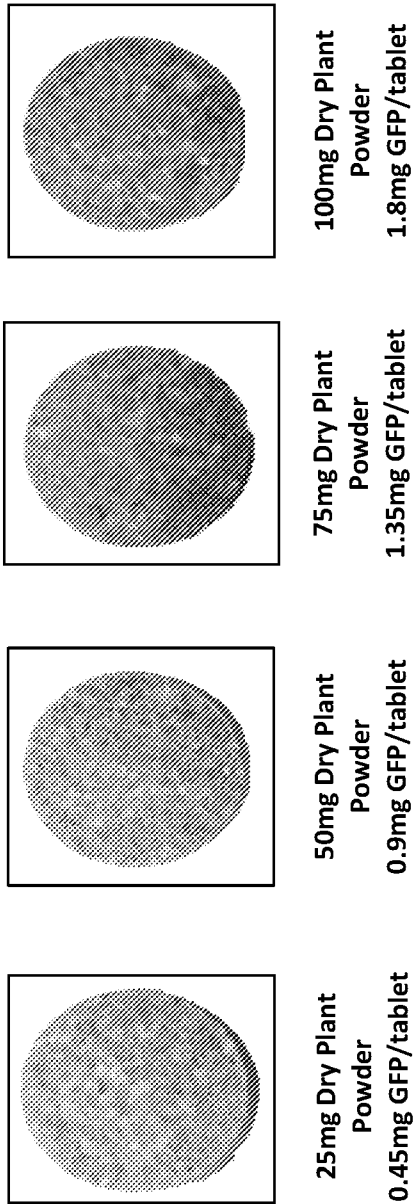


Figure 14

Chewing Gum tablet preparation



Lyophilized powder	25mg	50mg	75mg	100mg
GFP	0.45mg	0.9mg	1.35mg	1.8mg
Total Weight of the tablet	~2g	~2g	~2g	~2g
Weight of Sample taken for analysis and GFP concentration	250mg (56µg)	250mg (112µg)	250mg (168µg)	250mg (225µg)

Figure 15

Evaluation of gum tablet

	GFP Concentration (In 250mg)	
Gum tablet	Expected	Observed
25 mg	39 µg	20µg
50 mg	78.12 µg	41.3µg
75 mg	117.18 µg	82.03µg
100 mg	156.25 µg	130µg

Fluorescence

	GFP Concentration (In 250mg)	
Gum tablet	Expected	Observed
25 mg	56µg	11.6µg
50 mg	112µg	41.3µg
75 mg	168µg	66.8µg
100 mg	225µg	111µg

