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(54) Title: ANTI-BIOFILM SCREENING ASSAYS

(57) Abstract: Procedures are provided to evaluate the effectiveness of slow-acting antibacterial agents such as metal salts on reduction of bacterial oral biofilm. In the modified process, the exposure time of the agent is increased from the usual 3 minutes to 7-15 minutes, e.g., about 10 minutes, and the duration increased to 5-12 days, e.g., about 8 days instead of the conventional 3 days.

ANTI-BIOFILM SCREENING ASSAYS

BACKGROUND

[0001] Conventional methods of screening compounds for potential efficacy of oral care agents against bacteria typically assess the efficacy of test compounds and compositions in a suspension culture of defined species of bacteria, measuring bacterial proliferation as a function of optical density (OD) in the presence and absence of the test agent, over a short period of time, following a brief exposure to the test agent.

[0002] Such assays are very effective to identify potent and fast-acting antibacterial agents, but are poorly suited to identifying optimal antibacterial agents when the agents would have repeated application over longer periods of time (e.g., by daily or twice daily brushing or rinsing).

[0003] Moreover, activity against defined microorganisms in suspension culture may not adequately measure activity against the natural microflora, comprising a diversity of species in a biofilm. Bacteria in a biofilm are developmentally and phenotypically different from genetically identical bacteria in a free-floating suspension, and may react differently to antibacterial agents.

[0004] Therefore, there is a need for improved assays for identifying and evaluating antibacterial agents effective against oral biofilms.

SUMMARY

[0005] Procedures have been developed to evaluate the effectiveness of slow-acting antibacterial agents such as metal salts on reduction of bacterial oral biofilm. In the modified process, the exposure time of the agent is increased from the usual 3 minutes to 7-15 minutes, e.g., about 10 minutes, and the duration increased to 5-12 days, e.g., about 8 days instead of conventional 3 days. This process allows testing of comparatively slow acting antibacterial agents.

[0006] Further areas of applicability of the present invention will become apparent from the detailed description provided hereinafter. It should be understood that the detailed description and specific examples, while indicating the preferred embodiment of the invention, are intended for purposes of illustration only and are not intended to limit the scope of the invention.

DESCRIPTION OF THE INVENTION

[0007] The following description of the preferred embodiment(s) is merely exemplary in nature and is in no way intended to limit the invention, its application, or uses.

[0008] The invention provides, in one embodiment, a method (Method 1) of measuring the efficacy of a test composition (for example a toothpaste or mouthwash) against oral bacterial biofilm growth, comprising

a) Coating a dental sample, selected from a tooth section and a hydroxyapatite disc, with saliva and placing the sample in a flow of a buffered medium, wherein temperature, flow and pH adjusted to approximate conditions in the mouth and wherein the buffered medium comprises nutrients similar to those in the mouth;

b) Exposing the dental sample to a test composition, then reinstating the flow of buffered medium, wherein the period of exposure to a test compound is from 7-15 minutes, e.g. 8-12 minutes, e.g., about 10 minutes;

c) Repeating the preceding step from one to four times per day for 5-12 days, e.g., for 6-10 days, e.g., for about 8 days;

d) Vortexing the dental sample in anaerobic media to obtain a suspension and measuring the optical density thereof, wherein a lower optical density indicates that the test composition is more effective against oral biofilm growth.

For example, the invention provides

1.1. Method 1 wherein the period of exposure the dental sample to a test composition in step b) is for a period of about 10 minutes.

1.2. Method 1 or 1.1 wherein the step of repeating step b) is carried out from one to four times per day for about 8 days.

1.3. Any preceding method wherein the buffered medium comprises nutrients as required to support bacterial growth, e.g., comprising phosphate buffer, sugar, amino acids, and essential vitamins and minerals.

1.4. Any preceding method further comprising the step of culturing the suspension on a solid media and counting the number of colony forming units after a period of 2-5 days,

wherein a lower number of colony forming units indicates that the test composition is more effective against oral biofilm growth.

