

Melanin and bacteriocin from marine bacteria inhibit biofilms of foodborne pathogens

M Laxmi, Noble K Kurian, S Smitha and Sarita G Bhat*

Department of Biotechnology, Cochin University of Science and Technology
Kochi 682 022, India

Received 17 November 2014; revised 27 May 2015; accepted 9 June 2015

Biofilms are widespread and a bane in food based industry for being associated with the outbreaks of several food related diseases. Biofilms are also a cause for concern for their resistance to antimicrobial agents. In the present study, the biocontrol of biofilm forming food pathogens was achieved using two bioactive compounds, namely, melanin and bacteriocin, obtained from marine bacteria. Partially purified melanin and bacteriocin BL8 were observed to show great reduction in the biofilm formation of food pathogens, even in minute quantities, and showed high antibiofilm activity. Multiple antibiotic resistance (MAR) index showed the multiple resistance of nine food pathogens. FTIR spectrum of the melanin used in the study showed two peaks, which are the characteristic features of standard melanin IR spectrum. Scanning electron micrographs showed the variation in the microbial mass and biofilm formation before and after treatment with the two bioactive compounds, evidently showing their antibiofilm activity.

Keywords: Bacteriocin, biocontrol, biofilm, melanin

Introduction

Biofilms are designated as a 'city of microbes'¹ and are defined as communities of microorganisms attached to a surface. It is proved that microorganisms undergo profound changes during their transition from planktonic organisms to cells that are part of a very complex, surface-attached community. These changes are reflected in the new phenotypic characteristics developed by biofilm bacteria and occur in response to a variety of environmental signals. Studies till date suggest that the planktonic-biofilm transition is a complex and highly regulated process. Biofilm formation involves several steps, including an initial attachment, formation of microcolony and production of extracellular polymeric substances (EPS), followed by maturation². In food systems, the attachment of microorganisms leading to biofilm formation may not only be undesirable but also detrimental³. Biofilms with human pathogens can impair food safety and are, therefore, a significant threat to the food industry. Several recent outbreaks of food borne illnesses can be clearly attributed to biofilms⁴. The ability of biofilm forming microorganisms to act collectively to

make a microbial colony stronger and more resistant to conventional methods of sanitation and food safety methods is intimidating⁵. The most important biofilm associated microbes belong to the genera *Vibrio*, *Salmonella*, *Pseudomonas*, *Listeria*, *Bacillus*, *Escherichia* and *Clostridium*, to name a few.

Control of biofilms remains the most persistent challenge in the food industry⁴. With the emergence of resistance to the traditional antibiotics, the development of alternative methods of control has been the focus of interest. The different strategies currently used for biofilm control methods can be classified as physical, chemical and biological. Physical methods include super-high magnetic fields, ultrasound treatment, high pulsed-electrical fields⁶ etc. Chemical methods include the use of different detergents like EDTA, EGTA, per acetic acid and chlorinated compounds⁷, while biological methods include use of several enzymes and various bioactive compounds from bacteria⁸.

Melanin production is universal, with several different kinds being produced by different microorganisms. Melanins are brown to black colored complex natural pigments, widely distributed throughout all living forms in nature and the three main types being eumelanins (black or brown), pheomelanins (yellow red) and allomelanins⁹. They

*Author for correspondence:
Tel: +91-9846033486, Fax: +91-484-2577595,
saritagbhat@gmail.com

exhibit several biological functions, such as, photo protection, thermoregulation, act as free radical sinks, cation chelators and antibiotics. In plants, melanin is incorporated in the cell walls as strengtheners⁷, while in humans it not only determines skin colour but also protects skin against UV light injury¹⁰. In the microbial world, melanin acts as a protective agent against environmental stresses. For example melanin-producing bacteria are more resistant to antibiotics¹¹ and, in fungi too, melanins are involved in pathogenesis¹². Vasanthabharathi and coworkers reported that crude melanin from *Streptomyces* sp. showed antibacterial activity against *E. coli* and *Lactobacillus vulgaris*¹³. Melanin derived from *Auricularia auriculara*, an edible jelly mushroom, significantly inhibited biofilm formation of *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3¹¹.