Western blot

Figure 16

Chewing Simulator –

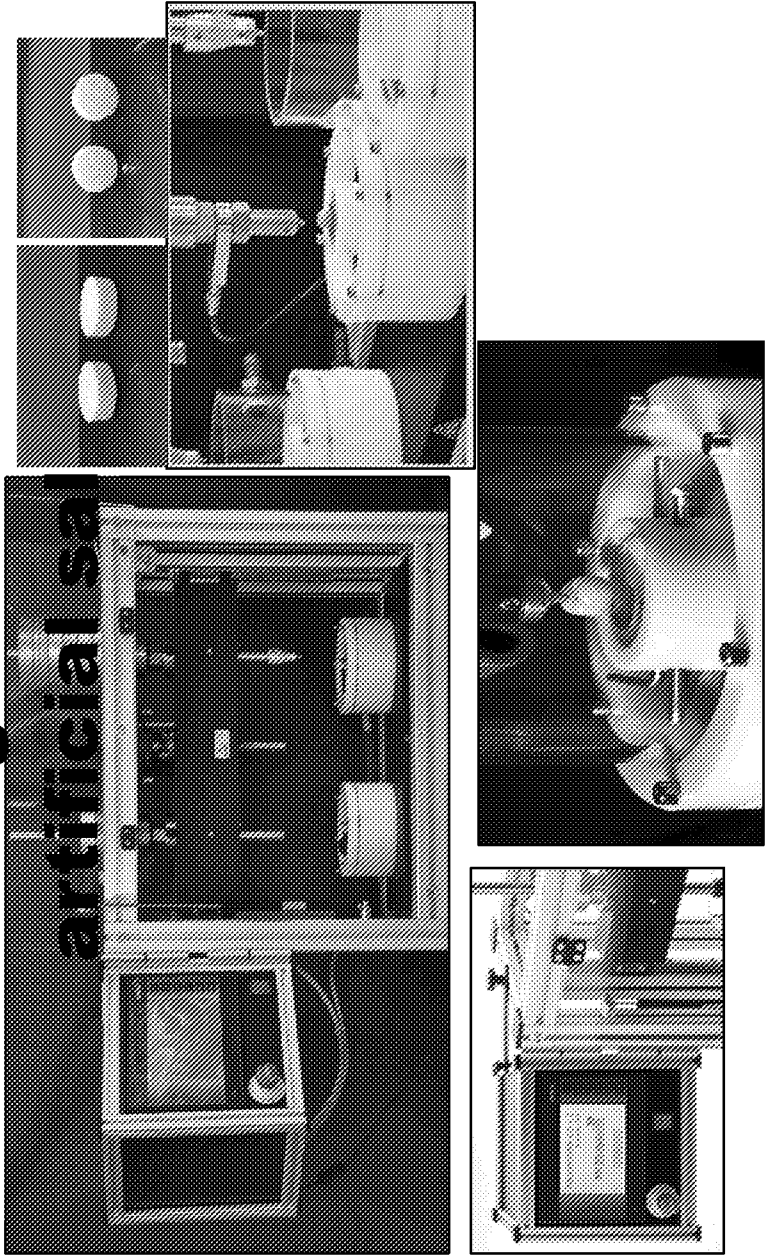


Figure 17

GFP release kinetics from gum tablets



Figure 18

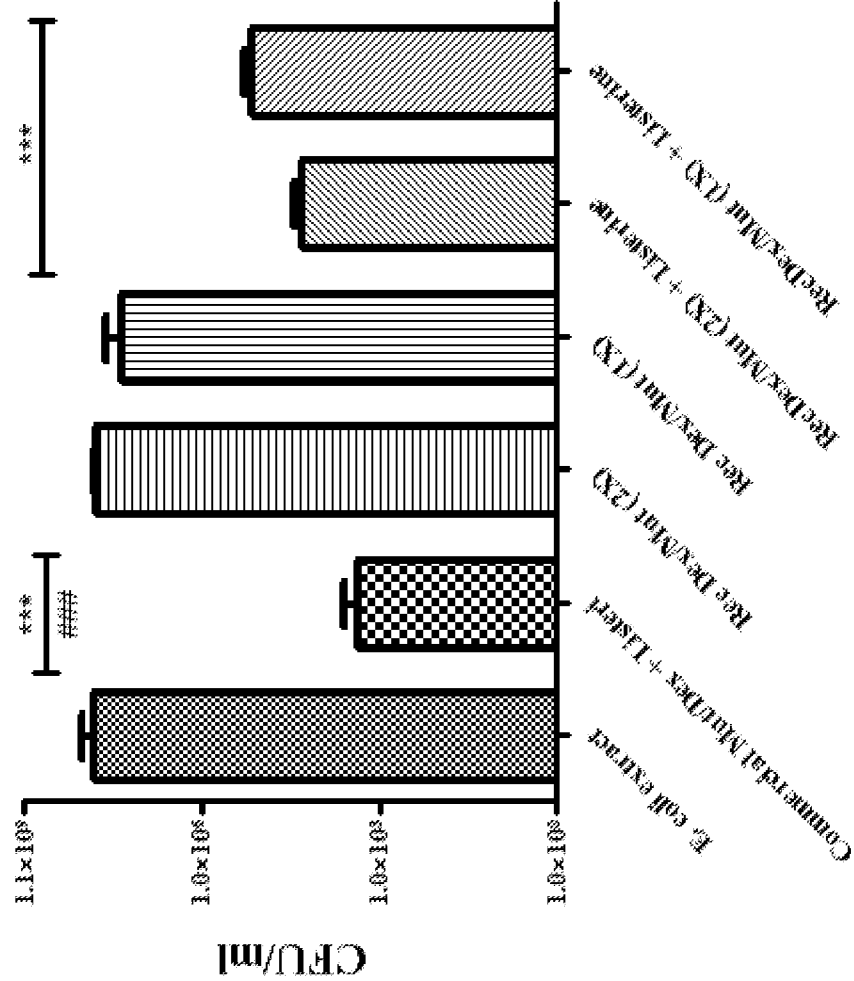


Fig.19A



**Vertically-
positioned**

**Saliva-coated
hydroxyapatite
(sHA)**

Fig. 19B

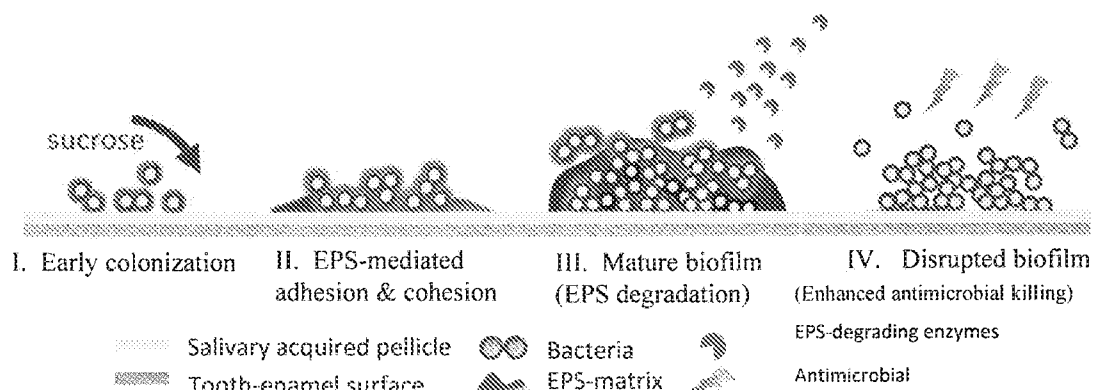


Fig. 19C

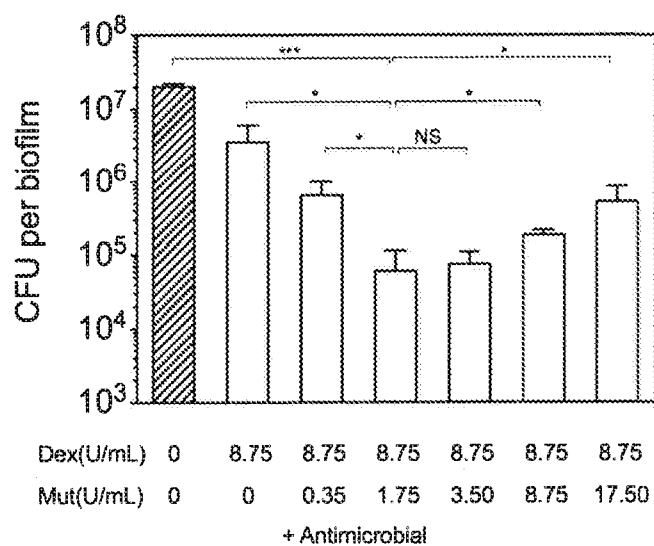
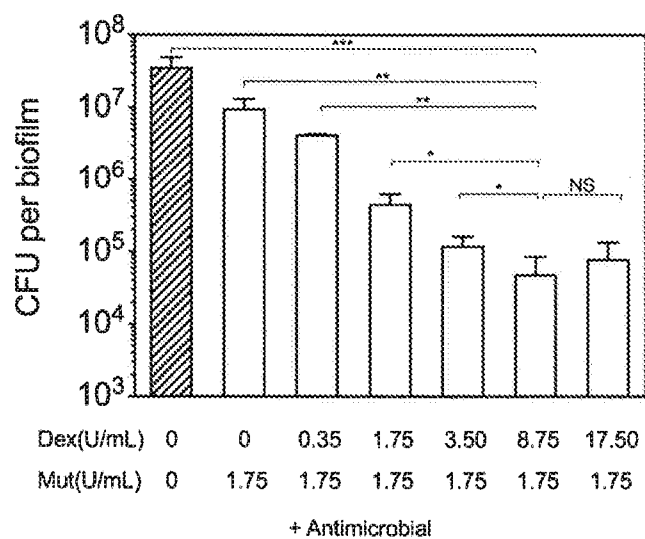


Fig. 19D.

