(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
2 July 2015 (02.07.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/100405 A1

(51) International Patent Classification:
C12N 1/20 (2006.01)

(21) International Application Number:
PCT/US2014/072331

(22) International Filing Date:
24 December 2014 (24.12.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/920,567 24 December 2013 (24.12.2013) US

(72) Inventor; and
(71) Applicant : **MAJEED, Muhammed** [US/US]; Sabinsa Corporation, 20 Lake Drive, East Windsor, NJ 08520 (US).

(72) Inventors: **NAGABHUSHANAM, Kalyanam**; Sabinsa Corporation, 20 Lake Drive, East Windsor, NJ 08520 (US). **ARUMUGAM, Sivakumar**; Sami Labs Limited, 19/1 And 19/2, I Main, Ii Phase, Peenya Industrial Area, Bangalore, Karnataka 560058 (IN). **ALI, Furqan**; Sami Labs Limited, 19/1 And 19/2, I Main, Ii Phase, Peenya Industrial Area, Bangalore, Karnataka 560058 (IN).

(74) Agent: **NAGABHUSHANAM, Kalyanam**; Sabinsa Corporation, 20 Lake Drive, East Windsor, NJ 08520 (US).

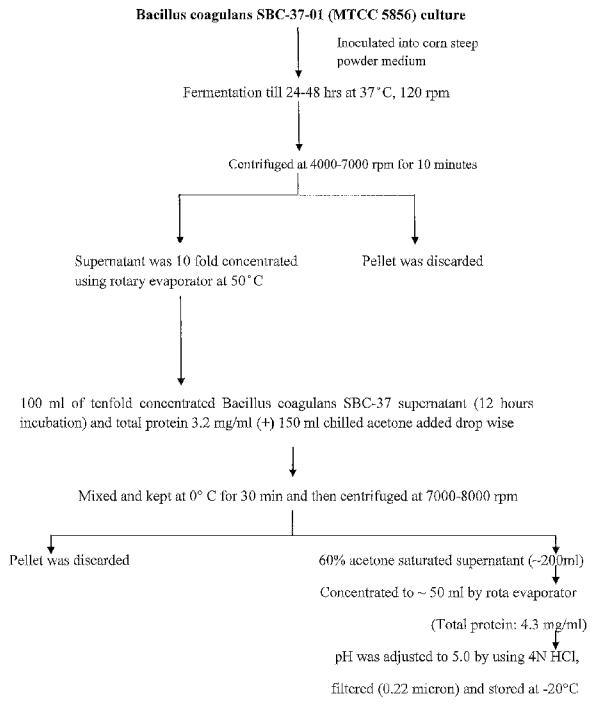
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,

[Continued on next page]

(54) Title: METHOD OF PRODUCING PARTIALLY PURIFIED EXTRACELLULAR METABOLITE PRODUCTS FROM BACILLUS COAGULANS AND BIOLOGICAL APPLICATIONS THEREOF

FIG. 1



(57) Abstract: Disclosed is a method for producing partially purified extracellular metabolite preparation from the probiotic bacterial strain Bacillus coagulans SBC-37 (Deposited in the Microbial Type Culture Collection and Gene Bank as strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains Bacillus coagulans ATCC 31284, Bacillus coagulans NBRC 3887 and Bacillus coagulans ATCC 7050. Also disclosed is the anti-microbial profile of said extracellular metabolite preparation against a panel of microbial pathogens, including synergistic anti-microbial effects of preparation when combined with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of Magnolia officinalis. The extracellular metabolite preparation alone or the combination of said extracellular metabolite preparation and preservative blend also inhibits microbial biofilm formation in a synergistic manner.

WO 2015/100405 A1

LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

METHOD OF PRODUCING PARTIALLY PURIFIED EXTRACELLULAR METABOLITE PRODUCTS FROM *BACILLUS COAGULANS* AND BIOLOGICAL APPLICATIONS THEREOF

CROSS-REFERENCE TO RELATED PATENT APPLICATION

This patent application is the non-provisional filing for provisional patent application US61920567 filed 24 December 2013 for the invention titled "METHOD OF PRODUCING PARTIALLY PURIFIED EXTRACELLULAR METABOLITE PRODUCTS FROM BACILLUS COAGULANS AND BIOLOGICAL APPLICATIONS THEREOF."

BACKGROUND OF THE INVENTION

[PARA 001] Field of the invention

[PARA 002] The invention in general relates to the anti-microbial effects of probiotic preparations. More specifically, the present invention relates to (1) a method for producing partially purified extracellular metabolite preparation from the probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050 and (2) the anti-microbial profile of said extracellular metabolite preparation against a panel of microbial pathogens, including synergistic anti-microbial effects of preparation when combined with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis.* The extracellular metabolite preparation alone or the

combination of said extracellular metabolite preparation and preservative blend is also shown to inhibit microbial biofilm formation in a synergistic manner.

[PARA 003] Description of prior art

[PARA 004] Extracellular products of *Bacillus coagulans* comprising a supernatant or filtrate of a culture *Bacillus coagulans* strain suitable for topical application to the skin or mucosal membranes of a mammal and thereby capable of being utilized to inhibit the growth of bacterium, yeast, fungi, virus, and combinations thereof is known in the art (US6905692, "Topical compositions containing probiotic Bacillus bacteria, spores, and extracellular products and uses thereof). The present invention pertains to further purification of extracellular components of cultures of probiotic *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) to obtain a concentrated extracellular metabolite preparation that exhibits enhanced anti-microbial effects when compared to the supernatant itself both alone and when combined with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis.*

[PARA 005] It is the principle objective of the present invention to disclose

1) A method for producing partially purified extracellular metabolite preparation from the probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050.

2) The anti-microbial profile of said extracellular metabolite preparation against a panel of microbial pathogens, including synergistic anti-microbial effects of preparation when combined with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis*.

3) The microbial biofilm inhibitory potential of said extracellular metabolite preparation alone or in combination with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis*.

[PARA 006] The present invention fulfills the aforesaid objectives and provides further related advantages.

SUMMARY OF THE INVENTION

[PARA 007] The present invention describes

(1) A method for producing partially purified extracellular metabolite preparation from the probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050;

(2) The anti-microbial profile of said extracellular metabolite preparation against a panel of microbial pathogens, including synergistic anti-microbial effects of preparation when combined with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of

monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis* and

(3) The microbial biofilm inhibitory potential of said extracellular metabolite preparation alone or in combination with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis*.

(4) The antimicrobial activity and anti-acidogenic effect against adherent *S. mutans* in water-insoluble glucans of said extracellular metabolite preparation alone or in combination with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis*.

[PARA 008] The present invention provides the following advantages.

1) Disclosure of a purification method for producing partially purified extracellular metabolite preparation from the probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050;
2) Disclosure of the anti-microbial profile of the partially purified against a panel of microbial pathogens, including synergistic anti-microbial effects of said partially purified extracellular preparation when combined with a synergistic preservative blend-comprising

from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis.*

3) Disclosure of the microbial biofilm inhibitory potential of said extracellular metabolite preparation alone or in combination with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis.*
4) Disclosure of the antimicrobial activity and anti-acidogenic effect against adherent *S. mutans* in water-insoluble glucans, of said extracellular metabolite preparation alone or in combination with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis*.

[PARA 009] Other features and advantages of the present invention will become apparent from the following more detailed description, taken in conjunction with the accompanying images, which illustrate, by way of example, the principle of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[PARA 0010] Fig.1 shows the process flowchart for a purification method to produce *partially purified* extracellular metabolite preparation from the probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 *and Bacillus coagulans* ATCC 7050.

[PARA 0011] Fig. 2 shows the representation of checkerboard broth micro-dilution method for synergistic anti-microbial studies.

[PARA 0012] Figs.3A-3B, 3C-3D, 3E-3F, 3G-3H, 3I-3J, 3K-3L, 3M-3N, 3O-3P show respectively, the graphical representation of the effect of natural preservative blend and extracellular metaboliltes of *B. coagulans* MTCC 5856 alone and in combination on the growth and viability of *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 25922, *Salmonella abony* NCIM 2257, *Streptococcus mutans* MTCC 1943, *Propionibacterium acnes* ATCC 11827, *Staphylococcus aureus* ATCC 29213, *Staphlococcus epidermidis* ATCC 14990 and *Bacillus cereus* ATCC 14579.

[PARA 0013] Figs. 4A, 4B, 4C, 4D and 4E show respectively, the graphical representations of the effect of natural preservative blend and extracellular metabolites of *B. coagulans* MTCC 5856 alone and in combination on the biofilm formation of *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 25922, *Streptococcus mutans* MTCC 1943,*Staphylococcus aureus* ATCC 29213 and *Staphylococcus epidermidis* ATCC 14990.

[PARA 0014] Figs.5A and 5B show respectively, the graphical representations of the effect of natural preservative blend and extracellular metabolites of *B. coagulans* MTCC 5856 on the growth/pH drop and the formation of water insoluble glucans of *S. mutans* MTCC 1943 biofilm in presence of 2% sucrose.

DETAILED DESCRIPTION OF THE MOST PREFERRED EMBODIMENT

[PARA 0015] In the most preferred embodiment the present invention relates to a purification method for producing partially purified extracellular metabolite preparation from the probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050, said purification method comprising the steps of:

1. Inoculating a culture of *Bacillus coagulans* MTCC 5856 exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050 into 1.0 liter of Glucose Yeast Extract Acetate broth medium (HiMedia, Mumbai India) or MRS broth containing 0.5% tween 80 or Corn steep powder media;

2. Allowing the fermentation in the inoculated medium of step 1 to proceed for 24-48 h at 37°C with 120 rpm;
3. Centrifuging the fermentation broth of step 2 at 4000-7000 rpm;
4. Concentrating supernatants 10 fold by using rotary evaporator at 50°C of step 3.
5. Adding 150 ml of chilled acetone drop by drop to 100 ml of tenfold concentrated supernatants of step 4, followed by mixing;
6. Incubating the mixture of step 5 at 0°C for 30 minutes followed by centrifuging at 7000-8000 rpm;
7. Discarding the pellet obtained in step 6 and collecting 60% acetone saturated supernatant (~200ml).
8. Concentrating the acetone saturated supernatant in step 7 to 50 ml by rotary evaporator.
9. Adjusting the pH to 5.0 by using 4N HCl, filtered (0.22 micron; Millex, Millipore, India) and stored at -20°C till further use.
10. Freeze drying/spray drying/tray drying the supernatant of step 8.

[PARA 0016] In another most preferred embodiment, the present invention relates to a process of microbial control, said process comprising the step of bringing into contact effective concentrations of a partially purified extracellular metabolite preparation from probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050 and a target microbial cell. In more specific embodiments, the microbial cell may be one selected from the group comprising *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Propionibacterium acnes, Bacillus cereus and Salmonella abony.*

[PARA 0017] In yet another most preferred embodiment, the present invention relates to a process of microbial control, said process comprising the step of bringing into contact a microbial cell with effective concentrations of a preparation consisting essentially of partially purified extracellular metabolite preparation from probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type

Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050 and a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis*. In more specific embodiments, the microbial cell may be one selected from the group comprising *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Propionibacterium acnes, Bacillus cereus and Salmonella abony.*

[PARA 0018] In yet another most preferred embodiment, the present invention also relates to a process of inhibiting microbial biofilm formation, said process comprising step of bringing into contact biofilm producing microbial cells and a preparation consisting essentially of partially purified extracellular metabolite preparation from probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050 alone or said preparation combined with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis*.