Bacteriocins produced by several species of bacteria are proteins/small peptides, which possess antibacterial activity and kill or inhibit the growth of other closely related bacteria by various mechanisms like increasing cell membrane permeability, inhibiting cell wall synthesis or by inhibiting DNase or RNase activity⁵. They were first characterized in Gram-negative bacteria and were found safe for use as natural preservatives in food industry¹⁴. Colicins, a type of bacteriocin, produced by *E. coli* were thoroughly studied by Lazdunski¹⁵. The term 'natural' is compromised when bacteriocin is obtained from genetically modified microorganism. Though nisin is currently the only bacteriocin approved for use as food preservative, several other bacteria are reported to produce bacteriocins that can safely be used in food industry¹⁶. The genus *Bacillus* includes representatives, such as, *B. subtilis* and *B. licheniformis*, those are 'generally recognized as safe' (GRAS)⁵ and thus find application in food processing environment, especially in the control of food pathogens and spoilage microorganisms. The qualification concerning 'qualified presumption of safety' (QPS) for *Bacillus* sp. is modified to 'absence of food poisoning toxins, absence of surfactant activities and absence of enterotoxic activities'¹⁵.

In the present study, ability for biofilm inhibition of nine strong biofilm forming food pathogens by melanin from *Providencia rettgeri* (BTKKS1) (GenBank acc. no.: KF515633) and a bacteriocin BL8 from *B. licheniformis* (BTHT8) (GenBank acc. no.: HM030819)¹⁶ was evaluated. *In vitro* biofilm formation and inhibition was tested using microtiter

plate assay with crystal violet staining. Multiple antibiotic resistance (MAR) index was calculated after demonstrating the presence of antibiotic resistance/sensitivity of the nine food pathogens. Scanning electron microscopy (SEM) was used to visualize biofilm inhibition by these two biomolecules.

Materials and Methods

Extraction and Purification of Melanin Pigment

Extraction and purification of melanin was performed according to Sajjan *et al*¹⁷ from *P. rettgeri* (BTKKS1), which was available in the culture collection of the Department of Biotechnology, Cochin University of Science and Technology (CUSAT), Kochi (Kerala), India. Cell free supernatant was acidified to pH 2 using 1 N HCl to precipitate melanin. Precipitate was continuously re-precipitated with 0.1 N HCl and further washed in ethanol to obtain pure melanin.

Chemical Analysis of Melanin

Solubility of melanin in deionized water, 1 N HCl, 1 N NaOH, 1N KOH, ethanol, acetone, chloroform, benzene, xylene, hexane and acetonitrile was evaluated. Reaction with oxidizing agents, such as, hydrogen peroxide (H₂O₂) and reducing agent sodium sulfite (Na₂SO₃) was determined¹⁸. All estimations were compared with synthetic melanin (Sigma) as standard.

FTIR Analysis of Pigment

FTIR was done as a part of melanin characterization to prove its structural similarity to the published reports of standard melanin¹⁹. IR spectrum was recorded at 4,000-400 cm⁻¹ using a Thermo Nicolet, Avatar 370 spectrophotometer.

Characterization of bacteriocin from *B. licheniformis*

The bacteriocin BL8 was extracted and partially purified from the strain *B. licheniformis* (BTHT8), which was also available in the culture collection of the Department of Biotechnology, CUSAT, Kochi. Bacteriocin BL8 was partially purified by ammonium sulphate fractionation and was subjected to glycine SDS-PAGE. The band exhibiting antimicrobial activity was electroeluted¹⁶.

Bacterial Strains and Growth Conditions

For the antibiofilm experiment, nine bacteria, *viz.*, *B. altitudinis* (KF460551), *B. pumilus* (KF460552), *P. aeruginosa* (KF460558), *Brevibacterium casei*

(KF573739), *Staphylococcus warneri* (KF573740), *Micrococcus luteus* (KF573741), *B. niacini* (KF573743), *Bacillus* sp. (KF573744) and *Geobacillus stearothermophilus* (KF573747) were cultured routinely in Tryptic soy broth (TSB) (Hi Media) at 37°C for 24 h. All the nine pathogens are reported to be strong biofilm producers²⁰.

Antibacterial Activity of Melanin and Bacteriocin BL8

The antibacterial activity of melanin and bacteriocin BL8 at different concentrations was tested against all the nine test pathogens using liquid broth assay²¹.

Antibiofilm Activity, Determination of Biofilm Inhibitory Concentration and Calculation of MAR Index

The quantification and inhibition of biofilm formation by melanin was tested using microtiter plate assay using crystal violet dye²². Based on the absorbance (A_{570}) produced by the bacterial films, the biofilm formation and inhibition was noted. All tests were interpreted thrice independently²³. Statistical evaluations were done by ANOVA, followed by Sign Test using StatsDirect statistical software (version 3.0, Cheshire, UK) computer program. The concentration of both compounds used for the assay was previously estimated and it was 100 µg/mL for melanin, while 2380 µg/mL for bacteriocin. The melanin and bacteriocin samples were serially diluted and checked for antibiofilm activity.