1.5. Method 1.4 wherein the culture on solid media is carried out in the presence of a metal salt, e.g., an iron or lead salt, which reacts with a volatile sulfur compound formed in step a-c, e.g., hydrogen sulfide, to form a black precipitate, e.g., a metal sulfide.

1.6. Method 1.5 wherein the solid media is an agar plate and the metal salt is lead acetate.

1.7. Method 1.4 or 1.5 wherein any black colony forming units are counted independently from the other colony forming units and wherein a lower number of black colony forming units indicates that the test composition is more effective against oral biofilm growth comprising bacteria that produce volatile sulfur compounds.

1.8. Any preceding method wherein the test composition is an oral care product.

1.9. Any preceding method wherein the test composition comprises at least 0.1% of a metal ion having a valence of +2 or +3, e.g. Zn^{2+} or $Sn(II)^{2+}$.

1.10. Method 1.9 comprising measuring the uptake of the metal ion from the test composition by the dental sample, wherein greater uptake of the metal ion by the dental sample corresponds to the greater efficacy of the oral care product against oral biofilm formation.

[0009] Buffered medium as used in step a) above may be any suitable medium as known in the art for allowing biofilm growth. See, for example, the various media described in Lemos, *et al.*, Methods Mol Biol. 2010; 666: 87–102, the contents of which are incorporated herein by reference. A typical medium thus comprises phosphate buffer, sugar, amino acids, and essential vitamins and minerals, and is adjusted to approximately pH and osmolality approximating natural saliva.

[0010] For example, one exemplary medium is made up of 58 mM K_2HPO_4, 15 mM KH_2PO_4, 10 mM $(NH_4)2SO_4$, 35 mM NaCl, 0.8% (wt/vol) glucose, 0.2% (wt/vol) Casamino Acids (CAA), and 100 mM $MnCl_2 \cdot 4H_2O$ (pH 7.4) and is supplemented with filter-sterilized vitamins (0.04 mM nicotinic acid, 0.1 mM pyridoxine HCl, 0.01 mM pantothenic acid, 1 μM riboflavin, 0.3 μM thiamin HCl, and 0.05 μM d-biotin), amino acids (4 mM l-glutamic acid, 1 mM L-arginine HCl, 1.3 mM l-cysteine HCl, and 0.1 mM l-tryptophan), and 2 mM $MgSO_2 \cdot 7H_2O$.

[0011] The dental samples as used herein are preferably saliva-coated hydroxyapatite discs. The saliva inoculates the discs with natural oral bioflora. Hydroxyapatite discs are commercially available, e.g., from Clarkson Chromatography Products, Inc. Hydroxyapatite, also called hydroxyapatite, is a naturally occurring mineral form of calcium apatite with the formula $Ca_5(PO_4)_3(OH)$. The formula is usually written $Ca_{10}(PO_4)_6(OH)_2$ to denote that the crystal unit cell comprises two entities. Dental enamel and dentin are comprised primarily of carbonated calcium-deficient hydroxyapatite.

[0012] Use of metal salts to detect the presence of bacteria producing volatile sulfur compounds is based on the fact that volatile sulfides such as hydrogen sulfide react with metal ions, for example $Fe(II)^{++}$ or $Pb(II)^{++}$, to form metal sulfides, which may be considered the salts of hydrogen sulfide. Metal sulfides generally have a dark color. Lead(II) acetate paper or media may be used to detect hydrogen sulfide, for example, because it turns grey or black in the presence of the gas as lead(II) sulfide is produced. Various media containing iron compounds also allow detection of hydrogen sulfide production, for example Sulfide-Indole-Motility (SIM) medium or triple sugar iron agar (TSIA), both of which contain $Fe(II)^{++}$, which reacts with hydrogen sulfide to produce FeS as a black precipitate.

[0013] As used throughout, ranges are used as shorthand for describing each and every value that is within the range. Any value within the range can be selected as the terminus of the range. In addition, all references cited herein are hereby incorporated by referenced in their entireties. In the event of a conflict in a definition in the present disclosure and that of a cited reference, the present disclosure controls.