[PARA 0019] The following examples are presented herewith to illustrate the exemplary embodiments of the present invention.

[PARA 0020] EXAMPLE 1

[PARA 0021] Microorganisms and culture conditions

[PARA 0022] The bacterial strains used in this study included *Streptococcus mutans* MTCC 1943, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 14990, *Escherichia coli* ATCC

25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella abony* NCIM 2257 and *Bacillus cereus* ATCC 14579. The reference strains were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA), MTCC (IMTECH, Chandigarh, India) and NCIM (National Collection of Industrial Microorganisms, Pune, India). *S. mutans* and *P. acnes* were maintained on brain–heart infusion agar (BHI; Difco Laboratories, Detroit, MI, USA) and reinforced clostridial agar (RCA; HiMedia, Mumbai, India) respectively. *S. aureus, S. epidermidis E. coli, P. aeruginosa, S. abony and B. cereus* were maintained on trypticase soy agar (Difco Laboratories) at 37°C. *S. mutans* and *P. acnes* were incubated anaerobically (80% N_2, 10% H_2 and 10% CO_2) at 37°C up to 48 h in anaerobic chamber (Coy Laboratory Products Inc, Michigan). *Bacillus coagulans* SBC37-01 used in the study was characterized and deposited to Microbial Type Culture Collection, Chandigarh, India and the strain was assigned as *Bacillus coagulans* MTCC 5856.

[PARA 0023] Technique-The checkerboard method for synergy study

[PARA 0024] This is the most frequently used method to access the antimicrobial combinations *in vitro*. The term "checkerboard" refers to the pattern (of tubes or microtiter plate wells) formed by multiple dilutions of two drugs being tested (Eliopoulos GM, Moellering RC: Antimicrobial combinations. In Antibiotics in laboratory medicine. Edited by Lorian V. Baltimore, The Williams & Wilkins Co; 1991:432-492). In the present study, the checker board consisted of columns in which each tube (or well) contains the same amount of the partially purified extracellular metabolite preparation from the probiotic bacterial strain *Bacillus coagulans* MTCC 5856 being diluted along the X-axis (rows) in which each tube (or well) contains the same amount of the preservative blend being diluted on the Y-axis (Fig 2). As a result each square in the checkerboard (which represents one tube/ well or plate) contained a unique combination of partially purified extracellular metabolite preparation from the probiotic bacterial strain *Bacillus coagulans* MTCC 5856 and preservative blend. The concentration range of preservative blend in the present study was 2000 µg/ml to 31.25 µg/ml and lower in some cases, whereas the partially purified extracellular metabolite preparations from the probiotic bacterial strain *Bacillus coagulans* MTCC 5856 was tested in the range of 8 % (v/v) to 0.015 % (v/v). This checkerboard technique can be performed with liquid or semisolid (agar)

media. BHI broth and RC broth were used for *S. mutans* and *P. acnes* respectively and plates were incubated anaerobically (80% N_2, 10% H_2 and 10% CO_2) at 37°C up to 48 h in anaerobic chamber. Mueller hinton broth (Difco) was used for *S. aureus, S. epidermidis E. coli, P. aeruginosa, S. abony and B. cereus* and plates were incubated at 37°C for 18 h.

[PARA 0025] Kill kinetics

[PARA 0026] Time–kill studies of the preservative blend and partially purified extracellular metabolite preparation from strain *Bacillus coagulans* MTCC 5856 were conducted against *P. aeruginosa, E. coli, S. aureus S. epidermidis, S. mutans, P. acnes, B. cereus* and *Salmonella abony* and evaluated using a time–kill curve method, as described previously (Eliopoulus GM, Moellering RCJ. Antimicrobial combinations. In: Lorian V, ed. Antibiotics in Laboratory Medicine, 4th edn. Baltimore: Williams & Wilkins, 1996; 330–6.). Bacterial suspension in its logarithmic phase (1×10^6 cfu/ml) was used as the inoculum. The preservative blend and partially purified extracellular metabolite preparation from strain *Bacillus coagulans* MTCC 5856 were tested alone and in combination at different concentrations, as determined in checkerboard assay. The cell population as cfu/ml was determined by a serial dilution method in triplicate on respective media and incubated in respective growth conditions. The viable count and absorbance (OD_{610}) were taken at 0 (untreated control), and 24 h of incubation at 37°C. Viable count was expressed in Log_{10} cfu/ml and absorbance was expressed in OD value at 610 nm.

[PARA 0027] Biofilm susceptibility assays

[PARA 0028] The biofilm inhibitory effect of preservative blend and partially purified extracellular metabolite preparation from the strain *Bacillus coagulans* MTCC 5856 alone and in combination were examined against *S. mutans, E. coli, S. aureus S. epidermidis* and *P. aerugenosa* by the microdilution method (Wei et al. Journal of Antimicrob. Chemother. 2006. **57:**1100–1109.). This method was similar to the checkerboard method for planktonic cells. For *S. mutans* biofilm assay, BHI broth supplemented with 2% sucrose was used. In case of other organisms, TSB supplemented with 2% glucose was used in the study. The bacterial suspensions were prepared from the overnight-grown culture, and the turbidity of the suspension was adjusted to an optical

density at 610 nm (A_{610}) of 0.7 (1 x 10^9 CFU/ml). The concentration range of preservative blend in the present study was 2000 µg/ml to 31.25 µg/ml and lower concentrations in some cases, whereas partially purified extracellular metabolite preparation from the strain *Bacillus coagulans* MTCC 5856 was tested in the range of 8 % (v/v) to 0.015 % (v/v). Forty microliters of fresh media broth was added to each well, followed by the addition of 60 µl of the above-mentioned suspension to each well of the plate. This resulted in the final inoculum of 6 x 10^7 CFU/ml in each well; After incubation at 37°C for 48 h, the culture supernatant from each well was decanted, and planktonic cells were removed by washing the wells with phosphate-buffered saline (PBS; pH 7.2). The biofilm was fixed with methanol for 15 min and then air dried at room temperature. The wells of the dried plate were stained with 0.1% (w/v) crystal violet (Sigma Chemical Co., St Louis, MO) for 10 min and rinsed thoroughly with water until the negative control wells appeared colorless. Biofilm formation was quantified by the addition of 200 µl of 95% ethanol to the crystal violet-stained wells and recording the absorbance at 595 nm (A_{595}). The percentage of biofilm inhibition was calculated using the equation (A_{595} of biofilm treated with test agent/A_{595} of non-treated control) ×100. Culture without test agent was used as the non-treated control.

[PARA 0029] Antimicrobial activity against adherent *S. mutans* in water-insoluble glucan

[PARA 0030] The formation of water-insoluble glucan by *S. mutans* MTCC 1943 was performed by a previously described method (Katsura et al., Antimicrob. Agents Chemother. 2001. 45:3009–3013). Briefly, aliquots of 100 µl of culture of *S. mutans* MTCC 1943 (~ 1 × 10^8 cells/ml) were inoculated into 10 ml of fresh BHI broth containing 2% sucrose (w/v) in the test tubes and incubated at 37°C for 24 h at an inclination of 30°. The fluid containing planktonic cells was gently removed. The water-insoluble glucan containing cells of *S. mutans* MTCC 1943 were gently washed with 10 ml of sterile water and resuspended in 10 ml of phosphate buffer (10 mM, pH 5.0) containing preservative blend and partially purified extracellular metabolite preparation from the strain *Bacillus coagulans* MTCC 5856 alone and in combination, followed by incubation at 37°C for 5 min. Chlorhexidine 0.12% (v/v) (Sigma Chemical Co., St Louis, MO) was used as internal reference in the study. The mixture was gently washed again with sterile saline (0.89% NaCl, w/v), followed by the resuspension of treated cells in 10 ml of BHI broth containing 2% sucrose (w/v). After incubation of cells at 37°C for 6, 12, 18, and 24 h, the acid produced by the culture was

measured by using a pH meter. The fluid containing free cells of *S. mutans* MTCC was gently removed. The water insoluble glucan was resuspended in 10 ml of 1 N NaOH solution and homogenized; the turbidity was measured at 610 nm.

[PARA 0031] RESULTS

[PARA 0032] Table 1 shows the fold reduction of human microbial pathogens when brought in contact with A. formulations containing just Partially Purified Extracellular Metabolites (PPEM) or Natural Preservative Blend (NPB); and B. formulations incorporating both Partially Purified Extracellular Metabolites (PPEM) and Natural Preservative Blend (NPB). It may be noted that the formulations incorporating both Partially Purified Extracellular Metabolites (PPEM) and Natural Preservative Blend (NPB) cause a significant eight fold decrease for pathogen *Pseudomonas aeruginosa* and four fold decrease for pathogen *Escherichia coli.*

Table 1. Results of checkerboard testing of the natural preservative blend and partially purified extracellular metabolites of probiotic stain *Bacillus coagulans* MTCC 5856 against human pathogens

S. No	Tested organisms	Partially Purified Extracellular Metabolites (PPEM)		Natural Preservative Blend (NPB)	
		MIC (%, v/v)		MIC (µg/ml)	
		alone	In combination with NPB (Fold reduction in MIC)	alone	In combination with PPEM (Fold reduction in MIC)
1	*P. aeruginosa* ATCC 9027	0.5	0.12 (4)	250	31.25 (8)
2	*E. coli* ATCC 25922	1.0	0.25 (4)	250	62.50 (4)
3	*S. abony* NCIM 2257	1.0	0.25 (4)	500	125 (4)
4	*S. mutans* MTCC 1943	4.0	2.0 (2)	15.62	7.81 (2)
5	*P. acnes* ATCC 11827	4.0	2.0 (2)	62.50	31.25 (2)
5	*S. aureus* ATCC 29213	2.0	1.0 (2)	31.25	15.62 (2)
6	*S. epidermidis* ATCC 14990	2.0	1.0 (2)	32.25	15.62 (2)
7	*B. cereus* ATCC 14579	4.0	2.0 (2)	125	31.25 (2)

[PARA 0033] While the invention has been described with reference to a preferred embodiment, it is to be clearly understood by those skilled in the art that the invention is not limited thereto. Rather, the scope of the invention is to be interpreted only in conjunction with the appended claims.

We claim,

1. A purification method for producing partially purified extracellular metabolite preparation from the probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050, said purification method comprising the steps of:
    1. Inoculating a culture of *Bacillus coagulans* MTCC 5856 exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050 into 1.0 liter of Glucose Yeast Extract Acetate broth medium or MRS broth containing 0.5% tween 80 or Corn steep powder media;
    2. Allowing the fermentation in the inoculated medium of step 1 to proceed for 24-48 h at 37°C with 120 rpm;
    3. Centrifuging the fermentation broth of step 2 at 4000-7000 rpm;
    4. Concentrating supernatants 10 fold by using rotary evaporator at 50°C of step 3.
    5. Adding 150 ml of chilled acetone drop by drop to 100 ml of tenfold concentrated supernatants of step 4, followed by mixing;
    6. Incubating the mixture of step 5 at 0°C for 30 minutes followed by centrifuging at 7000-8000 rpm;
    7. Discarding the pellet obtained in step 6 and collecting 60% acetone saturated supernatant (~200ml).
    8. Concentrating the acetone saturated supernatant in step 7 to 50 ml by rotary evaporator.

9. Adjusting the pH to 5.0 by using 4N HCl, filtered (0.22 micron; Millex, Millipore, India) and stored at -20°C till further use.
10. Freeze drying/spray drying/tray drying the supernatant of step 8.