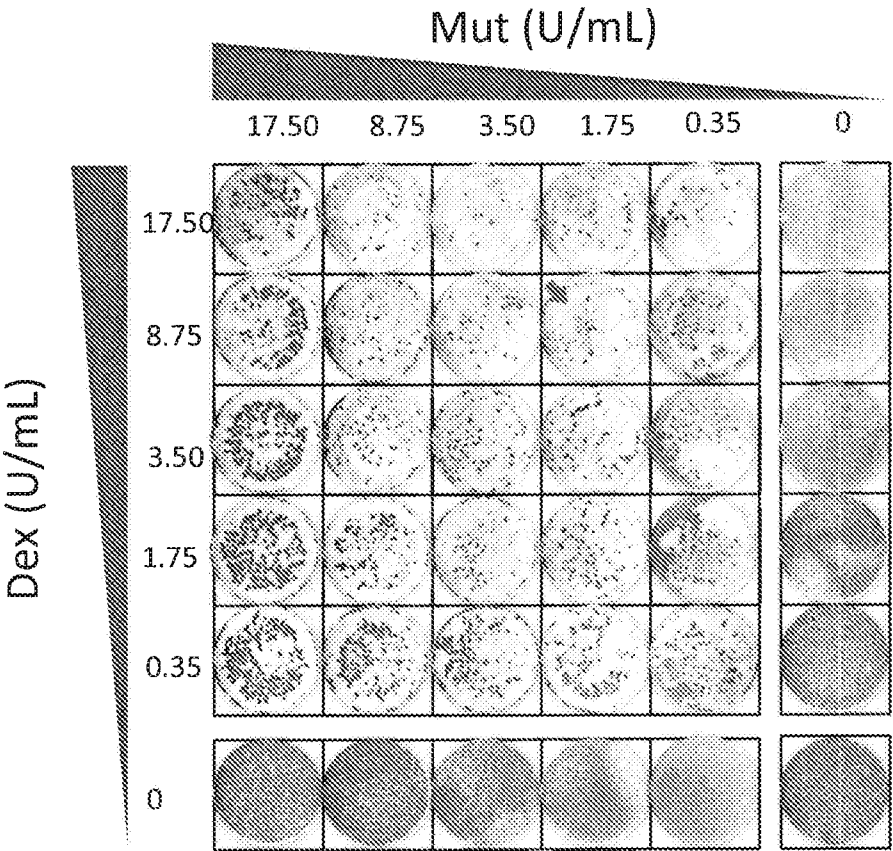


Fig. 19E

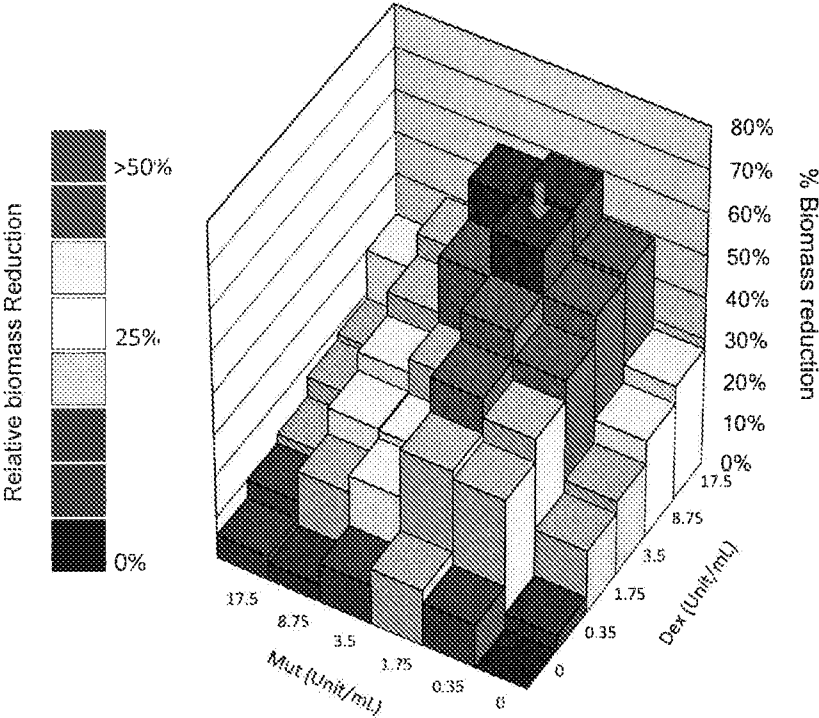


Fig. 20A

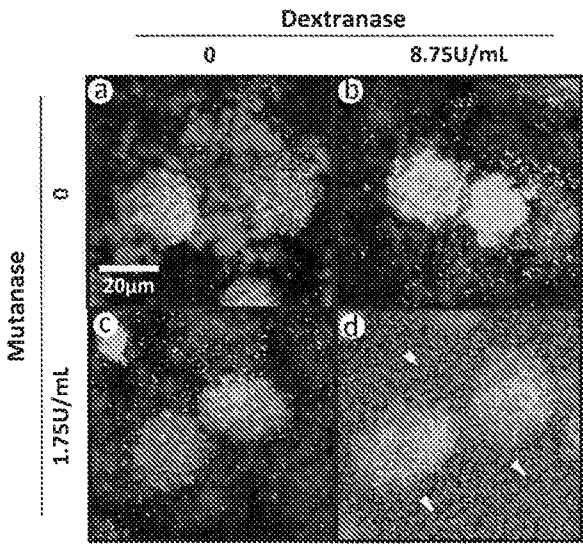


Fig. 20B

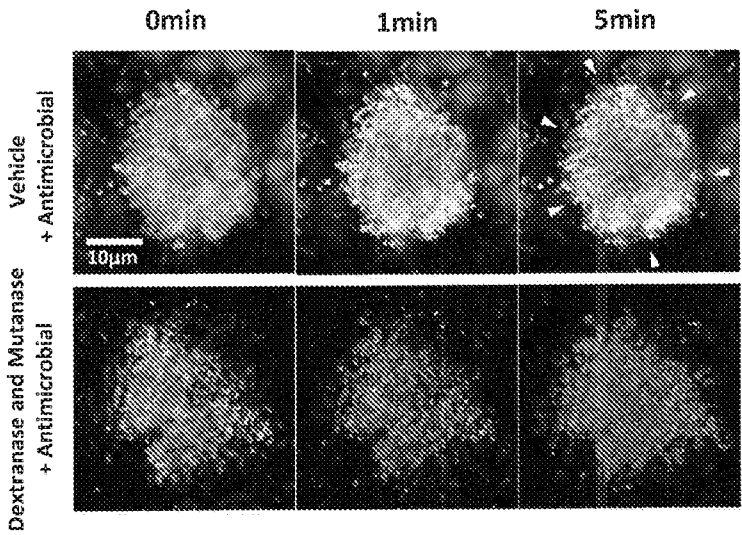


Fig. 20C

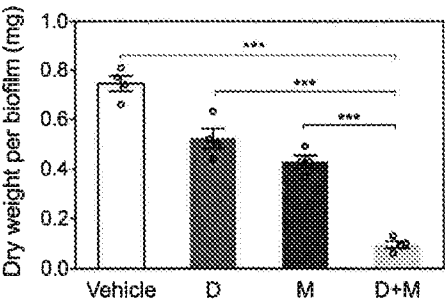