[0014] Unless otherwise specified, all percentages and amounts expressed herein and elsewhere in the specification should be understood to refer to percentages by weight. The amounts given are based on the active weight of the material.

EXAMPLES

[0015] Embodiments of the present invention are further described in the following examples. The examples are merely illustrative and do not in any way limit the scope of the invention as described and claimed.

Example 1: Conventional protocol design

[0016] A method of dynamic biofilm system based on conventional protocols is used to study the effect of actives on oral biofilm developed from salivary microflora. Saliva coated

hydroxyapatite (HAP) discs are placed in the flow cell and connected to a one flow medium passage. The medium flows from the reservoir through the flow cells and is removed as the waste. Untreated sample/placebo is used as a control to test the efficacy of the dentrifices. Treatment solutions of 1:10 diluted dentifrices (30 ml) are then drawn into the flow cells to contact the disks for 1 minute. The flow of media is reinstated to rinse the treated discs and further allow the growth of biofilm during the study period of three days. The treatment is repeated over a time interval of 24 hours with the flow adjusted to stimulate conditions in the human mouth.

[0017] The HAP discs are collected at the end of the study and introduced into anaerobic media, and vortexed to make the plaque on the disc thoroughly suspend in the solution. 300µl of the suspension is pipeted into the wells of a 96-well microplate, and the optical density (OD) at 610nm is read using a microplate reader. Means and standard deviation of the different treatments are calculated. The lower the OD, the lower the bacteria level, and the better the efficacy of the treatment.

[0018] The suspension from the flowcell is collected and diluted serially with anaerobic transfer media. Serial dilutions are plated on lead acetate plates and incubated anaerobically at 37°C for 2-3 days. The real colony forming units on the HAP disc are counted and the final result is reported as Log cfu/disc. The lower the value, the better the efficacy of the treatment.

[0019] The lead acetate reacts with sulfur to form a black deposit, so the black colonies on the plates can be counted independently as an evaluation for a product's efficacy against bacteria producing volatile sulfur compounds. This is of interest because bacteria which produce volatile sulfur compounds are thought to be major contributors to halitosis.

[0020] Prototype formulations are prepared as set forth in the table below.

Table 1: Comparison of different zinc prototype formulations

	Control	Option 1	Option 2	Option 3	Option 4	Option 5	Option 6
	(CAS)	1 % ZnO in chalk	1% ZnO + 0.5% Zn Citrate in chalk	2% Zn Citrate in silica	0.5% ZnO +2% Zn Citrate in silica	1% ZnO in silica	1% ZnO +0.5% Zn Citrate in silica
Ingredients	%	%	%	%	%	%	%
Sorbitol(70%)	21	21	21	22	22	55	55
PEG 600	-	-	-	2	2	2	2
Trimethyl glycine	-	-	-	-	-	1.25	1.25
Xanthan	0.2	0.3	0.3	0.3	0.3	0.3	0.3
Purified Water	28.9	28.1	27.59	44.59	45.09	15.54	15.04
Carboxymethyl cellulose TMS	0.72	0.75	0.75	-	-	-	-
Carboxymethyl cellulose 2000S	-	-	-	1.2	1.2	0.8	0.8
Sodium Saccharin	0.27	0.35	0.35	0.3	0.3	0.3	0.3
Sodium monofluorophosphate	0.76	0.76	0.76	0.76	0.76	0.76	0.76
Sodium bicarbonate	0.5	1.0	1.0	-	-	-	-
NaOH 50%	0.1	-	-	-	-	-	-
MFIL-P	2.5	0.5	0.5	5	4	4	4
Silica abrasive (ZEO 114)	-	-	-	10	10	10	10
Precipitated calcium carbonate (PCC)	18	-	-	-	-	-	-
Refined natural calcium carbonate (NCC)	22.5	-	-	-	-	-	-
NCC-India	-	35	35	-	-	-	-
Sodium lauryl sulfate	2.	2.0	2.0	2	2.	2	2
Benzyl alcohol	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Flavor	1	1.2	1.2	1.3	1.3	1.3	1.3
Tetrasodium pyrophosphate (TSPP)	0.5	1	1	1	1	0.5	0.5
Tetrapotassium pyrophosphate (TKPP)	-	1	1	2	2	-	-
Zinc oxide	-	1	1	-	0.5	1	1
Zinc citrate	-	-	0.5	2	2	-	0.5
Titanium dioxide	0.75	0.75	0.75	-	-	-	-
Pearlescent white pigment (Timiron®)	-	-	-	-	0.25	0.25	0.25
Small particle silica (AC43)	-	5	5	5	5	5	5
Total	100	100	100	100	100	100	100