2. A process of microbial control, said process comprising the step of bringing into contact effective concentrations of a partially purified extracellular metabolite preparation from probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050 and a target microbial cell.

3. The process of claim 2 wherein the microbial cell is one selected from the group comprising *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Propionibacterium acnes, Bacillus cereus and Salmonella abony.*

4. A process of microbial control, said process comprising the step of bringing into contact a microbial cell with effective concentrations of a preparation consisting essentially of partially purified extracellular metabolite preparation from probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050 and a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis*.

5. The process of claim 4 wherein the microbial cell is one selected from the group comprising *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Propionibacterium acnes, Bacillus cereus and Salmonella abony.*

6. A process of inhibiting microbial biofilm formation, said process comprising step of bringing into contact biofilm producing microbial cells and a preparation consisting essentially of partially purified extracellular metabolite preparation from probiotic bacterial strain *Bacillus coagulans*SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050 alone or said preparation combined with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis.*

7 The process of claim 6 wherein the biofilm producing microbial cell is one selected from the group comprising *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans.*

8. A process of preventing pH drop and further accumulation of biofilm of *Streptococcus mutans* in presence of sucrose, said process comprising step of bringing into contact biofilm/plaque producing *Streptococcus mutans* and a preparation consisting essentially of partially purified extracellular metabolite preparation from probiotic bacterial strain *Bacillus coagulans* SBC37-01 (Deposited in the Microbial Type Culture Collection and Gene Bank and was assigned the strain number MTCC 5856) exhibiting 99% genetic homology with the known bacterial strains *Bacillus coagulans* ATCC 31284, *Bacillus coagulans* NBRC 3887 and *Bacillus coagulans* ATCC 7050

alone or said preparation combined with a synergistic preservative blend comprising from about 61% w/w of thymol, about 38% of monolaurin and about 1% w/w of magnolol obtained from supercritical fluid extracts of *Magnolia officinalis.*

FIG. 1

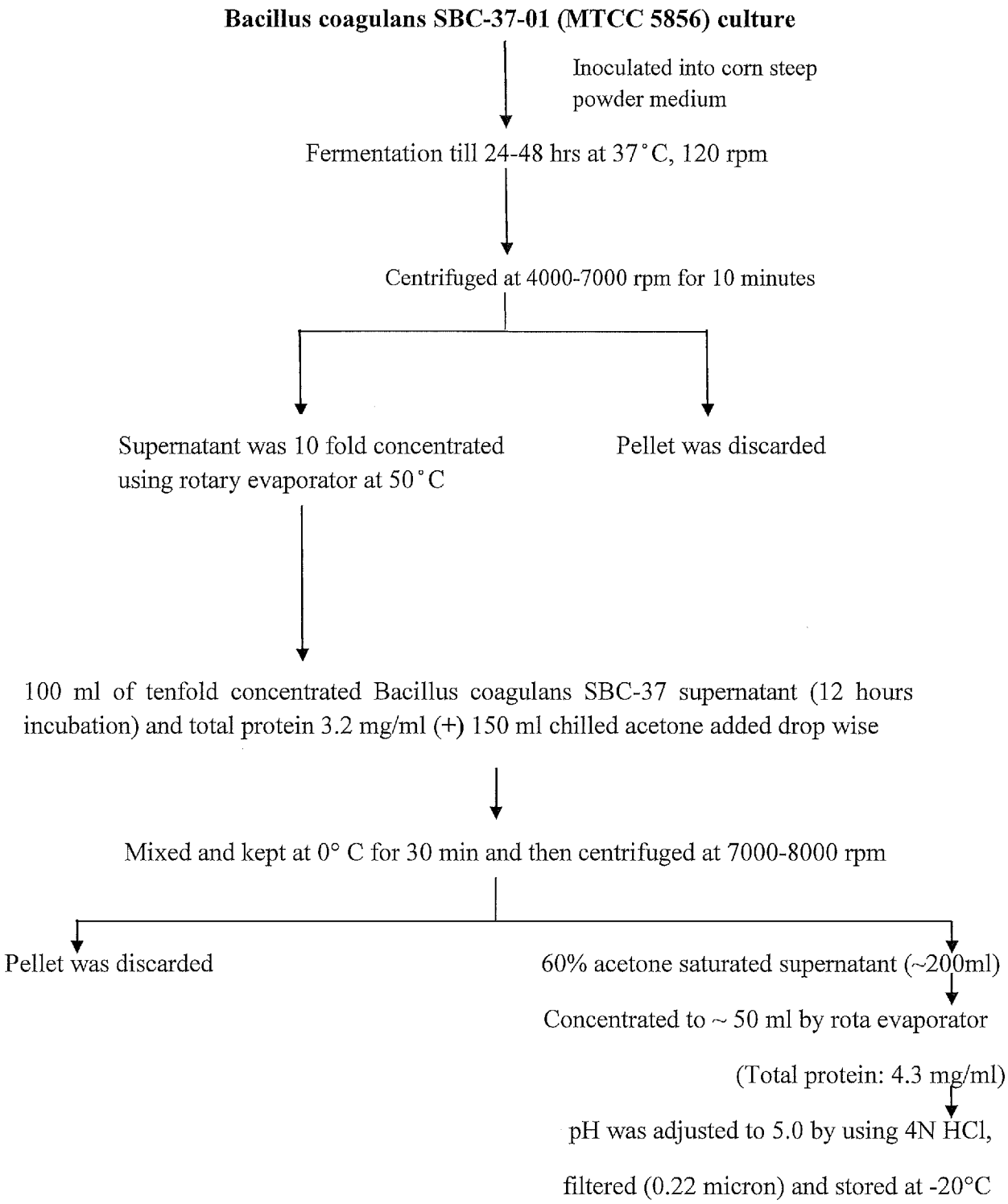

Bacillus coagulans SBC-37-01 (MTCC 5856) culture
Inoculated into corn steep powder medium
Fermentation till 24-48 hrs at 37°C, 120 rpm
Centrifuged at 4000-7000 rpm for 10 minutes
Supernatant was 10 fold concentrated using rotary evaporator at 50°C
Pellet was discarded
100 ml of tenfold concentrated Bacillus coagulans SBC-37 supernatant (12 hours incubation) and total protein 3.2 mg/ml (+) 150 ml chilled acetone added drop wise
Mixed and kept at 0° C for 30 min and then centrifuged at 7000-8000 rpm
Pellet was discarded
60% acetone saturated supernatant (~200ml)
Concentrated to ~ 50 ml by rota evaporator
(Total protein: 4.3 mg/ml)
pH was adjusted to 5.0 by using 4N HCl,
filtered (0.22 micron) and stored at -20°C

Fig.2

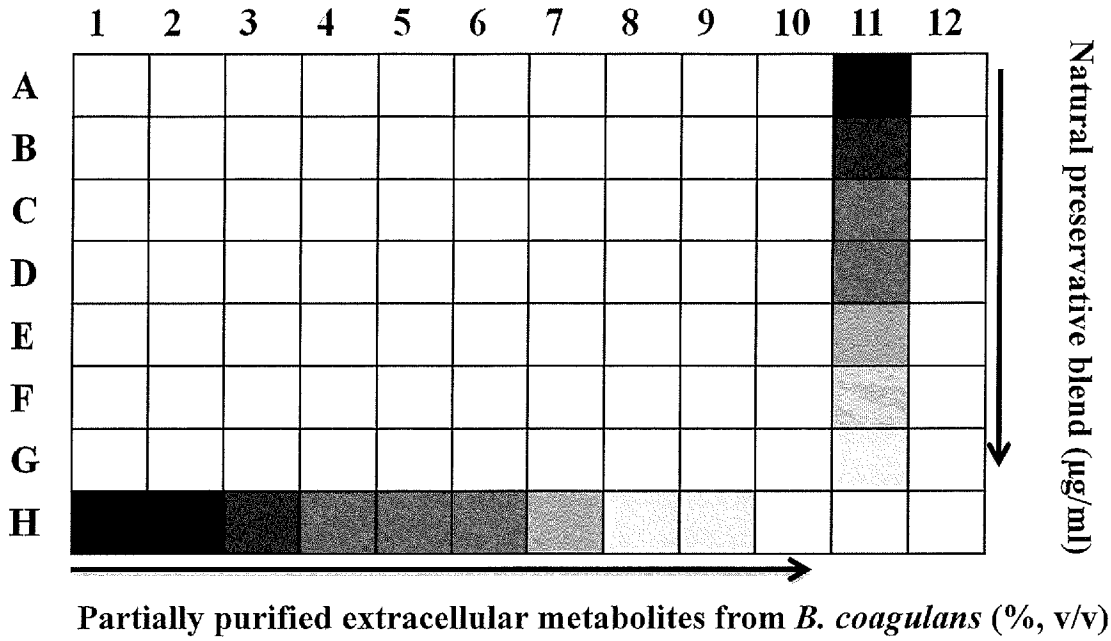

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Natural preservative blend (µg/ml)
Partially purified extracellular metabolites from B. coagulans (%, v/v)

Fig. 3A

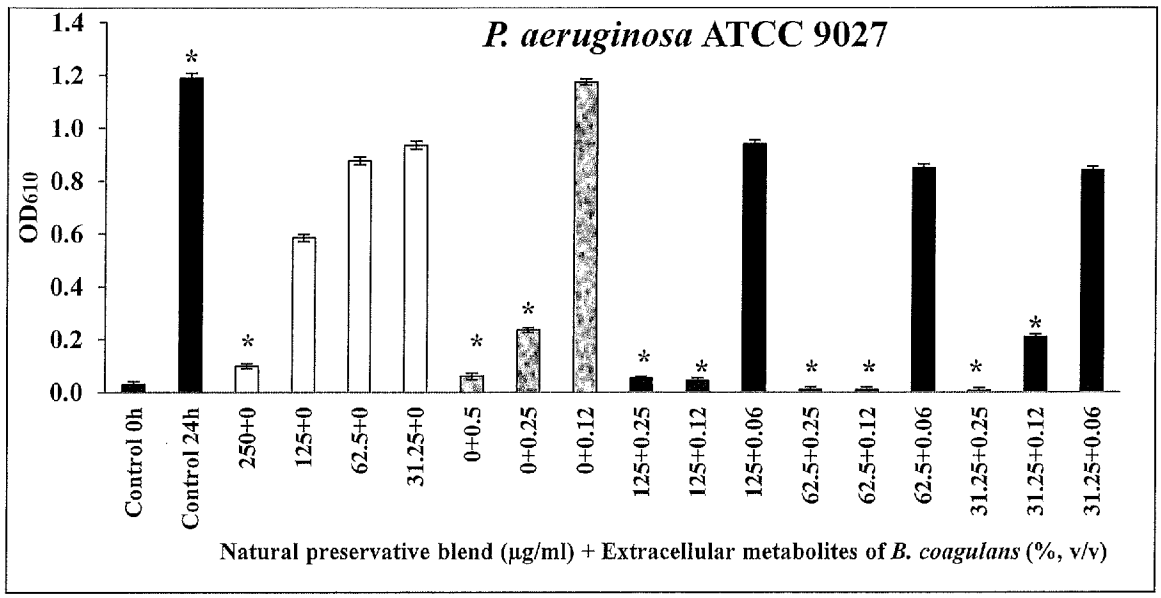

P. aeruginosa ATCC 9027
OD610
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control 0h
Control 24h
250+0
125+0
62.5+0
31.25+0
0+0.5
0+0.25
0+0.12
125+0.25
125+0.12
125+0.06
62.5+0.25
62.5+0.12
62.5+0.06
31.25+0.25
31.25+0.12
31.25+0.06
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3B