The biofilm inhibitory concentration (BIC) was determined. All strong biofilm producers²⁰ were tested for antibiotic sensitivity in accordance with the Kirby-Bauer method²⁴ with 12 different antibiotics (HiMedia, Mumbai, India) belonging to different classes, namely, ampicillin, azithromycin, cefixime, cefuroxime, ceftriazone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, norfloxacin, tetracycline and trimethoprim. The results were interpreted as per Clinical and Laboratory Standards Institute (CLSI). The MAR index was also calculated in accordance with the antibiogram drawn for all the biofilm forming food pathogens used in the study²⁵.

Scanning Electron Microscopy for Visualizing Biofilm Biocontrol by Melanin and Bacteriocin BL8

The SEM was used to visualize the effect of melanin on biofilm formation. The bacterial culture was inoculated and kept for biofilm formation in an incubator for 24 h at 37°C. Apart from control, the BIC of melanin and bacteriocin were added separately to all bacterial cultures and cultures were incubated as

above. Fixation for SEM was done with little modifications as shown in Table 1²⁶.

Results and Discussion

Extraction and Purification of Melanin

The melanin was extracted and purified from *P. rettgeri* (BTKKS1) (KF515633) and the concentration used for the assay was 100 µg/mL.

Chemical Reactivity of Melanin

Melanin from BTKKS1 strain was found to be soluble in alkaline solvents like sodium hydroxide and potassium hydroxide. However, the pigment showed least solubility in water and common organic solvents. Oxidizing (30% H₂O₂) and reducing (Na₂SO₃) agents decolorized the pigment.

IR spectrum showed a broad absorption peak around 3400 cm⁻¹, which indicated the presence of the -OH group. Peak around 1600 cm⁻¹ was attributed to aromatic ring C=C stretching. Both peaks are characteristic features of melanin IR spectrum. The spectrum also showed similarity with those in earlier reports²¹ and with the synthetic and sepia melanin standards included in the study. The data is shown in Fig. 1.

Antibacterial Activity and MIC of Melanin and Bacteriocin BL8

The antibacterial activity of melanin and bacteriocin BL8¹⁶ at different concentrations was tested against 9 food pathogens using liquid broth assay. MIC was calculated to be 100 µg/mL for melanin and 2380 µg/mL for bacteriocin where no visible growth was obtained (Tables 2 & 3).

Antibiofilm Activity and BIC of Melanin and Bacteriocin BL8

The antibiofilm activity was tested with the purified melanin (100 µg/mL) and bacteriocin BL8 (2380 µg/mL) samples after serial dilutions.

Table 1—Fixation procedure of Scanning Electron Microscopy

Steps	Solution	Time	Repetitions
1 Primary fixation	2.5% glutaraldehyde	1 h at RT*	-
2 Wash	0.1 M Sodium phosphate buffer (pH 7.3)	5-10 min	3-5
3 Dehydration	25% ethanol	5 min	-
	50% ethanol	5 min	-
	75% ethanol	5 min	-
	90% ethanol	5 min	-
	100% ethanol	5-10 min	2

*RT=Room temperature

Treatment with melanin (100 $\mu\text{g}/\text{mL}$) caused inhibitory effect ($p>0.5$) on the biofilm forming capability of 6 of the 9 isolates tested. It caused significant reduction of biofilm formed by

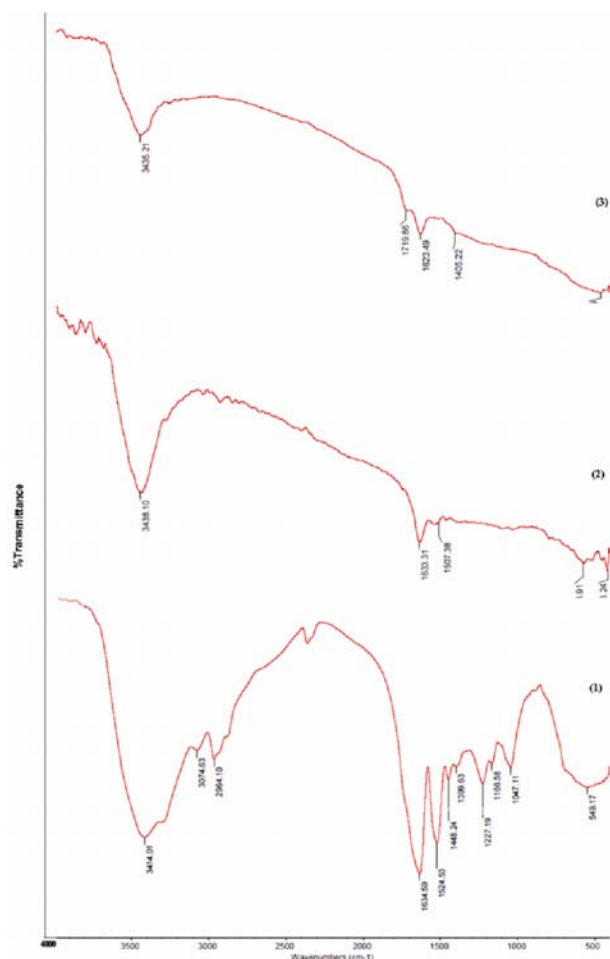


Fig. 1—FT-IR spectrum of melanin from *P. rettgeri* strain BTKKS1 in comparison to standards. [(1) BTKKS1 melanin; (2) Sepia melanin; & (3) Synthetic melanin.]