Fig. 20D

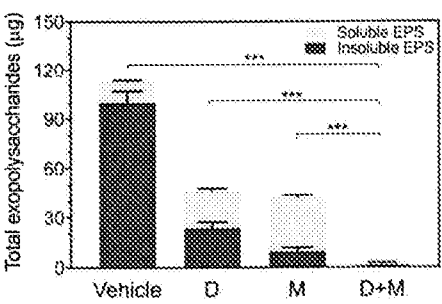


Fig. 20E

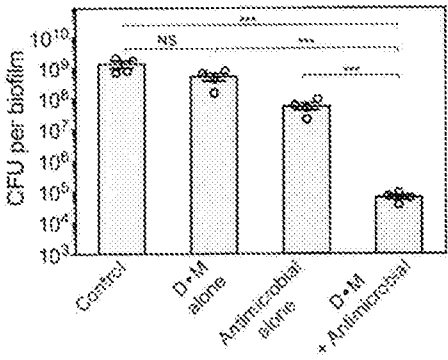
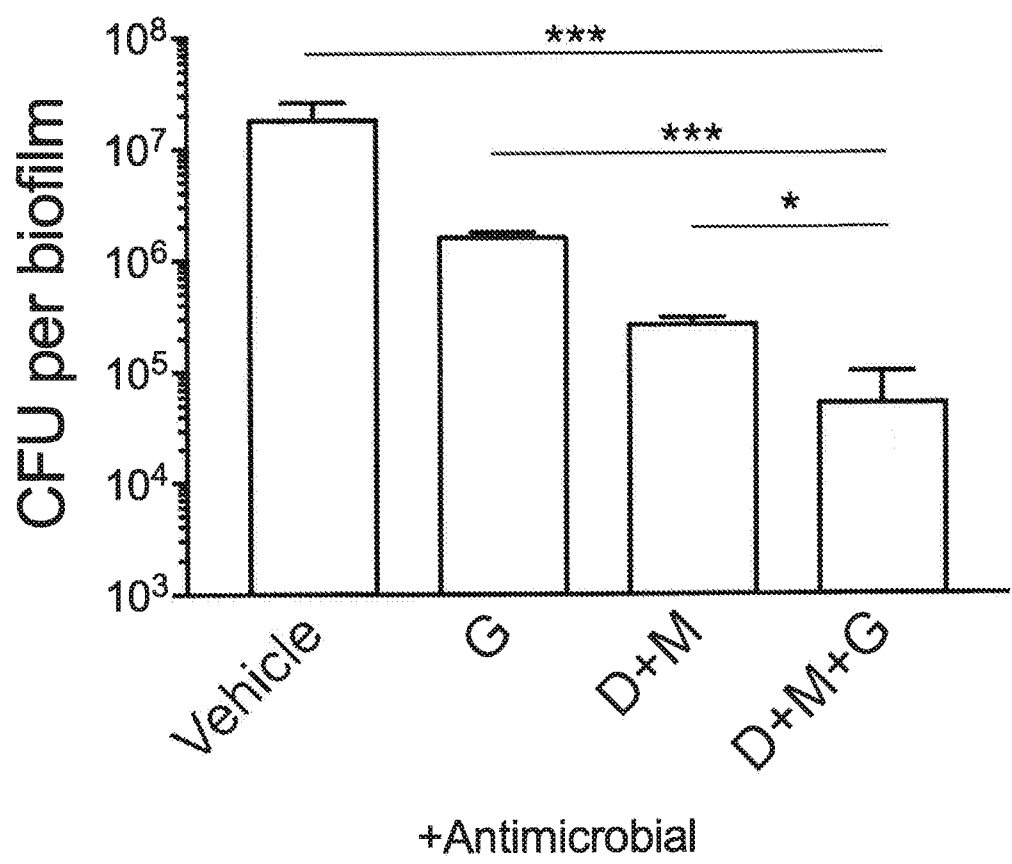


Fig. 20F



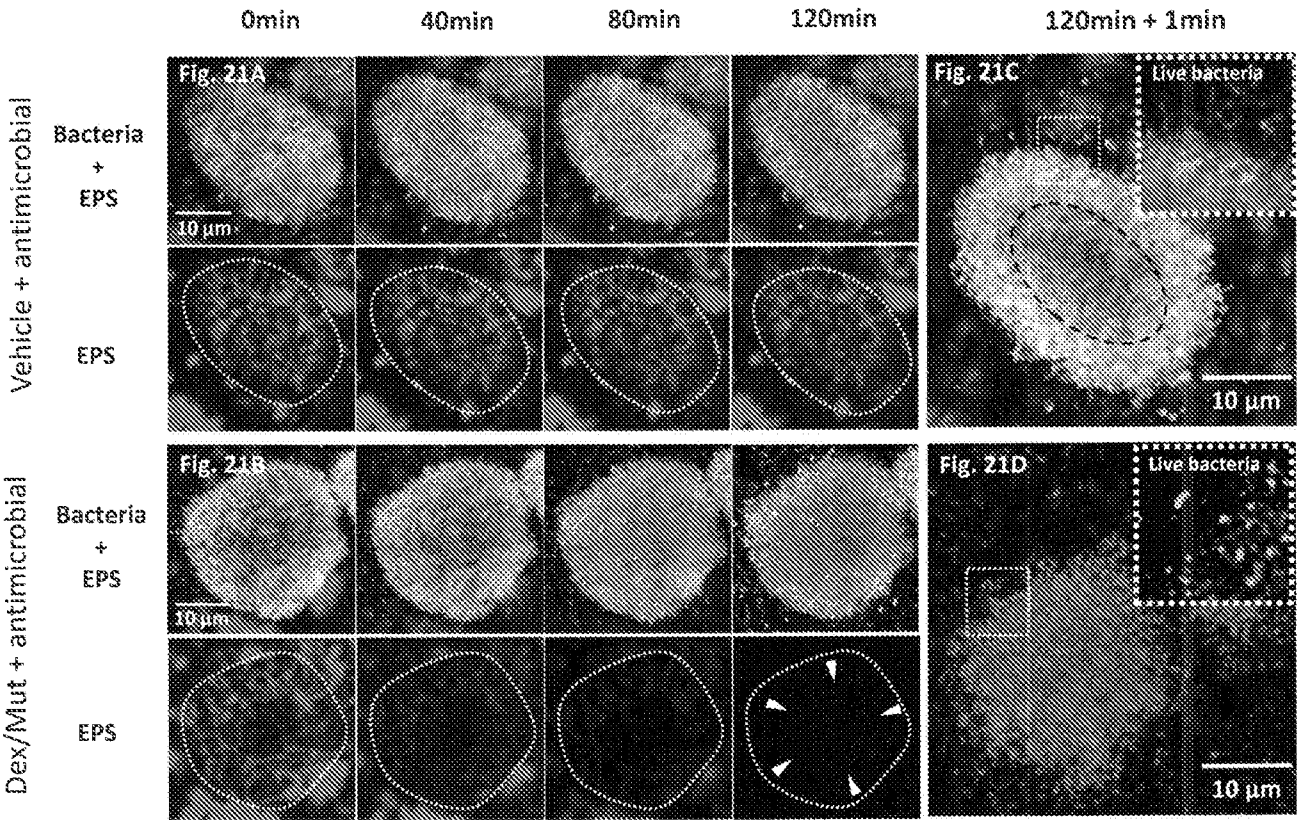
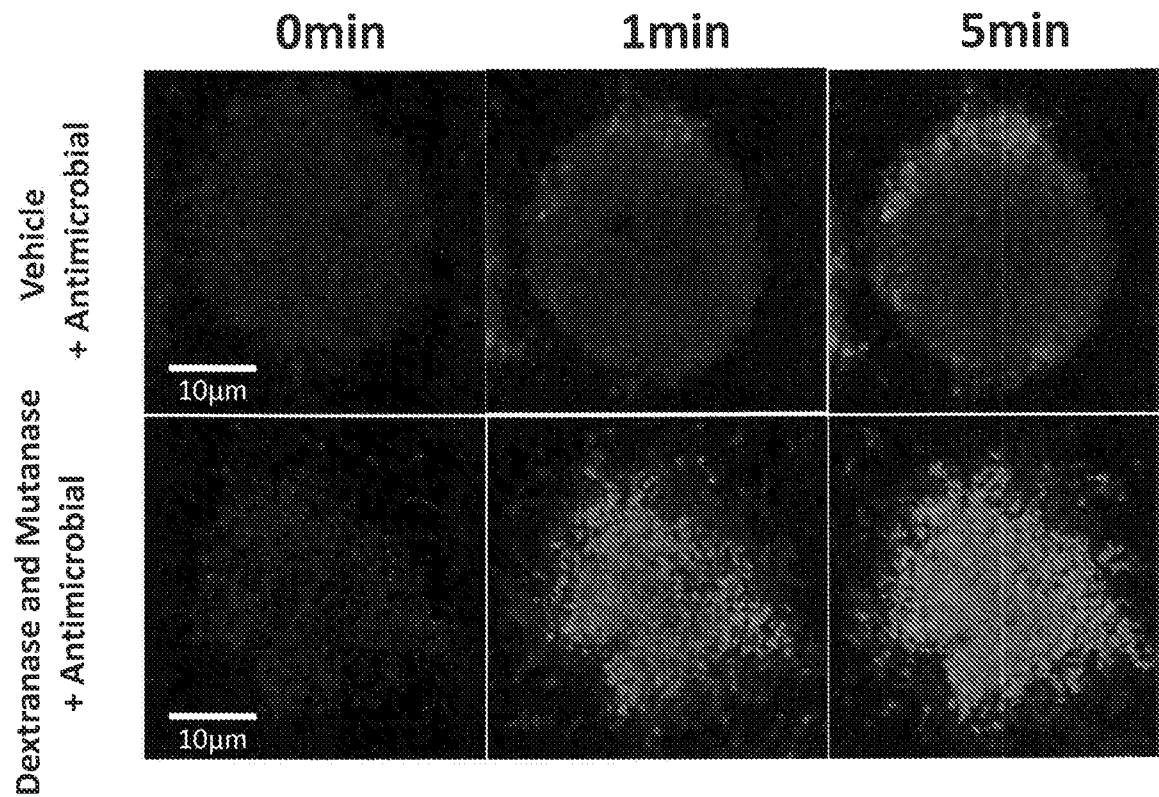