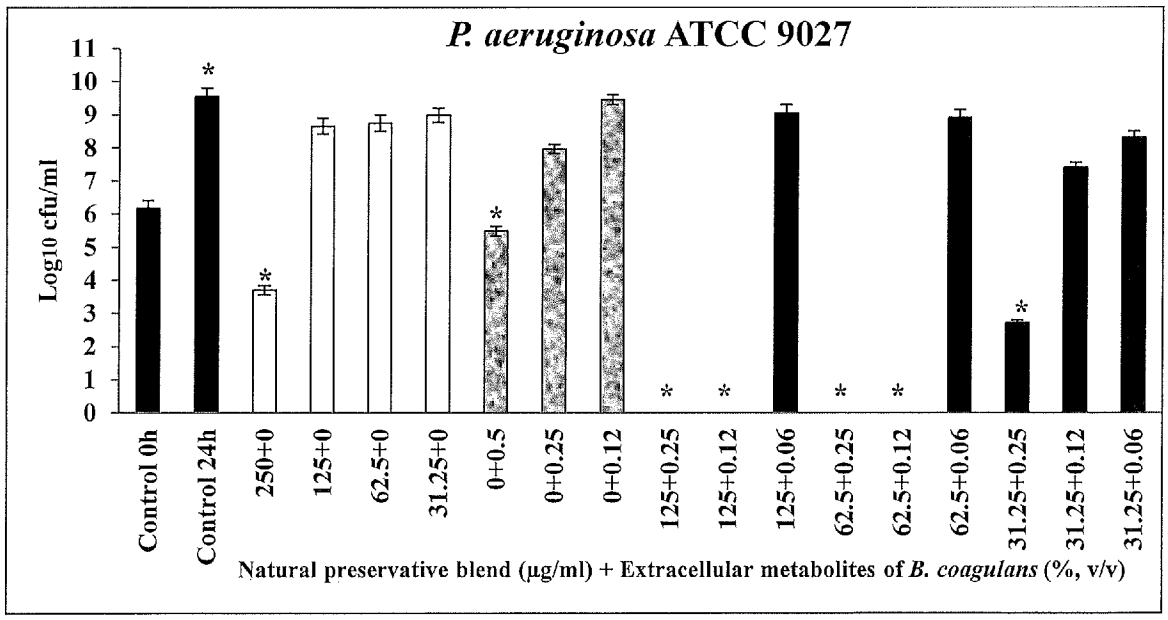

P. aeruginosa ATCC 9027
Log10 cfu/ml
11
10
9
8
7
6
5
4
3
2
1
0
Control 0h
Control 24h
250+0
125+0
62.5+0
31.25+0
0+0.5
0+0.25
0+0.12
125+0.25
125+0.12
125+0.06
62.5+0.25
62.5+0.12
62.5+0.06
31.25+0.25
31.25+0.12
31.25+0.06
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3C

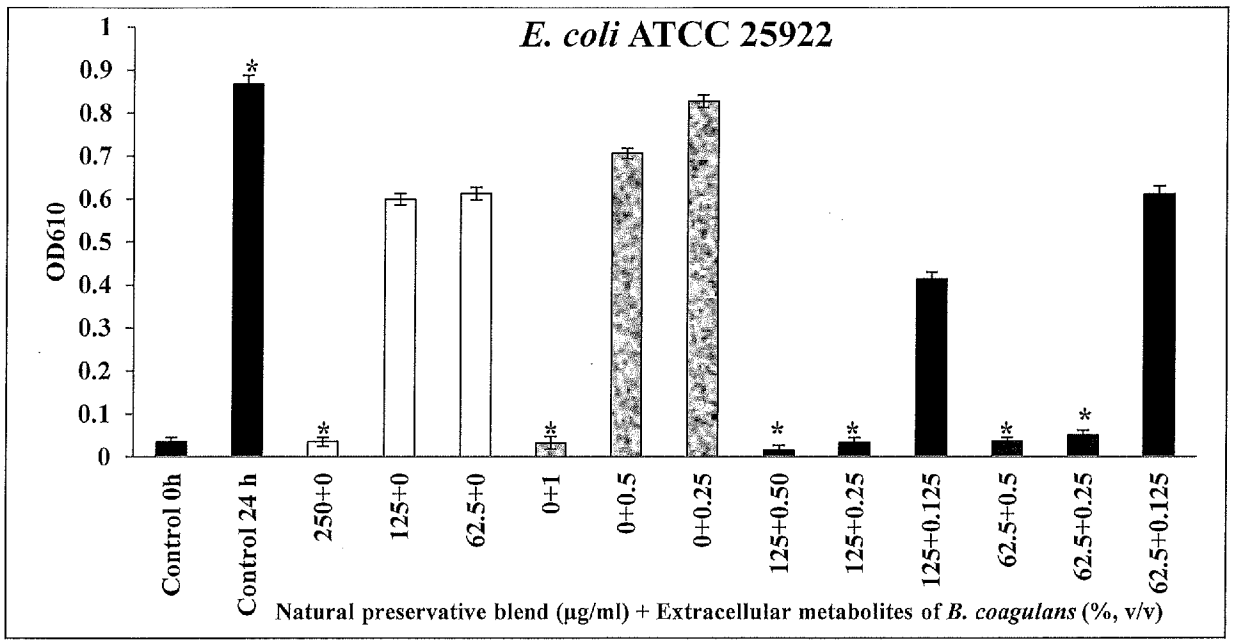

E. coli ATCC 25922
OD610
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control 0h
Control 24 h
250+0
125+0
62.5+0
0+1
0+0.5
0+0.25
125+0.50
125+0.25
125+0.125
62.5+0.5
62.5+0.25
62.5+0.125
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3D

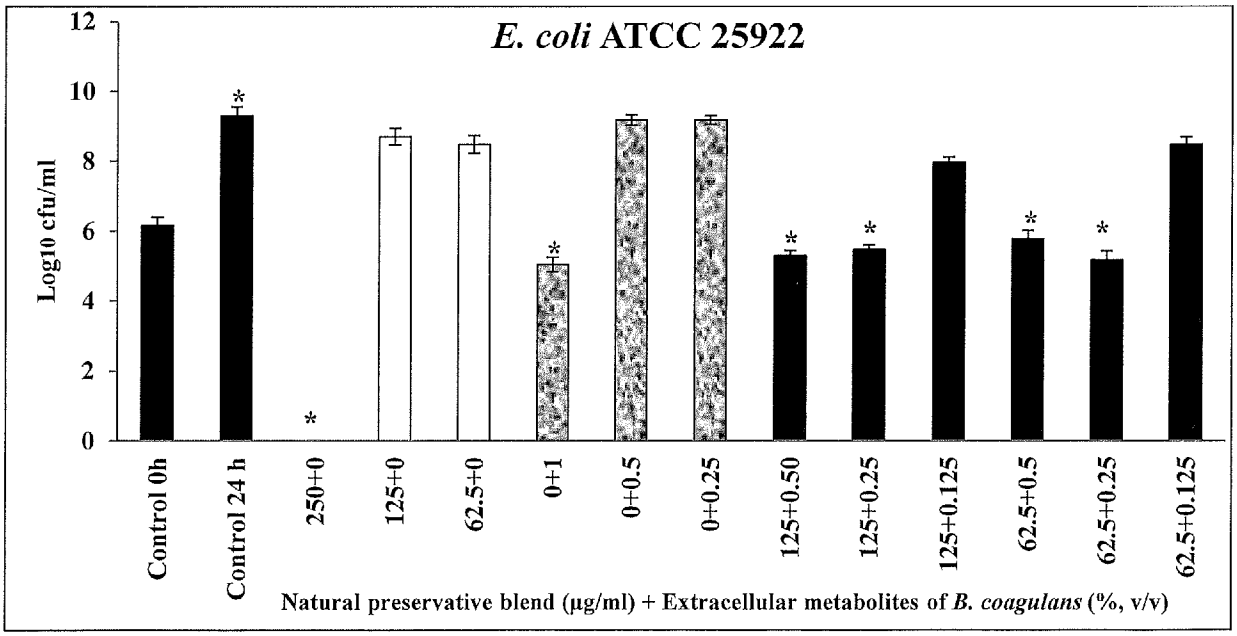

E. coli ATCC 25922
Log10 cfu/ml
12
10
8
6
4
2
0
Control 0h
Control 24 h
250+0
125+0
62.5+0
0+1
0+0.5
0+0.25
125+0.50
125+0.25
125+0.125
62.5+0.5
62.5+0.25
62.5+0.125
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig.3E

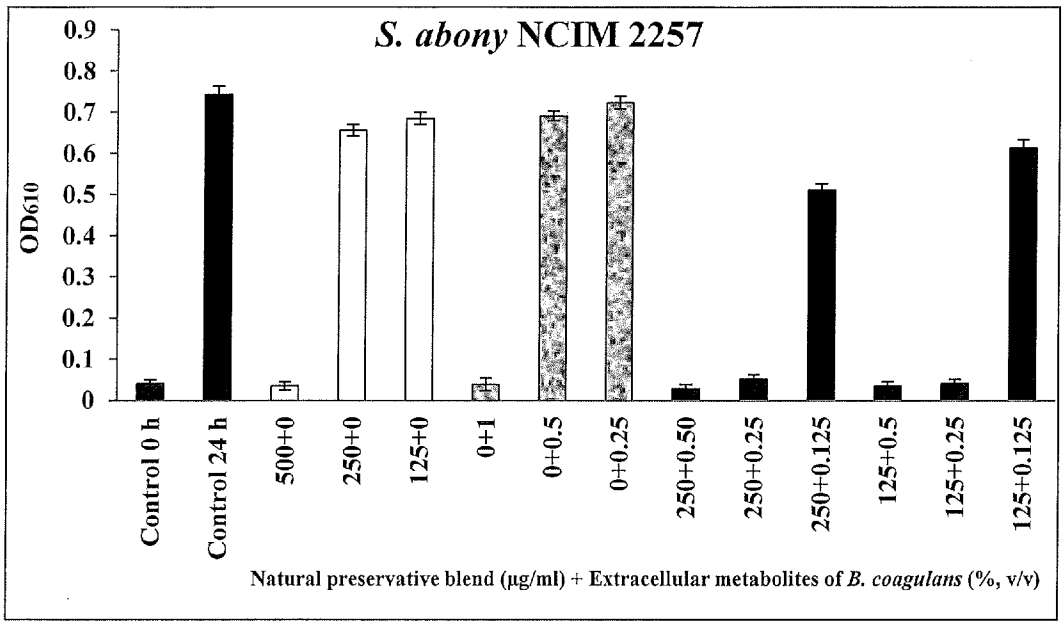

S. abony NCIM 2257
OD610
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control 0 h
Control 24 h
500+0
250+0
125+0
0+1
0+0.5
0+0.25
250+0.50
250+0.25
250+0.125
125+0.5
125+0.25
125+0.125
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3F