[0021] The results of the study with the above-described method (3 days) are outlined in Tables 2-4 below. The formulations tested include the prototype formulations, and also commercial toothpaste products Colgate Total® and Crest ProHealth, which contain triclosan and stannous fluoride respectively as antibacterial agents.

Table 2: OD at 610nm of the harvested HAP disc with three different inoculum sources (3 day study) – data grouped with Tukey method at 95% confidence level

	Subject 1	Subject 2	Subject 3	Mean
Sample Description	Avg.	Avg.	Avg.	
CAS control	0.43	0.59	0.33	0.45
Crest® ProHealth HW (2% Zinc citrate)	0.31	0.46	0.24	0.34
1% ZnO in chalk	0.24	0.47	0.19	0.30
360 WMH (2% Zn citrate)	0.25	0.33	0.23	0.27
1% ZnO + 0.5% Zn citrate in chalk	0.08	0.37	0.36	0.27
1% ZnO in silica	0.15	0.47	0.12	0.25
1% ZnO + 0.5% Zinc citrate in silica	0.23	0.28	0.22	0.24
Total® whitening (triclosan)	0.19	0.19	0.16	0.18

Table 3: Values of plate count on Neomycin Vancomycin agar (3 day study) – data grouped with Tukey method at 95% confidence level

	Subject 1	Subject 2	Subject 3	Mean
Sample Description	log CFU/disc	log CFU/disc	log CFU/disc	
CAS control	7.01	7.06	6.93	7.00
1% ZnO in chalk	6.66	6.80	6.87	6.78
1% ZnO + 0.5% Zn citrate in chalk	6.63	6.78	6.71	6.71
360 WMH (2% Zn citrate)	6.57	6.66	6.74	6.66
Crest® ProHealth HW (2% Zinc citrate)	6.59	6.64	6.54	6.59
1% ZnO in silica	6.53	6.40	6.54	6.49
1% ZnO + 0.5% Zinc citrate in silica	6.09	6.36	6.50	6.32
Total® whitening (triclosan)	5.26	4.92	5.94	5.38

CFU – colony forming units

Table 4: Values of plate count on Lead Acetate agar (3 day study) – data grouped with Tukey method at 95% confidence level

	Subject 1	Subject 2	Subject 3	Mean
Sample Description	log CFU/disc	log CFU/disc	log CFU/disc	
CAS control	6.75	6.88	6.82	6.82
1% ZnO in chalk	6.53	6.69	6.77	6.66
1% ZnO + 0.5% Zn citrate in chalk	6.39	6.63	6.48	6.50
Crest® ProHealth HW (2% Zinc citrate)	6.36	6.52	6.16	6.38
1% ZnO in silica	6.31	6.26	6.41	6.33
360 WMH (2% Zn citrate)	5.91	6.02	6.11	6.01
1% ZnO + 0.5% Zinc citrate in silica	5.72	5.84	5.94	5.83
Total® whitening (triclosan)	5.04	5.08	5.53	5.22

CFU – colony forming units

Example 2: Modified Dynamic Biofilm Protocol Design

[0022] The experiment is then modified to be more representative of the actual *in vivo* situation. Saliva coated HAP disks are placed in the flow cell and connected to a one flow medium passage. The medium flows from the reservoir through the flow cells and is removed to waste. Untreated sample/placebo is used as a control to test the efficacy of the dentrifices. Treatment solutions of 1:10 diluted dentifrices (30 ml) are then drawn into the flow cells to contact the disks for 10 minutes. The flow of media is reinstated to rinse the treatment and further allow the growth of biofilm during the study period. The treatment is repeated over a time interval of 24 hours with the flow adjusted to stimulate conditions in the human mouth.