Fig.21E



Magenta: dead cells (stained with PI)

Fig. 22A

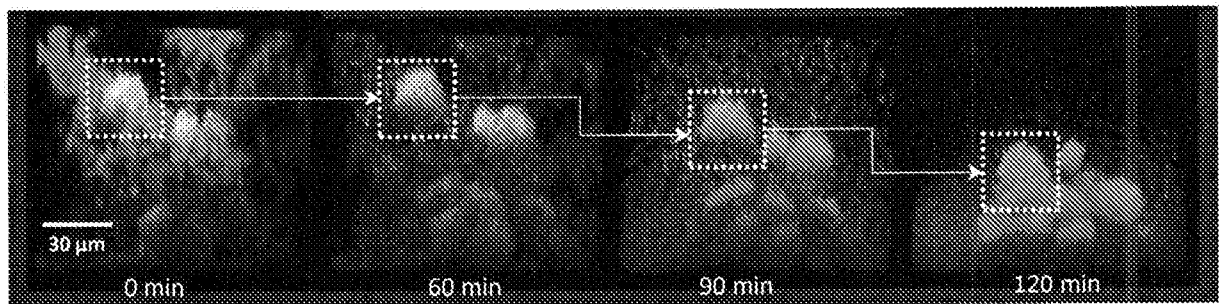


Fig. 22B

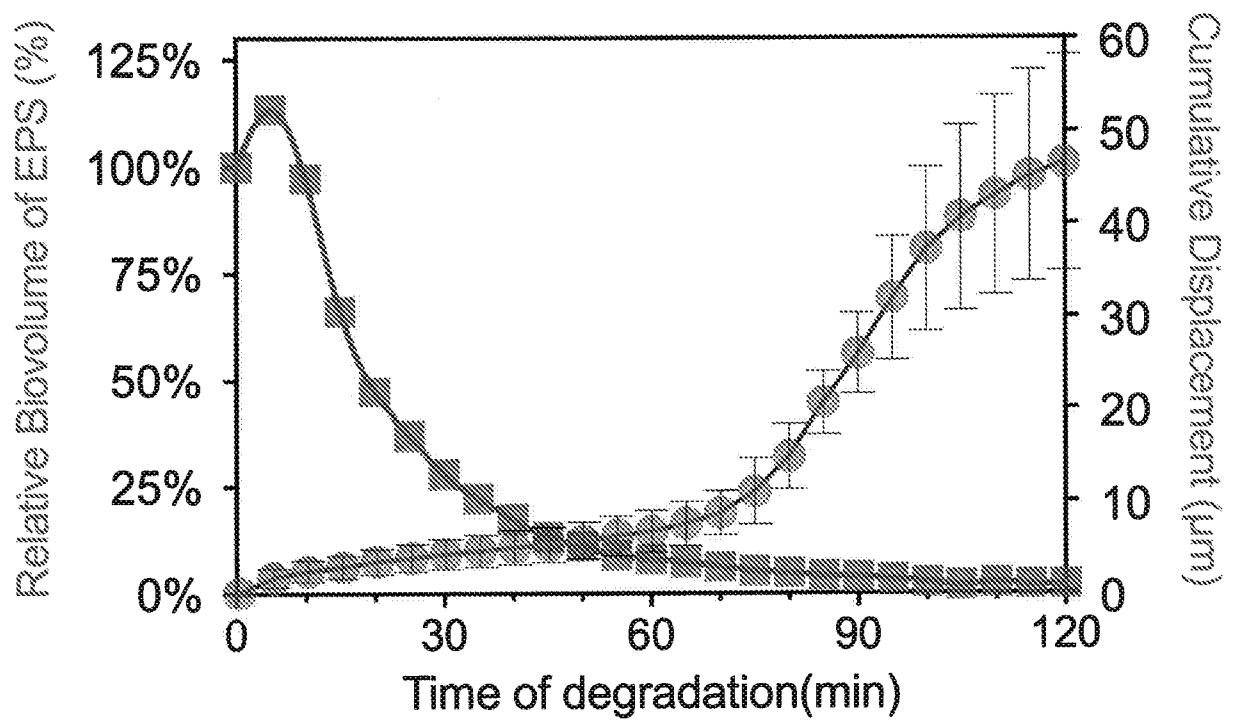


Fig. 22C

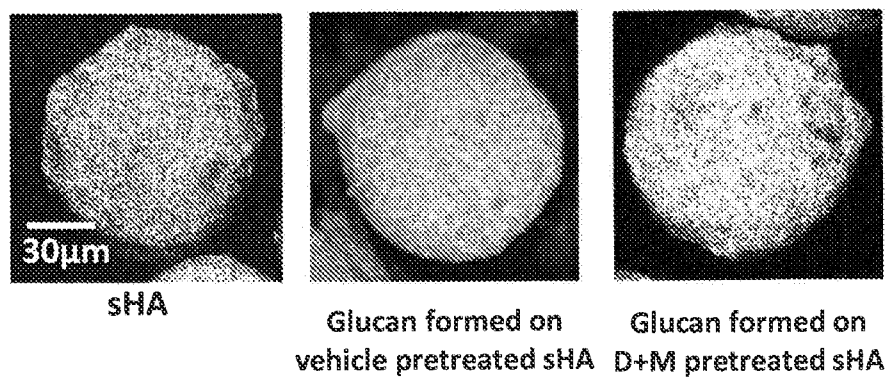


Fig. 23A

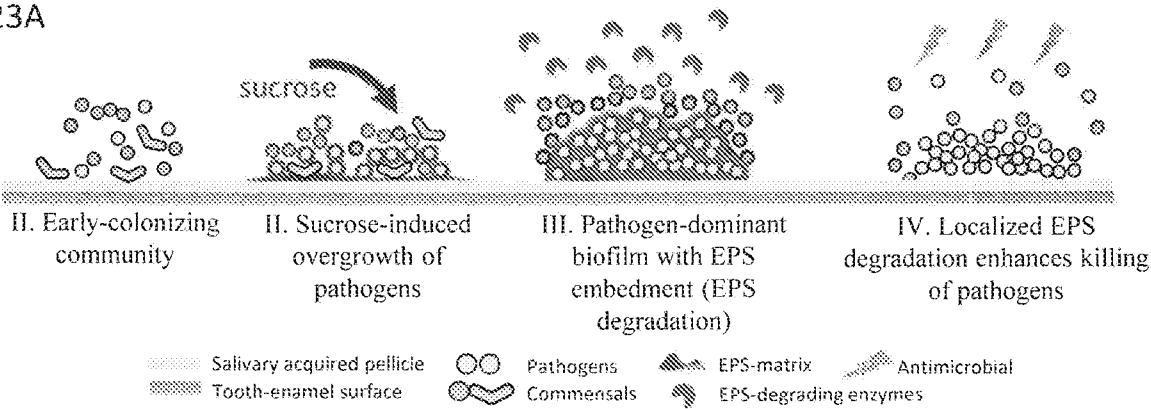


Fig. 23B

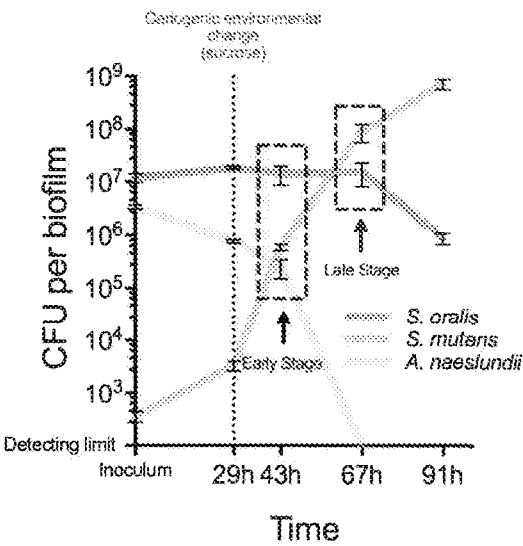


Fig. 23C

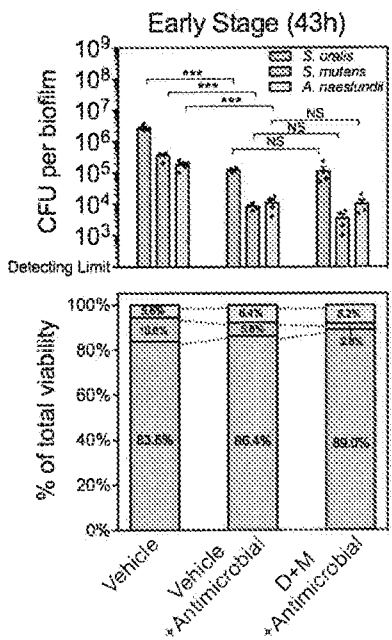


Fig. 23D

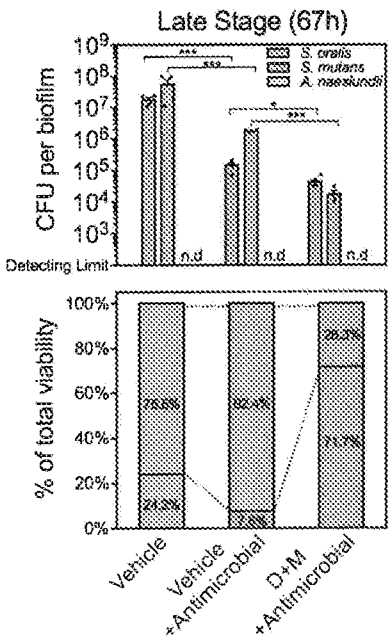


Fig. 23E

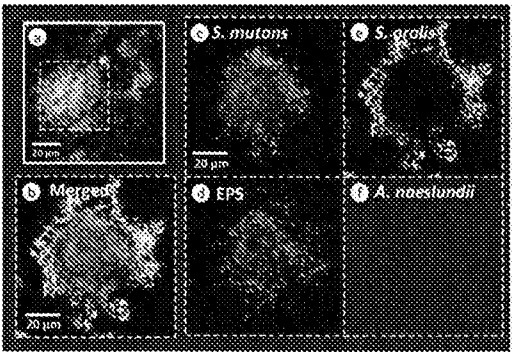


Fig. 23F

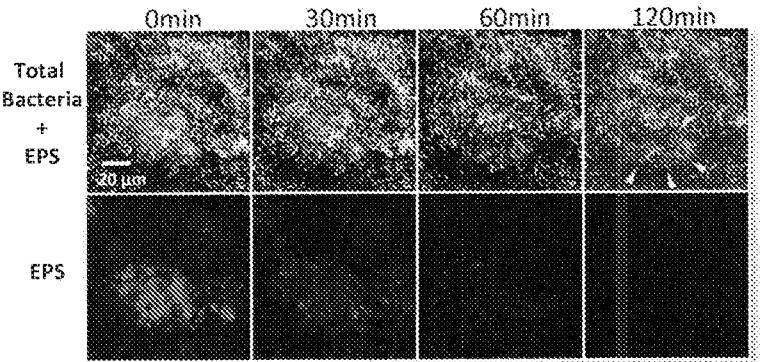


Fig. 24A

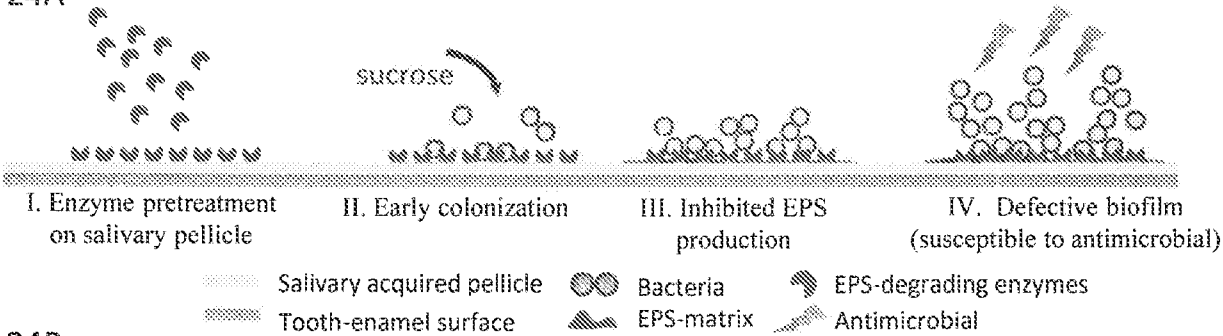


Fig. 24B

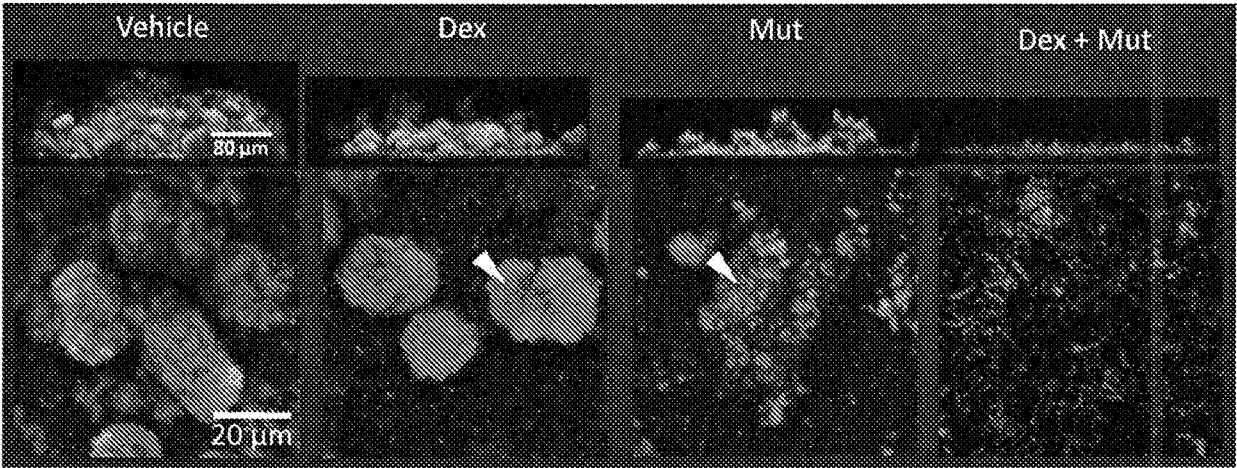


Fig. 24C

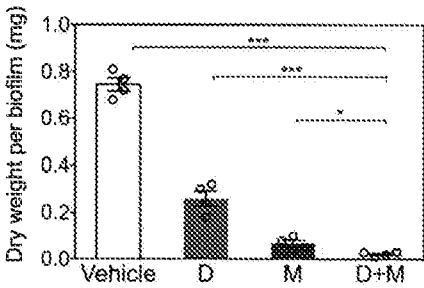


Fig. 24D

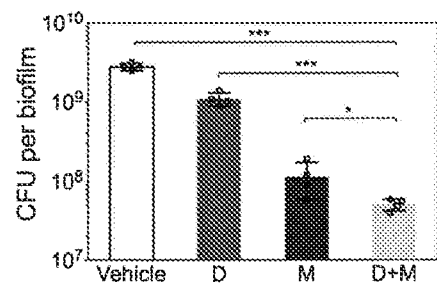


Fig. 24E

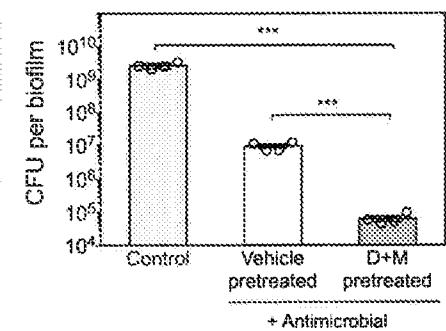


Fig. 24F

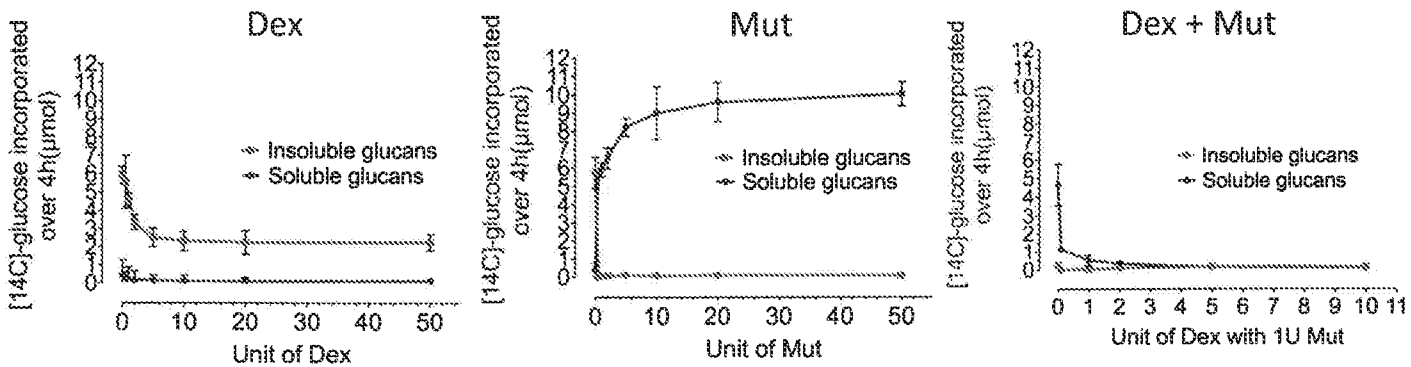


Fig. 25A

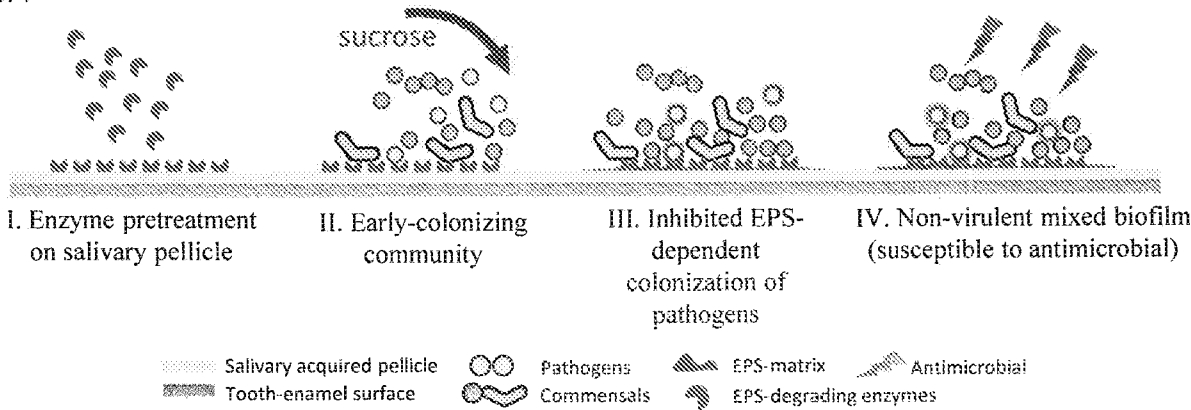


Fig. 25B

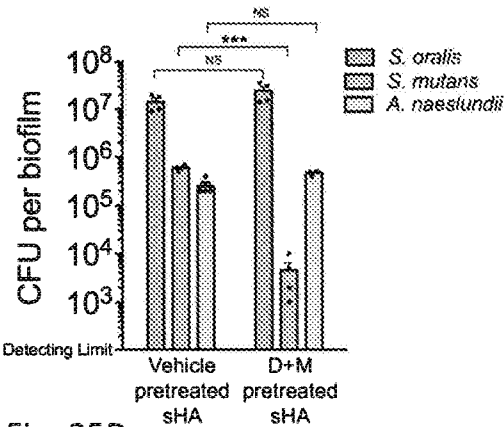


Fig. 25C

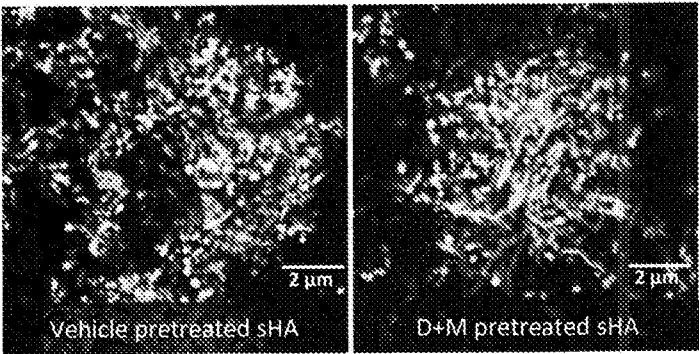


Fig. 25D

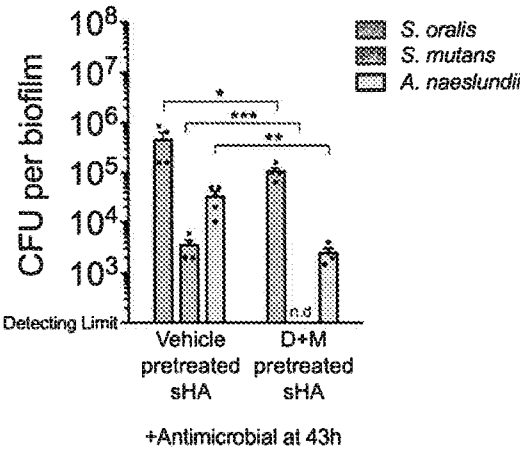
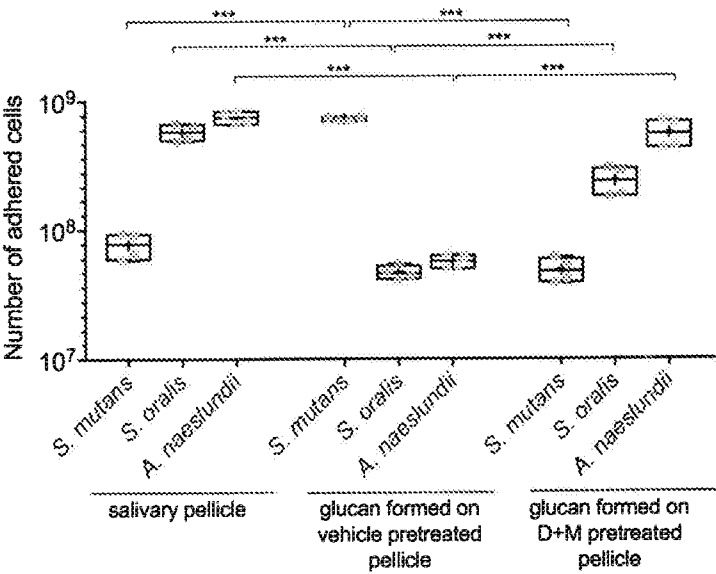


Fig. 25E



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/32549

A. CLASSIFICATION OF SUBJECT MATTER
 IPC(8) - A61K 38/00, A61K 38/04, A61K 38/08 (2018.01)
 CPC - A61K 2300/00, A61K 38/1729