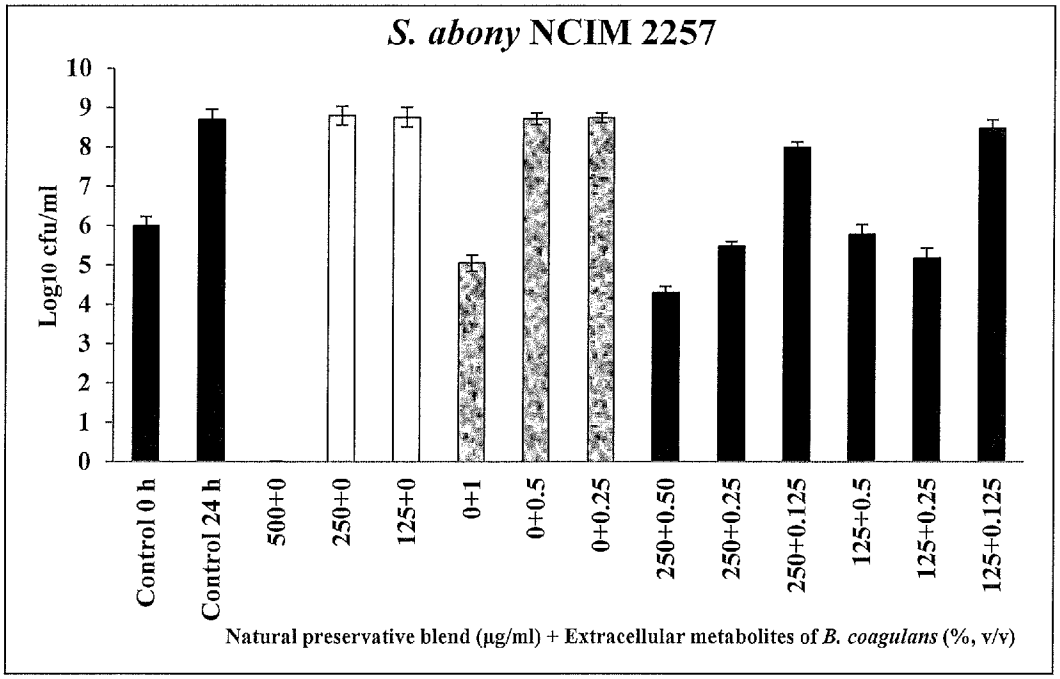

S. abony NCIM 2257
Log10 cfu/ml
10
9
8
7
6
5
4
3
2
1
0
Control 0 h
Control 24 h
500+0
250+0
125+0
0+1
0+0.5
0+0.25
250+0.50
250+0.25
250+0.125
125+0.5
125+0.25
125+0.125
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3G

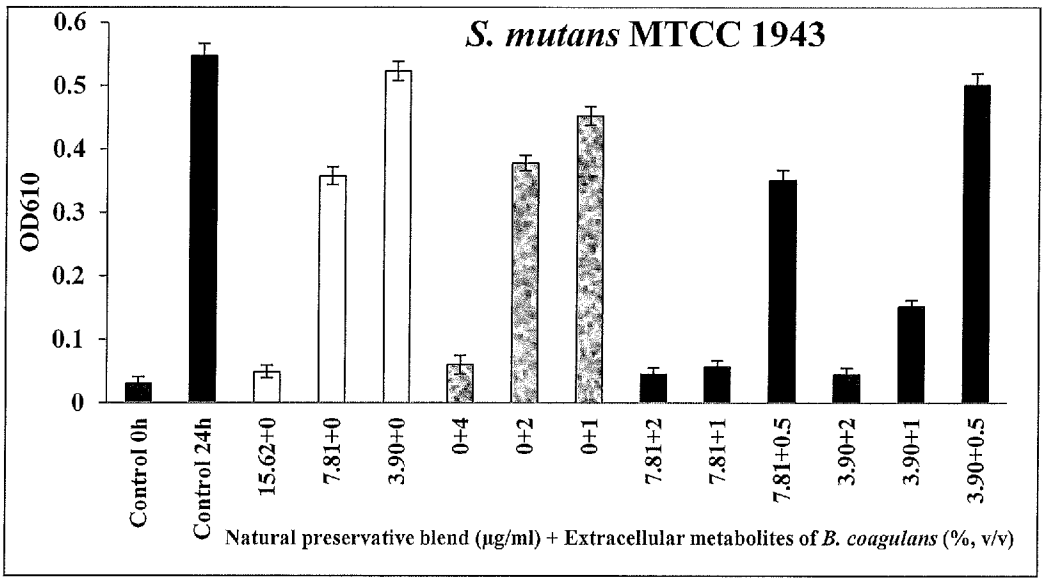

S. mutans MTCC 1943
OD610
0.6
0.5
0.4
0.3
0.2
0.1
0
Control 0h
Control 24h
15.62+0
7.81+0
3.90+0
0+4
0+2
0+1
7.81+2
7.81+1
7.81+0.5
3.90+2
3.90+1
3.90+0.5
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3H

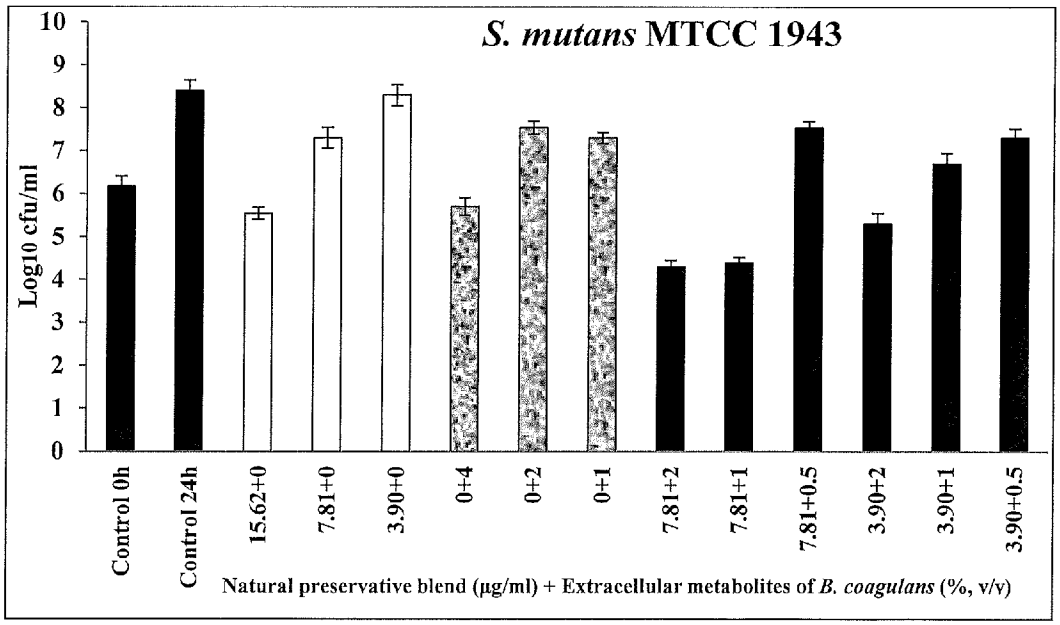

S. mutans MTCC 1943
Log10 cfu/ml
10
9
8
7
6
5
4
3
2
1
0
Control 0h
Control 24h
15.62+0
7.81+0
3.90+0
0+4
0+2
0+1
7.81+2
7.81+1
7.81+0.5
3.90+2
3.90+1
3.90+0.5
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3I

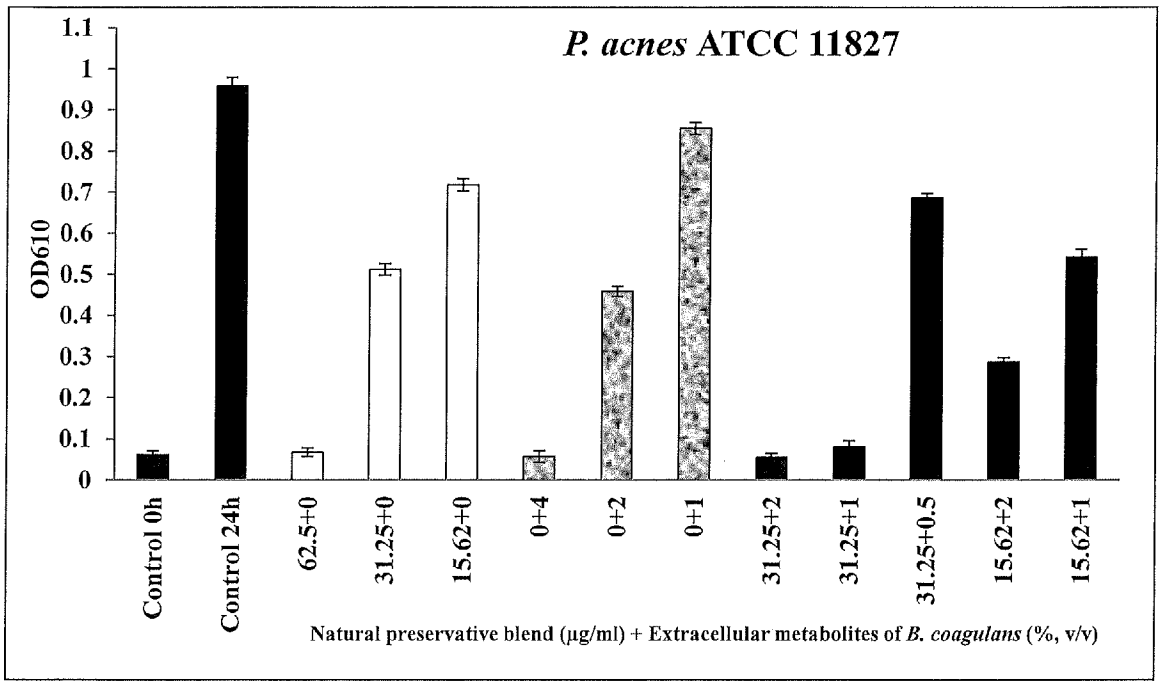

P. acnes ATCC 11827
OD610
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control 0h
Control 24h
62.5+0
31.25+0
15.62+0
0+4
0+2
0+1
31.25+2
31.25+1
31.25+0.5
15.62+2
15.62+1
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3J

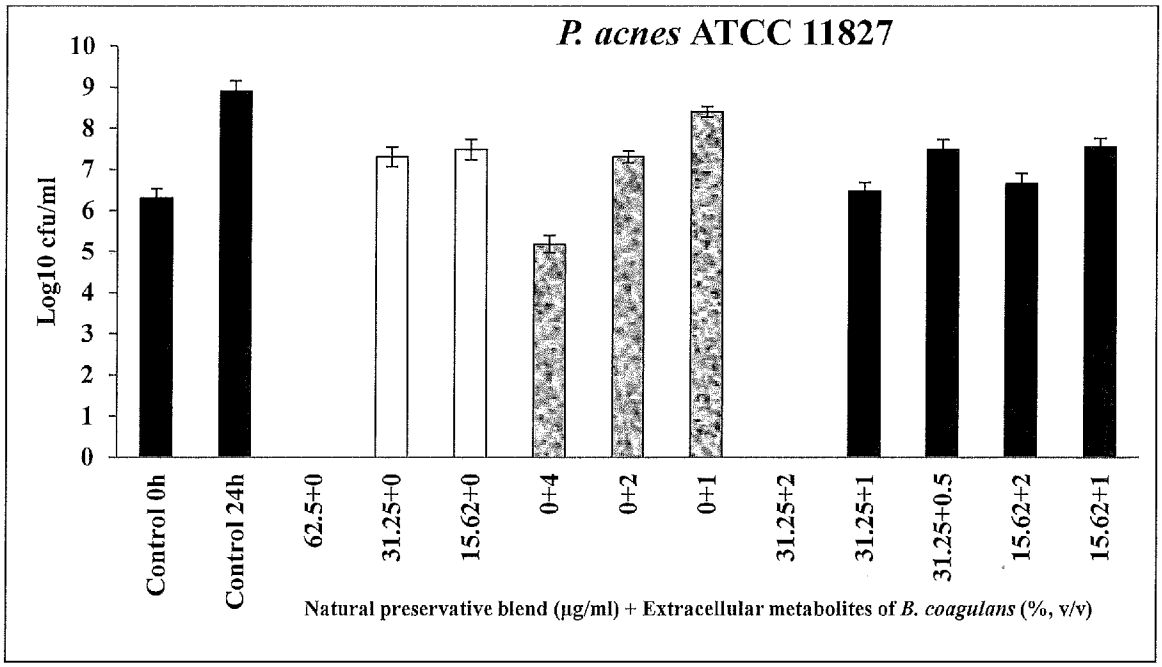

P. acnes ATCC 11827
Log10 cfu/ml
10
9
8
7
6
5
4
3
2
1
0
Control 0h
Control 24h
62.5+0
31.25+0
15.62+0
0+4
0+2
0+1
31.25+2
31.25+1
31.25+0.5
15.62+2
15.62+1
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3K