[0023] The treatment regime is increased to 8 days. The HAP disks are collected at the end of the study and introduced into anaerobic media, and vortexed to suspend the plaque on the disc thoroughly in the solution. 300µl of the suspension is pipetted into the wells of a 96-well microplate, and optical density (OD) is read at 610nm in the microplate reader. An Excel spreadsheet is used to calculate means and standard deviation of the different treatments. The lower the OD, the better the efficacy of the treatment.

[0024] The suspension from the flowcell is collected and diluted serially with anaerobic transfer media. Serial dilutions are plated on lead acetate plates and incubated anaerobically at 37°C for 2-3 days. The real colony forming units on the HAP disc are counted and the final result reported as Log CFU/disc. The lower the value, the better the efficacy of the treatment.

[0025] The black colonies on the plates are counted independently as an evaluation for the product's anti-VSC efficacy.

[0026] The following changes are made to the more conventional method of example 1 with respect to treatment- biofilm interaction, while keeping the working principle of the method constant. In the modified method the treament regime is increased to 8 days in place of 3 days. The contact time is also increased to 10 minutes from 1 minute. The rest of the procedure is repeated as is with harvesting after completion of 8 days. The differentiation between in the individual values of the results is found to be significant.

[0027] The results are summarized in Tables 5-7 below:

Table 5: OD at 610nm of the harvested HAP disc with three different inoculum sources (8 day study) – data grouped with Tukey method at 95% confidence level

	Subject 1	Subject 2	Subject 3	Mean
Sample Description	Avg.	Avg.	Avg.	
CAS control	1.50	1.36	1.44	1.43
Crest® ProHealth HW (2% Zinc citrate)	1.12	1.18	1.20	1.17
1% ZnO in chalk	1.10	1.04	0.94	1.03
1% ZnO + 0.5% Zn citrate in chalk	0.82	0.76	0.92	0.84
360 WMH (2% Zn citrate)	0.64	0.58	0.66	0.63
1% ZnO in silica	0.51	0.62	0.68	0.60
1% ZnO + 0.5% Zinc citrate in silica	0.35	0.48	0.42	0.42
Total® whitening (triclosan)	0.25	0.31	0.34	0.30

Table 6: Values of plate count on Neomycin Vancomycin agar (8 day study) – data grouped with Tukey method at 95% confidence level

	Subject 1	Subject 2	Subject 3	Mean
Sample Description	log CFU/disc	log CFU/disc	log CFU/disc	
CAS control	7.32	7.46	7.55	7.44
Crest® ProHealth HW (2% Zinc citrate)	7.00	6.64	6.54	6.73
1% ZnO in chalk	6.28	6.24	6.49	6.34
1% ZnO + 0.5% Zn citrate in chalk	5.95	6.12	6.20	6.09
360 WMH (2% Zn citrate)	5.85	6.02	6.35	6.08
1% ZnO in silica	5.72	5.98	6.04	5.91
1% ZnO + 0.5% Zinc citrate in silica	5.67	5.75	5.59	5.67
Total® whitening (triclosan)	4.95	4.86	5.02	4.94

CFU – colony forming units

Table 7: Values of plate count on Lead Acetate agar (8 day study) – data grouped with Tukey method at 95% confidence level

	Subject 1	Subject 2	Subject 3	Mean
Sample Description	log CFU/disc	log CFU/disc	log CFU/disc	
CAS control	6.13	6.24	6.49	6.28
1% ZnO in chalk	5.82	5.74	5.95	5.84
Crest® ProHealth HW (2% Zinc citrate)	5.72	5.56	5.63	5.63
1% ZnO + 0.5% Zn citrate in chalk	5.58	5.46	5.60	5.55
360 WMH (2% Zn citrate)	5.65	5.46	5.37	5.49
1% ZnO in silica	5.11	5.35	5.16	5.21
1% ZnO + 0.5% Zinc citrate in silica	4.88	4.95	5.04	4.96
Total® whitening (triclosan)	4.76	4.92	4.06	4.56