B. altitudinis (57%), *P. aeruginosa* (65%), *B. casei* (75%), *S. warneri* (69%), *B. niacini* (37%) and *Bacillus* sp. (55%). However, biofilm formation by *B.pumilus*, *M. luteus* and *G. stearothermophilus* could not be controlled (Fig. 2). The minimum concentration at which melanin inhibited the biofilm formation for all the isolates (BIC) was found to be 16×10^{-2} $\text{ng}/\mu\text{L}$ after proper dilutions (10^{-1} - 10^{-4}).

Fig. 3 shows the percentage of biofilm inhibition ($p>0.5$) by bacteriocin BL8 where the bacteriocin was observed to inhibit the biofilm formation of all the 9 strains of food pathogens used in the study. There was a greater reduction of the biofilm formation by all organisms under study ranging from 61% in *B. casei* to 83% in *Bacillus* sp. The BIC of bacteriocin BL8 was calculated to be 3.8 $\text{ng}/\mu\text{L}$ after appropriate dilutions (10^{-1} - 10^{-4}) for all test organisms under study.

The melanin from different bacteria and fungi are reported to have antibiofilm activity^{27,28}. In this study, melanin from marine *P. rettgeri* proved to be effective in controlling biofilm formed by most food pathogens tested. Remarkably, melanin was also successful in controlling biofilm formed by *P. aeruginosa*, which is the most pathogenic among all the isolates tested and a strong biofilm producer in the food industry. It was shown to be resistant to multiple antibiotics²⁰. The melanins are reported to be safe for consumption in minor quantities and thus provide an effective strategy for biocontrol of biofilms.

There are also several reports that bacteriocins are safe antimicrobials for food preservation¹⁴. Marugg *et al*²⁹ reported a method of inhibiting bacteria using novel lactococcal bacteriocin. Nisin compositions are also used as food preservatives³⁰. Pediocin, enterocin, lactocin and several other bacteriocins are also reported to be safe for consumption through food¹⁵.

Table 2—Antibacterial activity of melanin of different concentrations against nine foodborne pathogens using broth assay

Test organism	Positive control ($\times 10^8$ cfu/mL)	On addition of melanin (25 $\mu\text{g}/\text{mL}$) ($\times 10^4$ cfu/mL)	On addition of melanin (50 $\mu\text{g}/\text{mL}$) (cfu/mL)	On addition of melanin (100 $\mu\text{g}/\text{mL}$) (cfu/mL)
<i>Bacillus altitudinis</i> (BTMW1)	3.1 \pm 0.1414	1.25 \pm 0.2121	65 \pm 0.1414	0
<i>B. pumilus</i> (BTMY2)	2.7 \pm 0.2828	2.1 \pm 0.1414	198 \pm 0.2828	50 \pm 0.2121
<i>Pseudomonas aeruginosa</i> (BTRY1)	3.6 \pm 0.2828	2.8 \pm 0.1414	97 \pm 0.0707	0
<i>Brevibacterium casei</i> (BTDF1)	2.4 \pm 0.2121	1.9 \pm 0.0707	78 \pm 0.2121	0
<i>Staphylococcus warneri</i> (BTDF2)	1.35 \pm 0.2121	0.97 \pm 0.0070	39 \pm 0.1414	0
<i>Micrococcus luteus</i> (BTDF3)	2.05 \pm 0.0707	1.75 \pm 0.0707	169 \pm 0.1414	40 \pm 0.2828
<i>B. niacini</i> (BTDP3)	2.15 \pm 0.2121	1.91 \pm 0.0707	89 \pm 0.2828	0
<i>Bacillus</i> sp. (BTSD1)	3.6 \pm 0.1414	2.85 \pm 0.0707	94 \pm 0.0707	0
<i>Geobacillus stearothermophilus</i> (BTFF2)	2.2 \pm 0.2828	1.85 \pm 0.0707	177 \pm 0.2121	20 \pm 0.0141

*MIC of melanin=100 $\mu\text{g}/\text{mL}$ (at which no visible growth is obtained)

Table 3—Antibacterial activity of bacteriocin BL8 at different concentrations against nine foodborne pathogens by broth assay