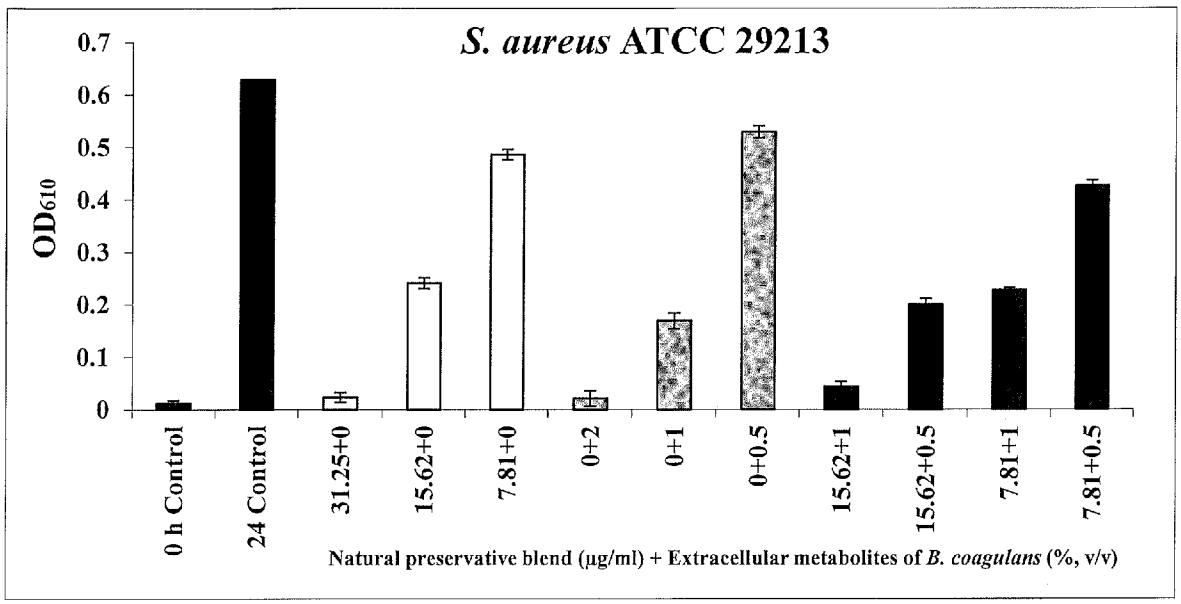

S. aureus ATCC 29213
OD610
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 h Control
24 Control
31.25+0
15.62+0
7.81+0
0+2
0+1
0+0.5
15.62+1
15.62+0.5
7.81+1
7.81+0.5
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3L

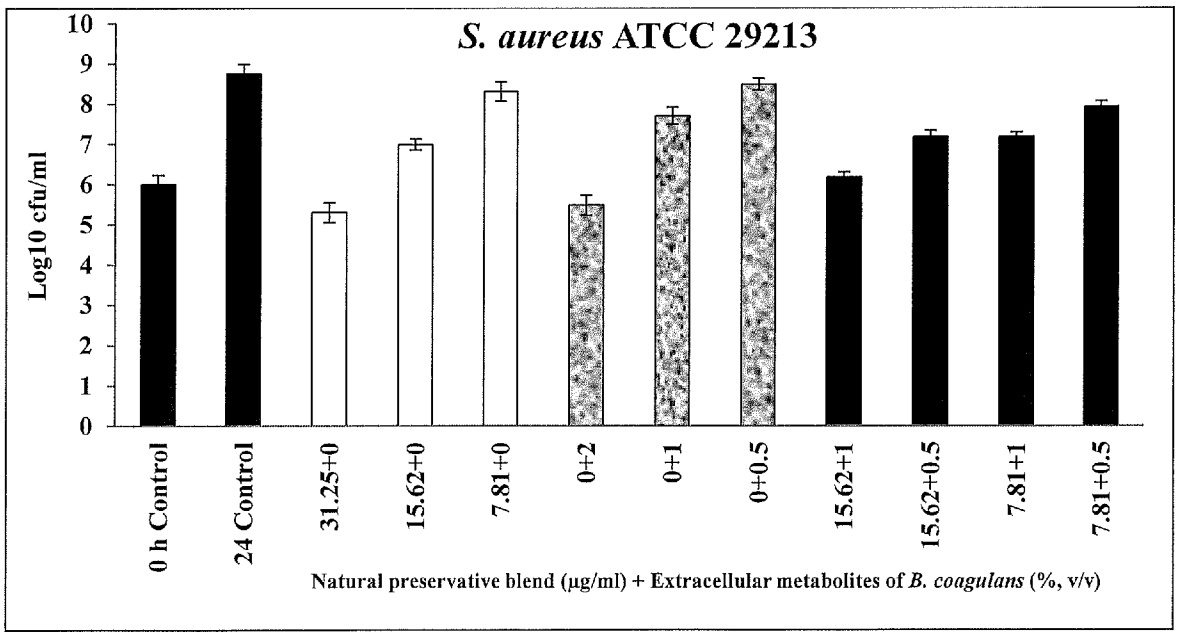

S. aureus ATCC 29213
Log10 cfu/ml
10
9
8
7
6
5
4
3
2
1
0
0 h Control
24 Control
31.25+0
15.62+0
7.81+0
0+2
0+1
0+0.5
15.62+1
15.62+0.5
7.81+1
7.81+0.5
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3M

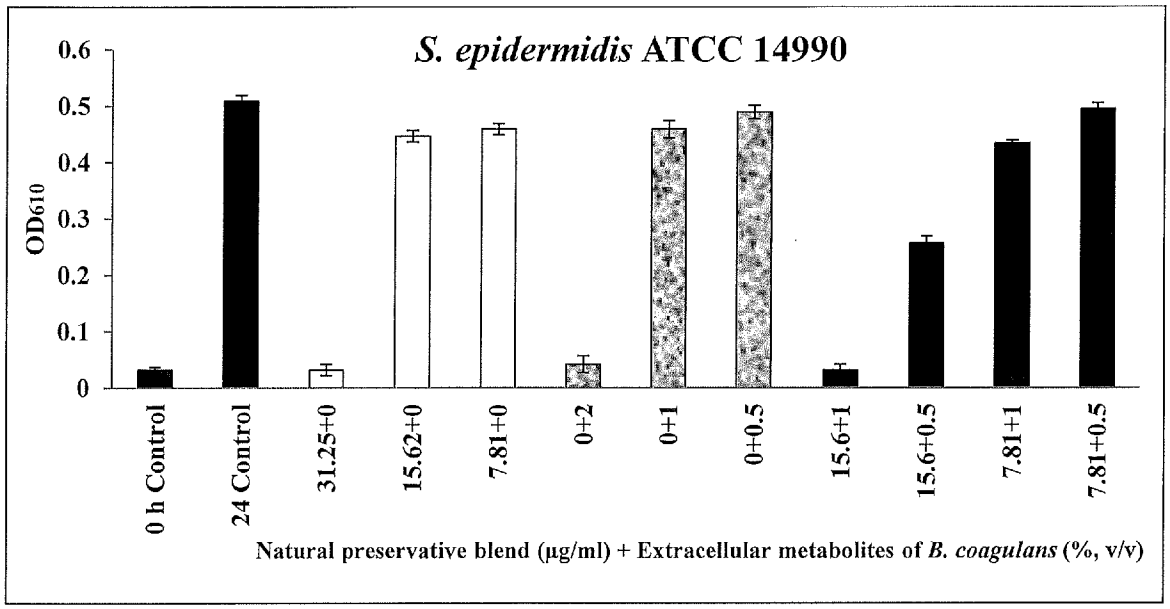

S. epidermidis ATCC 14990
OD610
0.6
0.5
0.4
0.3
0.2
0.1
0
0 h Control
24 Control
31.25+0
15.62+0
7.81+0
0+2
0+1
0+0.5
15.6+1
15.6+0.5
7.81+1
7.81+0.5
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3N

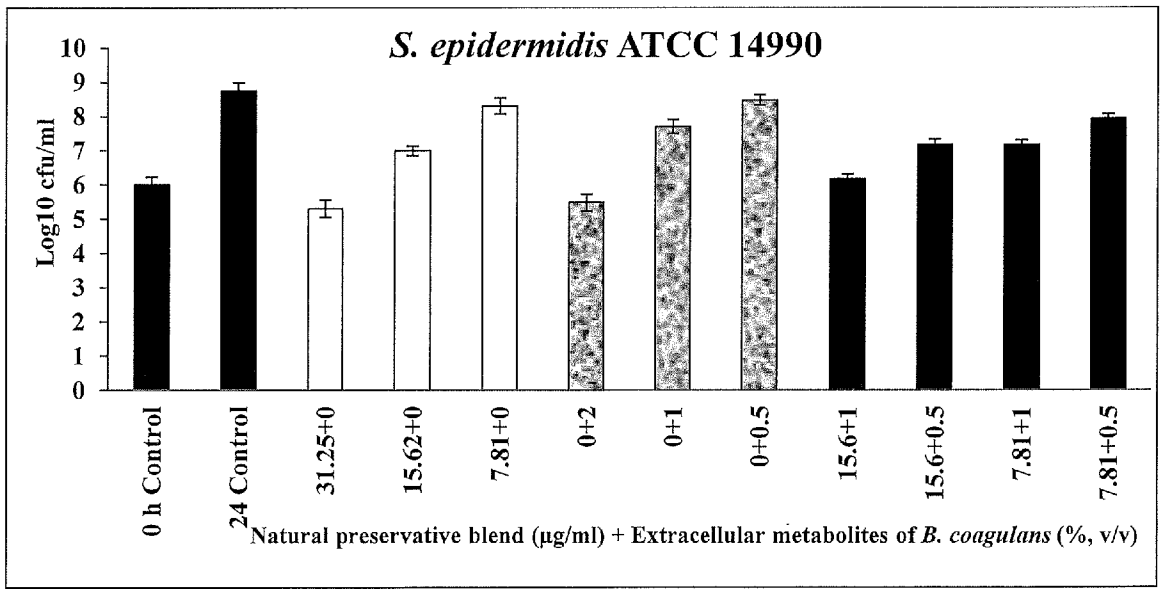

S. epidermidis ATCC 14990
Log10 cfu/ml
10
9
8
7
6
5
4
3
2
1
0
0 h Control
24 Control
31.25+0
15.62+0
7.81+0
0+2
0+1
0+0.5
15.6+1
15.6+0.5
7.81+1
7.81+0.5
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3O