CFU – colony forming units

[0028] The modified method of this example is not only closer to the natural situation, but it does a better job of distinguishing the relative antibacterial activities of the different

formulations. While the commercial Total ® formulation, which contains a potent antibacterial agent (triclosan) was always the most effective, the different formulations with different metal salts proved to have good activities over time, and as seen from the Tukey analysis, these differences allow separation and ranking of the different formulations, which was not possible using conventional testing, which had these formulations as statistically indistinguishable.

Example 3: Comparative evaluation between data for 3 days (conventional) and 8 days (modified) study

[0029] The mean data for each prototype was compared using each of the two methods. The modified method is able to effectively differentiate statistically between the positive control (Total whitening (triclosan)) and the negative control (CAS control), and moreover is able to distinguish between different prototypes using different Zn compound combinations. This allows better selection and optimization of lead formulation based on efficacy.

Table 8: Mean values of OD at 610nm for conventional and modified method

	3 day study	8 day study
Sample Description		
CAS control	0.45	1.43
Crest® ProHealth HW (2% Zinc citrate)	0.34	1.17
1% ZnO in chalk	0.30	1.03
1% ZnO + 0.5% Zn citrate in chalk	0.27	0.84
360 WMH (2% Zn citrate)	0.27	0.63
1% ZnO in silica	0.25	0.60
1% ZnO + 0.5% Zinc citrate in silica	0.24	0.42
Total® whitening (triclosan)	0.18	0.30

Table 9: Mean values of plate count on NV agar with conventional and modified method

	3 day study	8 day study
Sample Description		
CAS control	7.00	7.44
Crest® ProHealth HW (2% Zinc citrate)	6.59	6.73
1% ZnO in chalk	6.78	6.34
1% ZnO + 0.5% Zn citrate in chalk	6.71	6.09
360 WMH (2% Zn citrate)	6.66	6.08
1% ZnO in silica	6.49	5.91
1% ZnO + 0.5% Zinc citrate in silica	6.32	5.67
Total® whitening (triclosan)	5.38	4.94

CFU – colony forming units

Table 10: Mean values of plate count on Lead Acetate agar with conventional and modified method

	3 day study	8 day study
Sample Description		
CAS control	6.82	6.28
1% ZnO in chalk	6.66	5.84
Crest® ProHealth HW (2% Zinc citrate)	6.38	5.63
1% ZnO + 0.5% Zn citrate in chalk	6.50	5.55
360 WMH (2% Zn citrate)	6.01	5.49
1% ZnO in silica	6.33	5.21
1% ZnO + 0.5% Zinc citrate in silica	5.83	4.96
Total® whitening (triclosan)	5.22	4.56

CFU – colony forming units

Example 4: Correlation between Zn uptake and biofilm reduction data for conventional and modified method

[0030] The average amount of Zn uptake on HAP is a direct measure of the ability of the compound to reduce biofilm *in vivo*. Over a short interval, zinc citrate has a higher uptake, but with a longer time interval, the uptake of zinc oxide increases. Thus, the available zinc is enhanced with multiple treatments of zinc oxide, resulting in increased biofilm reduction.

Table 11: Average values of Zn uptake on HAP using AAS with both methods

	Uptake µg/HAP	
Sample Description	3 day study	8 day study
Crest® ProHealth HW (2% Zinc citrate)	52	17
360 WMH (2% Zn citrate)	47	56
1% ZnO in chalk	77	99
1% ZnO + 0.5% Zn citrate in chalk	77	116
1% ZnO in silica	85	186
1% ZnO + 0.5% Zinc citrate in silica	73	190

As can be seen from the data in Table 11, the two samples with the best degree of zinc uptake also correlated with the two best samples in the 8 day biofilm protocols (i.e. 1% ZnO in silica and 1% ZnO + 0.5% Zinc citrate in silica)

[0031] As those skilled in the art will appreciate, numerous changes and modifications may be made to the embodiments described herein without departing from the spirit of the invention. It is intended that all such variations fall within the scope of the appended claims.