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,741,487 A (Asai et al.) 21 April 1998 (21.04.1998); col1, col2, col5, col8, col14, col17, col19, col23	1-2, 4, 6-10, 24-25
---		3, 5, 11, 22-23
Y	US 2004/0136924 A1 (Boyd et al.) 15 July 2004 (15.07.2004); Title, para[0047], para[0048], para[0071]	3, 5, 11
Y	US 2012/0171128 A1 (Ramirez) 05 July 2012 (05.07.2012); para[0040], para[0048]	22-23
A	US 2012/0315260 A1 (Ivanova et al.) 13 December 2012 (13.12.2012); para[0208]	1, 7
A	US 2012/0128599 A1 (Schaeffer-Korbylo et al.) 24 May 2012 (24.05.2012); entire document	1-11, 22-25

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

16 July 2018

Date of mailing of the international search report

14 SEP 2018

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/32549

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-11, 22-25

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/32549

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-11, 22-25 drawn to a composition comprising an effective amount of one or more biofilm degrading enzymes selected from mutanase, dextranase and glucoamylase and one or more essential oils selected from the group consisting of menthol, thymol, eucalyptol, methyl salicylate, and combinations of two or more thereof in a carrier suitable for oral delivery, wherein said carrier is selected from the group consisting of mouthwash, mouth rinse, mouth spray, toothpaste, chewing gum, tooth gel, sub-gingival gel, mousse, foam, chewable tablet, dentifrice, lozenge, and dissolvable strip

Group II: Claims 12-21 drawn to a method of degrading unwanted biofilm or inhibiting biofilm deposition on the surface of the oral cavity, comprising administering an effective amount of one or more biofilm degrading enzymes selected from the group consisting of mutanase, dextranase, glucoamylase, and combinations of two or more thereof, and one or more essential oils selected from the group consisting of menthol, thymol, eucalyptol, methyl salicylate, and combinations of two or more thereof, to a surface of the oral cavity having said biofilm, said enzymes degrading said film and said essential oil(s) exerting a bactericidal effect

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group II requires a method of degrading unwanted biofilm or inhibiting biofilm deposition on the surface of the oral cavity, not required by Group I

Shared Common Features

Groups I-II share technical feature of a composition comprising an effective amount of one or more biofilm degrading enzymes selected from mutanase, dextranase and glucoamylase and one or more essential oils selected from the group consisting of menthol, thymol, eucalyptol, methyl salicylate, and combinations of two or more thereof.

However, these shared technical feature does not represent a contribution over prior art, because the shared technical feature as being anticipated by US 5,741,487 A to Asai et al. (hereinafter 'Asai'). Asai discloses a composition comprising an effective amount of two biofilm degrading enzymes mutanase and dextranase, and one essential oil of menthol (col19, Table 1: "1-menthol 0.7%", "Dextranase 3000 U/g", "RM1 enzyme 300 U/g"; col17, ln65-67 "An animal test was done for examining the caries preventive effect of the three mutanases: RM1, RM4 and M7 enzymes", col1, ln19-22 "It is known in the art that dextranase capable of cleaving α -1,6 links and mutanase capable of cleaving α -1,3 links are effective for suppressing dental plaque formation; plaque is biofilm, see US 2012/0315260 A1 to Ivanova et al. (hereinafter 'Ivanova'), para[0208] "Since the bacterial biofilm is the essence of the dental plaque").

As the technical features were known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups. Groups I-II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note: claim 13 is improperly depending from claim 11. For the purposes of completing this ISR, claim 13 is assumed to depend from claim 12.