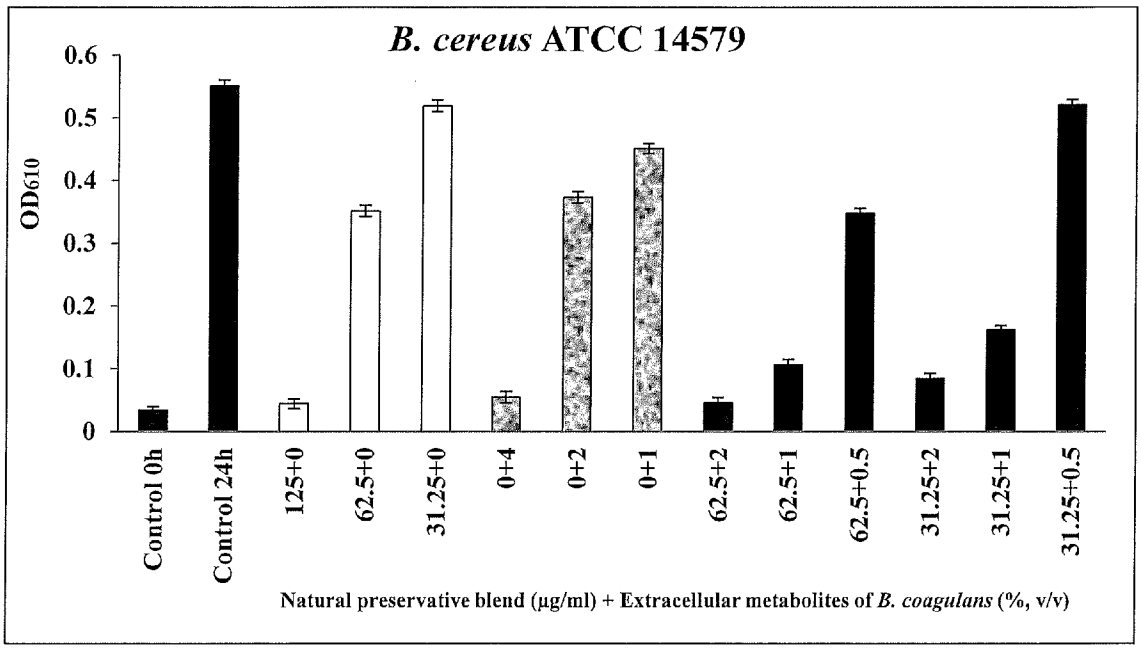

B. cereus ATCC 14579
OD610
0.6
0.5
0.4
0.3
0.2
0.1
0
Control 0h
Control 24h
125+0
62.5+0
31.25+0
0+4
0+2
0+1
62.5+2
62.5+1
62.5+0.5
31.25+2
31.25+1
31.25+0.5
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 3P

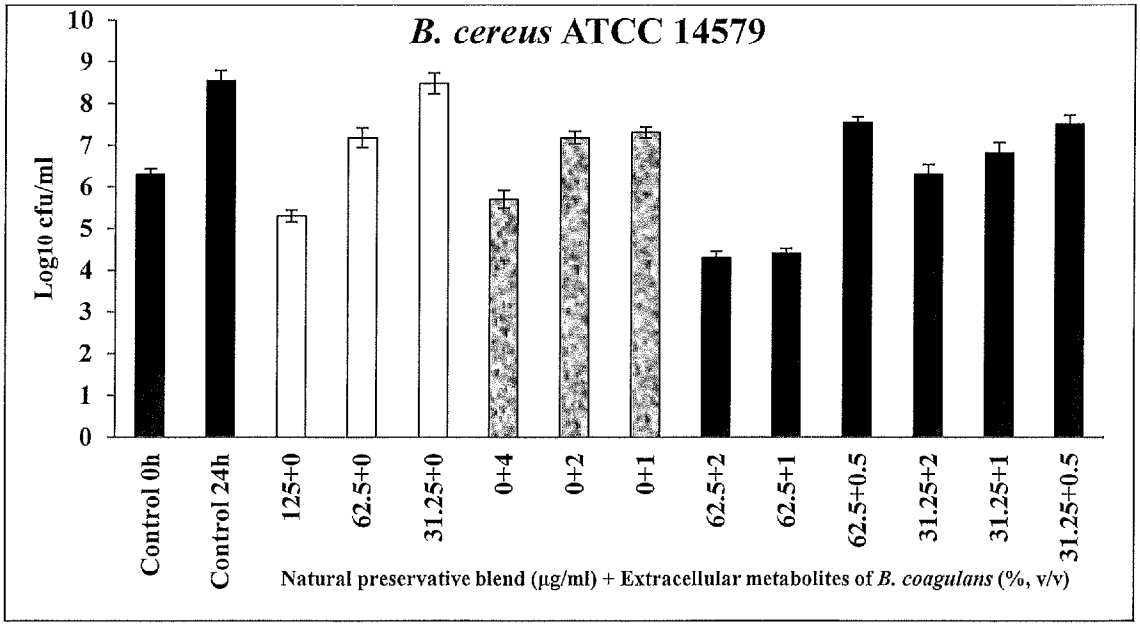

B. cereus ATCC 14579
Log10 cfu/ml
10
9
8
7
6
5
4
3
2
1
0
Control 0h
Control 24h
125+0
62.5+0
31.25+0
0+4
0+2
0+1
62.5+2
62.5+1
62.5+0.5
31.25+2
31.25+1
31.25+0.5
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 4A

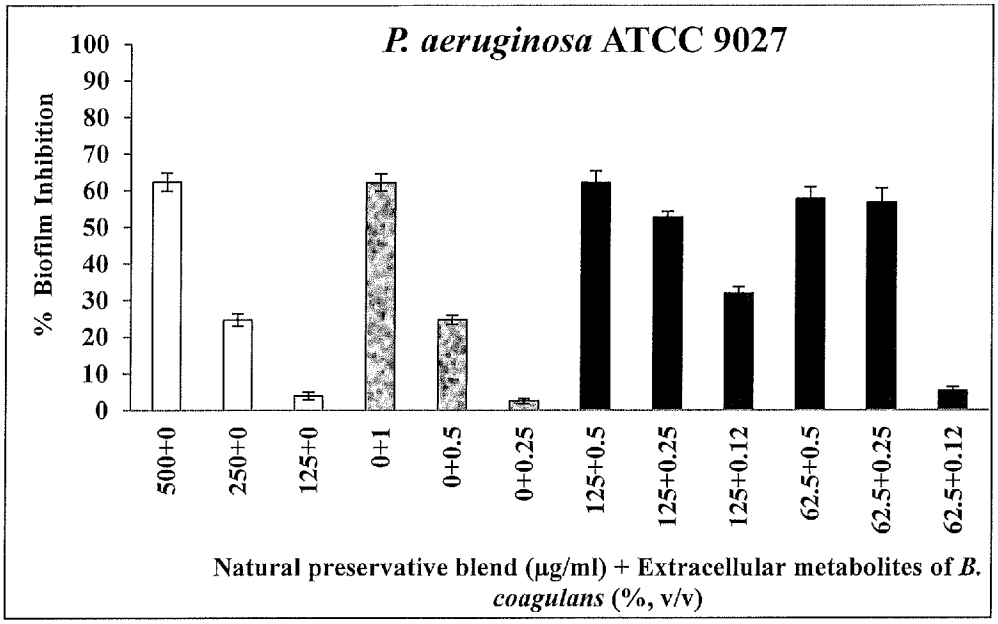

P. aeruginosa ATCC 9027
% Biofilm Inhibition
100
90
80
70
60
50
40
30
20
10
0
500+0
250+0
125+0
0+1
0+0.5
0+0.25
125+0.5
125+0.25
125+0.12
62.5+0.5
62.5+0.25
62.5+0.12
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 4B

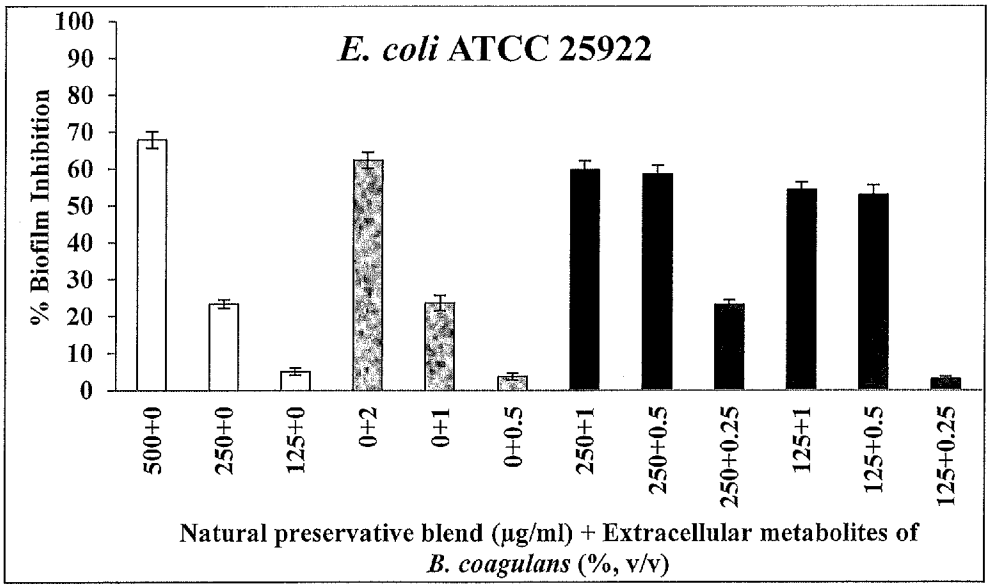

E. coli ATCC 25922
% Biofilm Inhibition
100
90
80
70
60
50
40
30
20
10
0
500+0
250+0
125+0
0+2
0+1
0+0.5
250+1
250+0.5
250+0.25
125+1
125+0.5
125+0.25
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 4C

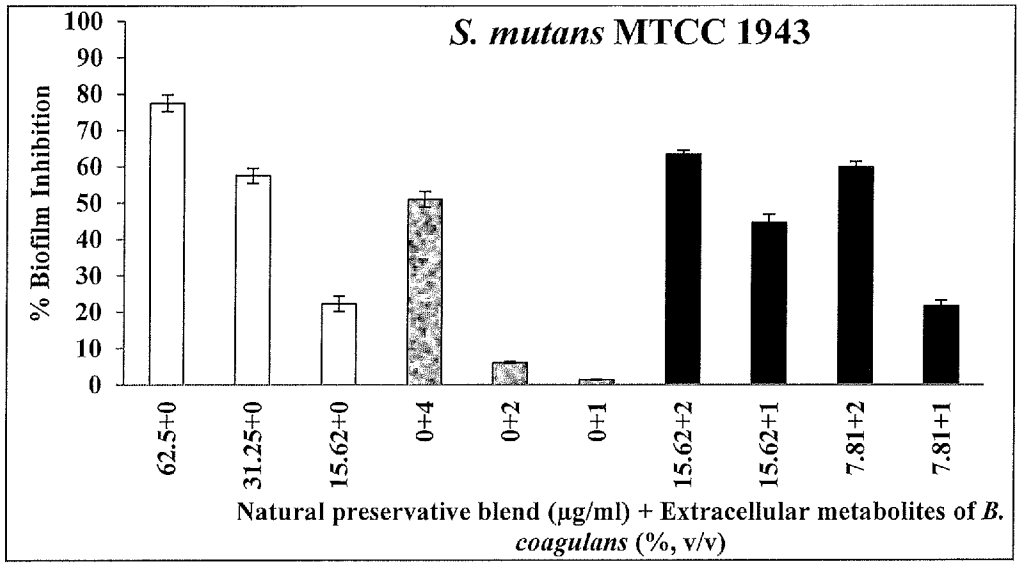

S. mutans MTCC 1943
% Biofilm Inhibition
100
90
80
70
60
50
40
30
20
10
0
62.5+0
31.25+0
15.62+0
0+4
0+2
0+1
15.62+2
15.62+1
7.81+2
7.81+1
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 4D