Test organism	Positive control ($\times 10^{-8}$ cfu/mL)	On addition of bacteriocin (1500 μ g/mL) ($\times 10^{-4}$ cfu/mL)	On addition of bacteriocin (2000 μ g/mL) (cfu/mL)	On addition of bacteriocin (2380 μ g/mL) (cfu/mL)	On addition of bacteriocin (2500 μ g/mL) (cfu/mL)
<i>Bacillus altitudinis</i> (BTMW1)	3.10 \pm 0.1414	0.945 \pm 0.2121	120 \pm 0.0141	0	0
<i>B. pumilus</i> (BTMY2)	2.70 \pm 0.2828	0.970 \pm 0.0141	150 \pm 0.0212	0	0
<i>Pseudomonas aeruginosa</i> (BTRY1)	3.60 \pm 0.2828	0.870 \pm 0.0141	70 \pm 0.0707	0	0
<i>Brevibacterium casei</i> (BTDF1)	2.40 \pm 0.2121	2.050 \pm 0.0707	300 \pm 0.0707	0	0
<i>Staphylococcus warneri</i> (BTDF2)	1.35 \pm 0.2121	0.880 \pm 0.0141	95 \pm 0.0212	0	0
<i>Micrococcus luteus</i> (BTDF3)	2.05 \pm 0.0707	1.815 \pm 0.0212	85 \pm 0.0141	0	0
<i>B. niacini</i> (BTDP3)	2.15 \pm 0.2121	0.930 \pm 0.0707	110 \pm 0.0212	0	0
<i>Bacillus</i> sp. (BTSD1)	3.60 \pm 0.1414	1.915 \pm 0.0212	90 \pm 0.0707	0	0
<i>Geobacillus stearothermophilus</i> (BTFF2)	2.20 \pm 0.2828	1.310 \pm 0.0141	80 \pm 0.0141	0	0

*MIC of bacteriocin=2380 μ g/mL (at which no visible growth is obtained)

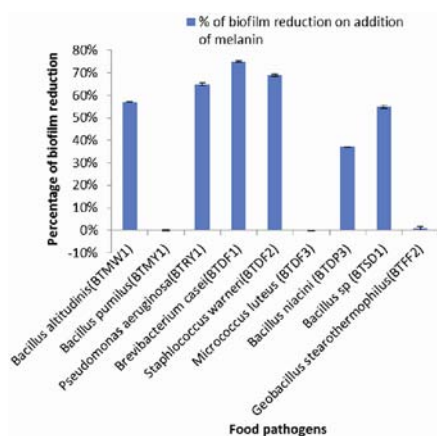


Fig. 2—Effect of melanin on *in vitro* biofilm formation by various food pathogens.

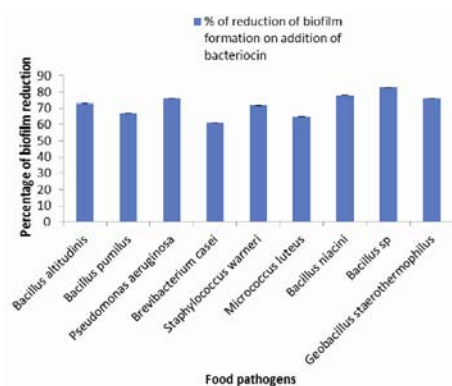


Fig. 3—Effect of bacteriocin BL8 on *in vitro* biofilm formation by various food pathogens.

MAR Index

The test organisms were all found to be multiple drug resistant²² and the MAR index was calculated to be >0.2 (20%), which indicates that these organisms originated from an environment where several antibiotics were used. It also showed the high

Table 4—Multiple antibiotic resistance (MAR) index of the nine strong biofilm producers studied

Isolate	No. of antibiotics to which isolate was resistant (a)	Total no. of antibiotics to which isolate was subjected (b)	MAR index (a/b)
<i>Bacillus altitudinis</i> (BTMW1)	10	12	0.83
<i>B. pumilus</i> (BTMY1)	4	12	0.33
<i>Pseudomonas aeruginosa</i> (BTRY1)	10	12	0.83
<i>Brevibacterium casei</i> (BTDF1)	5	12	0.42
<i>Staphylococcus warneri</i> (BTDF2)	8	12	0.66
<i>Micrococcus luteus</i> (BTDF3)	6	12	0.50
<i>B. niacini</i> (BTDP2)	4	12	0.33
<i>Bacillus</i> sp. (BTSD1)	5	12	0.42
<i>Geobacillus stearothermophilus</i> (BTFF2)	2	12	0.17

resistance pattern of these biofilm forming food pathogens. Table 4 shows the MAR index values of the nine food pathogens and Fig. 4 depicts the MAR index expressed in percentage.