We Claim:

1. A method of measuring the efficacy of a test composition against oral bacterial biofilm formation, comprising

   a) Coating a sample selected from a tooth section and a hydroxyapatite disc with saliva and placing the sample in a flow of a buffered medium, with temperature, flow and pH adjusted to approximate conditions in the mouth;

   b) Exposing the sample to a test composition, then reinstating the flow of buffered medium, wherein the period of exposure to a test compound is from 7-15 minutes;

   c) Repeating the preceding step from one to four times per day for 5-12 days;

   d) Vortexing the sample in anaerobic media to obtain a suspension and measuring the optical density thereof, wherein the optical density is a measure of the degree of bacterial growth.

2. The method of claim 1 wherein the period of exposure the sample to a test composition in step b) is for a period of about 10 minutes.

3. The method of any preceding claim wherein the step of repeating step b) is carried out from one to four times per day for about 8 days.

4. The method of any preceding claim wherein the buffered medium comprises nutrients as required to support bacterial growth.

5. The method of any preceding claim further comprising the step of culturing the suspension on a solid media and counting the number of colony forming units after a period of 2-5 days, wherein the number of colony forming units is a measure of the degree of bacterial growth.

6. The method of claim 5 wherein the culture on solid media is carried out in the presence of a metal salt which reacts with volatile sulfur compounds formed in steps a-c to form a black precipitate.

7. The method of claim 6 wherein the solid media is an agar plate and the metal salt is lead acetate.

8. The method of claim 6 or 7 wherein any black colony forming units are counted independently from the other colony forming units and wherein the number of black

colony forming units corresponds to the degree of growth of bacteria which produce volatile sulfur compounds.

9. The method of any preceding claim wherein the test composition is an oral care product.

10. The method of any preceding claim wherein the test composition comprises at least 0.1% of a metal ion having a valence of +2 or +3.

11. The method of any preceding claim wherein metal ion having a valence of +2 or +3 is Zn^{2+}.

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2013/000183

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/569 A61Q11/00
ADD.

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)
G01N A61Q

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

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

EPO-Internal, WPI Data, EMBASE, BIOSIS, COMPENDEX, INSPEC, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HERLES S ET AL: "Chemostat flow cell system: an in vitro model for the evaluation of antiplaque agents.", JOURNAL OF DENTAL RESEARCH NOV 1994, vol. 73, no. 11, November 1994 (1994-11), pages 1748-1755, XP008165974, ISSN: 0022-0345 abstract ----- -/--	1-11

[X] Further documents are listed in the continuation of Box C.

[X] 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)

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 November 2013	09/12/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	van der Kooij, M

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2013/000183

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S.L. KINNIMENT ET AL: "The effect of chlorhexidine on defined, mixed culture oral biofilms grown in a novel model system", JOURNAL OF APPLIED BACTERIOLOGY, vol. 81, no. 2, 1 August 1996 (1996-08-01) , pages 120-125, XP055089944, ISSN: 0021-8847, DOI: 10.1111/j.1365-2672.1996.tb04488.x abstract; figure 1 page 121, column 1, paragraph 3 - column 2, paragraph 4	1-11
A	WO 2012/024897 A1 (HAWLEY & HAZEL CHEMICAL COMPANY ZHONGSHAN LTD [CN]; CHEN WANG HUA [CN]) 1 March 2012 (2012-03-01) claims 1,6,18,27; examples 1-3	1-11
A	HOROWITZ A ET AL: "Hydrogen sulfide production in the periodontal environment.", JOURNAL OF PERIODONTOLOGY JUL 1973, vol. 44, no. 7, July 1973 (1973-07), pages 390-395, XP008165980, ISSN: 0022-3492 the whole document	5-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IN2013/000183

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012024897 A1	01-03-2012	CN 101935611 A	05-01-2011
		WO 2012024897 A1	01-03-2012