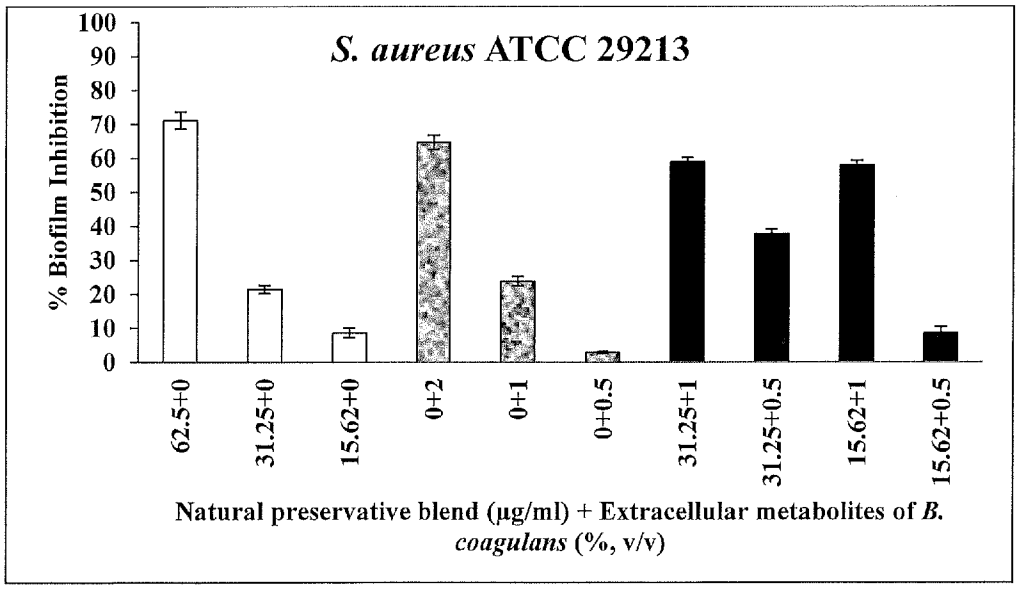

S. aureus ATCC 29213
% Biofilm Inhibition
100
90
80
70
60
50
40
30
20
10
0
62.5+0
31.25+0
15.62+0
0+2
0+1
0+0.5
31.25+1
31.25+0.5
15.62+1
15.62+0.5
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 4E

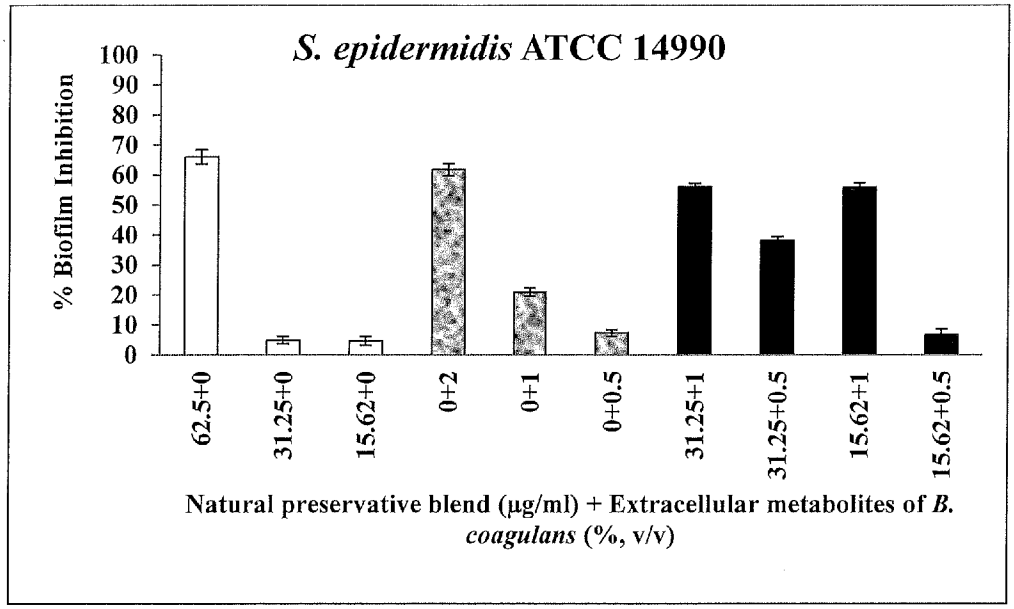

S. epidermidis ATCC 14990
% Biofilm Inhibition
100
90
80
70
60
50
40
30
20
10
0
62.5+0
31.25+0
15.62+0
0+2
0+1
0+0.5
31.25+1
31.25+0.5
15.62+1
15.62+0.5
Natural preservative blend (µg/ml) + Extracellular metabolites of B. coagulans (%, v/v)

Fig. 5A

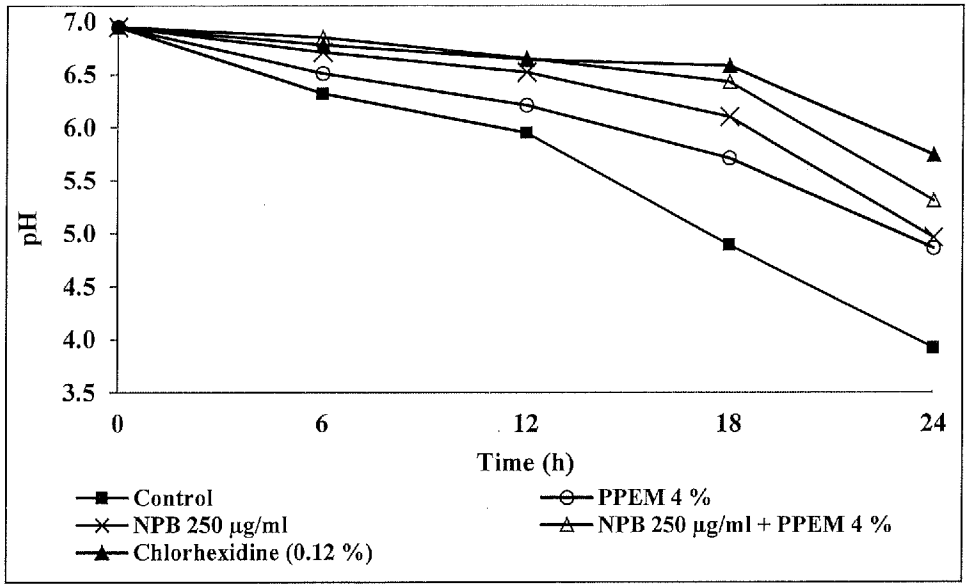
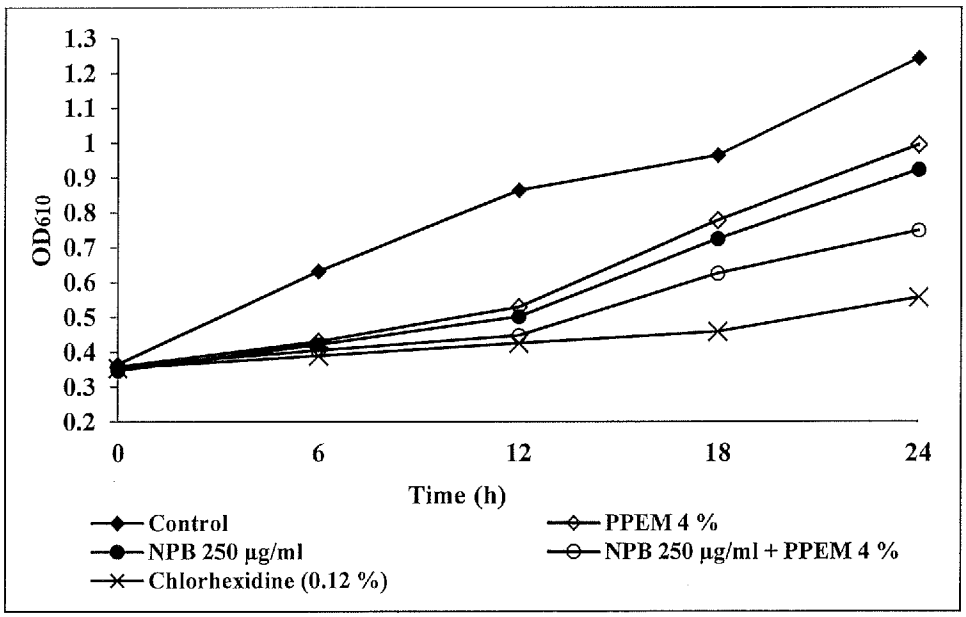

7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
pH
0
6
12
18
24
Time (h)
Control
PPEM 4 %
NPB 250 µg/ml
NPB 250 µg/ml + PPEM 4 %
Chlorhexidine (0.12 %)

Fig. 5B


1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
OD610
0
6
12
18
24
Time (h)
Control
PPEM 4 %
NPB 250 µg/ml
NPB 250 µg/ml + PPEM 4 %
Chlorhexidine (0.12 %)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/072331

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 1/20 (2015.01)
CPC - C12N 1/20 (2015.01)
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)
IPC(8) - C07C 59/52, 59/64, 65/21; C12N 1/20, 1/38, C12N 3/00; C12R 1/07 (2015.01)
CPC - C02F 3/00; C12N 1/005, 1/20, 1/38, 3/00 (2015.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C02F 3/00; C12N 1/005, 1/20, 1/38, 3/00 (2015.01) (keyword delimited)
US Classes - 435/242, 252.1, 252.5; 562/475, 478, 576; 568/337

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

PatBase, Orbit, Google Patents, Google Scholar, Google.
Search terms used: bacillus coagulans, ATCC, lactospore, MTCC 5856, SBC37-01, L. sporogenes, probiotic, sami labs, sabinsa

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, Y	US 2014/0308733 A1 (CALPIS CO LTD et al) 16 October 2014 entire document	1-8
A	WO 2012/079973 A1 (UNILEVER et al) 21 June 2012 (21.06.2012) entire document	1-8
A	US 2012/0251512 A1 (FARMER et al) 04 October 2012 (04.10.2012) entire document	1-8
A	MAJEED et al "Bacillus coagulans: Probiotic of choice," NutraCos Perbiotics/Probiotics, March/April 2012, Pgs. 19-21 entire document	1-8
A	"LACTOSPORE," Sabinsa Corporation, Pgs. 1-44, 31 August 2007 (31.08.2007). Retrieved from the Internet:<http://www.sabinsa.com/products/probiotics-synbiotics/lactospore/lactospore-booklet.pdf> on 09 March 2015 (09.03.2015). entire document	1-8
A	RIAZI et al. "Characterization of lactosporin, a novel antimicrobial protein produced by Bacillus coagulans ATCC 7050," Journal of Applied Microbiolog, Vol. 106, (2009), Pgs. 1370–1377. entire document	1-8

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

* 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	Date of mailing of the international search report
09 March 2015	29 APR 2015

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)