Scanning Electron Microscopy for Visualizing Biocontrol of Biofilm by Melanin and Bacteriocin BL8

The scanning electron microscopy was utilized to observe the effect of melanin and bacteriocin BL8 on the biofilm formation of all test isolates. Fig. 5 shows the scanning electron micrographs of untreated and melanin-and bacteriocin-treated samples. Significant observable reduction in microbial number and also in the biofilm formation could be seen in the test (melanin- & bacteriocin-treated) samples in comparison to the control (melanin & bacteriocin BL8 untreated) samples.

Thus, in this study, two biomolecules sourced from marine bacteria were proved to be potent biofilm inhibitors. In an effort to find viable sources of anti-biofilm agents, many researchers have extracted and analyzed natural products from countless plants and marine organisms³¹. Many of the compounds are secondary metabolites generated by the host organism in response of external stresses, such as, competition for space and potential predators³². Marine biofouling is mainly due to the biofilm formation by several marine microorganisms. In the marine environment, antimicrobial and antifouling metabolites are vital for many sessile organisms to insure that they do not form hazardous biofilms on their exposed surfaces,

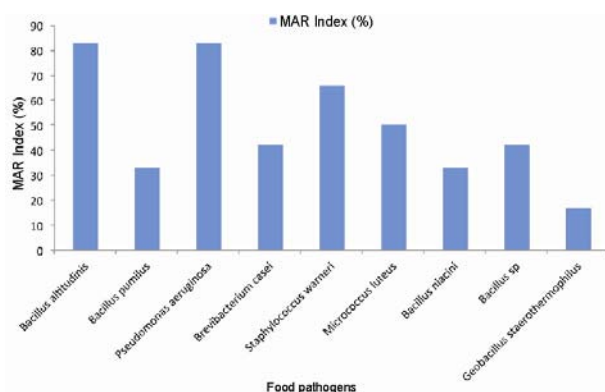


Fig. 4— Showing MAR index in percentage.

especially given that an overwhelming majority of the planet's microbial biomass prefers to be in a biofilm state³³. Of all the species studied, marine sponges (Phylum: Porifera) have been the most valuable. Sponges have been the source of > 30% of marine natural products, generating a diverse array of molecules that not only have antimicrobial and antibiofilm capabilities, but also have potential cancer therapeutics³⁴. Despite this, very few metabolites and their derivatives can serve as biofilm modulators in a non-microbicidal manner. Different marine bacteria and fungi are therefore promising alternatives for marine drugs against marine biofouling³⁵

In the present study, bacteriocin BL8 from marine *B. licheniformis* inhibited biofilm production of all food pathogens included in the study. *B. licheniformis* is already reported to be safe for use in food industry³⁶ and can thus be used as an effective biocontrol mechanism for biofilms in common foods. The scanning electron micrographs also showed significant decrease of biofilms on treatment with both melanin and bacteriocin in the study. It can be observed that both melanin and bacteriocin in small quantities inhibited bacterial growth as well as biofilm formation. The significance of the study is that nanogram quantities of the compounds helped to control biofilm formation.

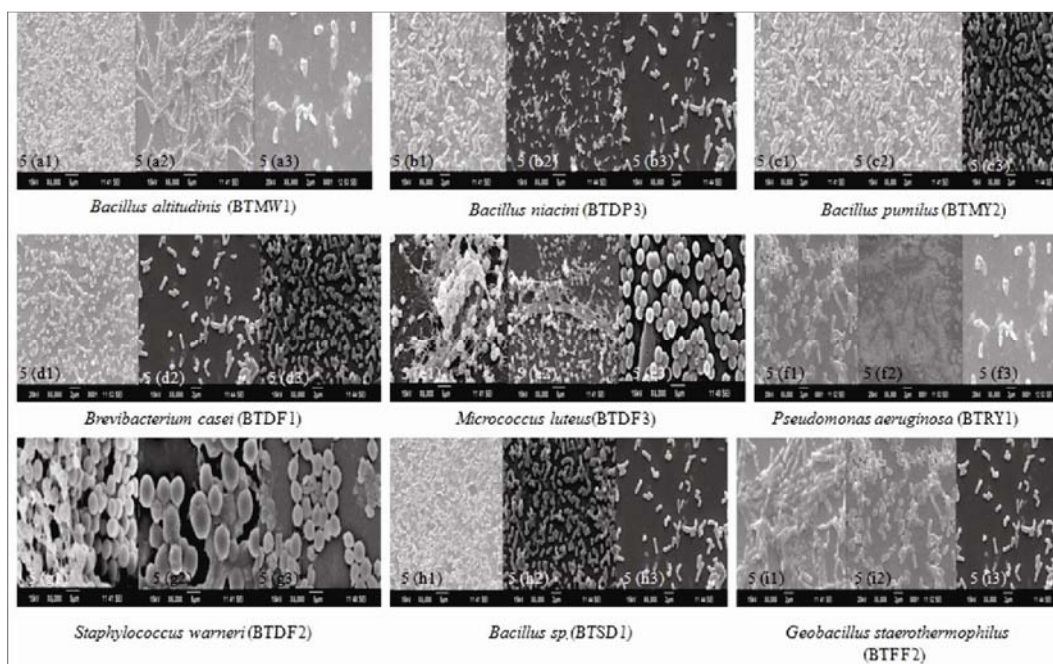


Fig. 5—Scanning electron micrographs showing inhibition of biofilm produced by nine pathogens on treatment with melanin and bacteriocin BL8. [(a1-i1) Control (melanin & bacteriocin untreated); (a2-i2) Melanin treated; & (a3-i3) Bacteriocin BL8 treated.]

Conclusion

The biofilms in food industries are mainly produced by pathogenic microorganisms and are therefore a source of contamination. As the demand for fresh, ready to eat and processed foods increase, several efforts are essential to accomplish biofilm removal in food industries. Even though conventional control strategies are used even today, a more economical and environment friendly control strategies are inevitable, to satisfy the requirement of industrial food safety. One of the alternatives is the use of bioactive compounds isolated from the microorganisms itself. It should be taken into account that it shall provide a desirable cost-effective result and do not cause any adverse effect on human health as well as the environment.

Further studies on the application of these biomolecules on the stronger biofilm producers in the food industry can challenge the current antibiofilm therapies in food safety as mentioned. The study revealed the importance of using bioactive compounds to inhibit the biofilm formation of food pathogens instead of the amplified use of the antibiotics and could help change the era of multiple resistances of the pathogens in the food industry. Both compounds used in the study can be considered as a weapons to add to the current antibiofilm strategies.

Acknowledgements

The first author acknowledges the Department of Science and Technology (DST), Government of India, New Delhi for the INSPIRE Junior Research fellowship.

References

- 1 Watnick P & Kolter R, Biofilm, city of microbes, *J Bacteriol*, 182 (2000) 2675-2679.
- 2 Kokare C R, Chakraborty S, Khopade A & Mahadik K R, Biofilm: Importance and applications, *Indian J Biotechnol*, 8 (2009) 159-168.
- 3 Annous B A, Burke A & Sites J E, Surface pasteurization of whole fresh cantaloupes inoculated with *Salmonella* Poona or *Escherichia coli*, *J Food Prot*, 67 (2004) 1876-1885.
- 4 Van Houdt R & Michiels C W, Biofilm formation and the food industry, a focus on the bacterial outer surface, *J Appl Microbiol*, 109 (2010) 1117-1131.
- 5 Vasanthabharathi V, Lakshminarayanan R & Jayalakshmi S, Melanin production from marine Streptomyces, *Afr J Biotechnol*, 10 (2011) 11224-11234.
- 6 Qian P-Y, Thiyagarajan V, Lau S C K & Cheung S C K, Relationship between bacterial community profile in biofilm and attachment of the acorn barnacle *Balanus amphitrite*, *Aquat Microb Ecol*, 33 (2003) 225-237.
- 7 Wirtanen G & Mattila-Sandholm T, Measurement of biofilm of *Pediococcus pentosaceus* and *Pseudomonas fragi* on stainless steel surfaces, *Coll Surf B: Biointerfaces*, 2 (1994) 33-39.
- 8 Gowrishankar S, Duncun M N & Karutha P S, Coral-associated bacteria as a promising antibiofilm agent against methicillin-resistant and -susceptible *Staphylococcus aureus* biofilms, *Evid-Based Complement Alternat Med*, 40 (2012) 862374.
- 9 Plonka P M & Grabacka M, Melanin synthesis in microorganism—Biotechnological and medical aspects, *Acta Biochim Pol*, 53 (2006) 429-443.
- 10 Riley P, Melanin, *Int J Biochem Cell Biol*, 29 (1997) 1235-1239.
- 11 Huang H C & Chang T M, Antioxidative properties and inhibitory effect of *Bifidobacterium adolescentis* on melanogenesis, *World J Microbiol Biotechnol*, 28 (2012) 2903-2912.
- 12 Li B, Li W, Chen X, Jiang M & Dong M, *In vitro* antibiofilm activity of the melanin from *Auricularia auricula*, an edible jelly mushroom, *Ann Microbiol*, 62 (2012) 1523-1530.
- 13 Butler M J & Day A W, Fungal melanins: A review, *Can J Microbiol*, 44 (1998) 1115-1136.
- 14 Cleveland J, Mantiville T J, Ness I F & Chiknids M L, Bacteriocins: Safe antimicrobials for food preservation, *Int J Food Microbiol*, 71 (2001) 1-20.
- 15 Lazdunski C J, Pore-forming colicins: Synthesis, extracellular release, mode of action, immunity, *Biochimie*, 70 (1988) 1291-1296.
- 16 Smitha S & Bhat S G, Thermostable bacteriocin BL8 from *Bacillus licheniformis* isolated from marine sediment, *J Appl Microbiol*, 114 (2013) 688-694.
- 17 Sajjan S S, Anjaneya O, Guruprasad B K, Anand S N, Suresh B M *et al*, Properties and functions of melanin pigment from *Klebsiella* sp. GSK, *Korean J Microbiol Biotechnol*, 41 (2013) 60-69.
- 18 Fava F, Gioia D D & Merchetti L, Characterization of a pigment produced by *Pseudomonas fluorescens* during 3-chlorobenzoate co-metabolism, *Chemosphere*, 27 (1993) 825-835.
- 19 Ravishankar J P, Muruganandam V & Suryanarayanan T S, Isolation and characterization of melanin from a marine fungus, *Bot Mar*, 38 (1995) 413-416.
- 20 Laxmi M & Sarita G B, Diversity characterization of biofilm forming microorganisms in food sampled from local markets in Kochi, Kerala, India, *Int J Recent Sci Res*, 5 (2014) 1070-1075.
- 21 Jiang L, Comparison of disk diffusion, agar dilution, and broth microdilution for antimicrobial susceptibility testing of five chitosans. M Sc Thesis, Louisiana State University, Louisiana, 2011.
- 22 Rode T M, Lansruds S, Holck A & Moretro T, Different patterns of biofilm formation in *Staphylococcus aureus* under food related stress conditions, *Int J Food Microbiol*, 116 (2007) 372-383.
- 23 Stepanovic S, Vukovic D, Dakic I, savic B & Milena S V, A modified microtiter plate test for quantification of staphylococcal biofilm formation, *J Microbiol Methods*, 40 (2000) 175-179.
- 24 Bauer A W, Kirby W M, Sherris J C & Turck M, Antibiotic susceptibility testing by a standardized single disk method, *Am J Clin Pathol*, 45 (1966) 493-496.

- 25 Subramani S & Vignesh S, MAR index study and MDR character analysis of a few Golden Staph isolates, *Asian J Pharm Lif Sci*, 2 (2012) 151-154.
- 26 Lembke C, Podbielski A, Hidalgo-Grass C, Jonas L, Hanski E *et al*, Characterization of biofilm formation by clinically relevant serotypes of group A streptococci, *Appl Environ Microbiol*, 72 (2006) 2864-2875.
- 27 Nosanchuk J D & Casadevall A, Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds, *Antimicrob Agents Chemother*, 50 (2006) 3519-3524.
- 28 Soundra Rani M H, Ramesh T, Subramanian J & Kalaiselvam M, Production and characterization of melanin pigment from halophilic black yeast *Hortaea werneckii*, *Int J Pharm Res Rev*, 2 (2013) 9-17.
- 29 Marugg J D, Gonzalez C F, Kunka B S, Ledebor A M, Pucci M J *et al*, Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, and bacteriocin from *Pediococcus acidilactici* PAC1.0, *Appl Environ Microbiol*, 58 (1992) 2360-2367.
- 30 Blackburn P, Polak J, Gusik S & Rubino S, Nisin compositions for use as enhanced, broad range bactericides. International Patent No. PCT/US89/02625; WO 89/12399, 1998.
- 31 Donia M & Hamann M T, Marine natural products and their potential applications as anti-infective agents, *Lancet Infect Dis*, 3 (2003) 338-348.
- 32 Wulff J L, Ecological interactions of marine sponges, *Can J Zool*, 84 (2006) 146-166.
- 33 Costerton W, Veeh R, Shirliff M, Pasmore M & Ehrlich G, The application of biofilm science to the study and control of chronic bacterial infections, *J Clin Invest*, 112 (2003) 1466-1477.[Erratum in *J Clin Invest*, 117 (2007) 278]
- 34 Proksch P, Defensive roles for secondary metabolites from marine sponges and sponge-feeding nudibranchs, *Toxicon*, 32 (1994) 639-655.
- 35 Faulkner D J, Marine natural products, *Nat Prod Rep*, 19 (2002) 1-49.
- 36 Cladera-Olivera F, Caron G R & Brandelli A, Bacteriocin-like substance production by *Bacillus licheniformis* strain P40, *Lett Appl Microbiol*, 38 (2004) 251-256.