



Bactericidal Effects of *Exiguobacterium* sp GM010 Pigment Against Food-Borne Pathogens

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Bacterium producing yellowish-orange pigment was identified (morphological, biochemical, and 16S rRNA) as *Exiguobacterium* sp GM010. The UV-visible spectrum of *Exiguobacterium* sp GM010 extract showing λ max at 465 nm revealed orange pigment characteristic. Pigment showed broad spectrum antibacterial action against gram positive and gram negative food-borne pathogens. The SYTO9 and propidium iodide (PI) staining revealed the cell membrane damage of food-borne pathogens under confocal laser scanning microscope (CLSM) indicating the bactericidal effect. This was evidenced by the fourier transform infrared (FTIR) spectrum, showing characteristic functional groups that mainly included hydroxyl, carbonyl, and carboxylic groups causing a system of delocalized electrons leading to destabilization of membrane and decrease in membrane potential that resulted in bactericidal effect. The pigment of *Exiguobacterium* sp GM010 were non-toxic against *Artemia franciscana* and can be a promising source to control the food-borne pathogens in food industries.

Keywords: *Exiguobacterium* sp GM010, pigment, marine bacteria, antimicrobial, food-borne pathogens

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INTRODUCTION

Food-borne diseases have become one of the most widespread public health problems. About two-thirds of all the outbreaks were due to consumption of microorganisms contaminated food and water. World Health Organization (WHO) estimates, unsafe food causes 600 million cases of food-borne diseases and 4,20,000 deaths annually, including children (Bajpai et al., 2013; Fukuda, 2015; World Health Organization, 2020). Hence, food safety is a major concern not only for developing countries but also for the developed countries. A study was conducted to characterize the prevalence and diversity of food-borne pathogens, recommended to minimize the risk of contamination in fields (Strawn et al., 2013). Recent reports have identified the prevalence of bacterial pathogens in quality export seafood (Bandeekar, 2015) causes massive economic losses to the food industry.

To reduce the health risks and economic losses, the foods are treated with antimicrobial agents as preservative. There has been a major focus on the development of safe and efficient natural broad spectrum antimicrobials that can replace synthetic alternatives (Kim et al., 2013; Al-zoreky and Al-Taher, 2015). The marine microorganisms are excellent source of bioactive compounds, where antimicrobial compounds stand in the majority (Burgess et al., 1999; Nithyanand et al., 2011; Wiese and Imhoff, 2019). Many marine microorganisms have been isolated from different marine habitats to discover new bioactive compounds (Blunt et al., 2012, 2014). These bioactive compounds have a considerable importance (Newman and Cragg, 2007) for drug discovery industry due to their

TABLE 1 | Antimicrobial activity of pigment of *Exiguobacterium* sp GM010 against food-borne pathogens.

Strain/antibiotics	Inhibition zone (mm)*							
	Gram-positive				Gram-negative			
	<i>B. cereus</i> ATCC 11778	<i>B. subtilis</i> ATCC 06633	<i>S. aureus</i> FRI722	<i>M. luteus</i> ATCC 9341	<i>L. monocytogenes</i> Scott A	<i>E. coli</i> EFR02	<i>P. aeruginosa</i> ATCC 15442	<i>Klebsiella</i> sp
<i>Exiguobacterium</i> sp GM010	7.16 ± 0.28	7.66 ± 0.57	8.5 ± 0.5	10.83 ± 0.36	14.33 ± 0.57	6.66 ± 0.28	8.16 ± 0.28	8.1 ± 0.0.28
AMP	10	16	12	16	18	10	8	11
TET	18	18	16	21	21	12	16	16
CIP	16	19	14	23	18	14	15	14

*Zone of inhibition including the disc.

AMP, Ampicillin; TET, Tetracycline; CIP, Ciprofloxacin are standard broad spectrum antibiotics.

structural diversity and biological activity. More than 22,000 bioactive compounds from marine organisms have been reported (Demain and Sanchez, 2009) that includes both prokaryotes and eukaryotes. The *Streptomyces* sp was widely studied microbial species from Indian coastal waters as a source of antibiotics (Chandramohan, 1997; Valliappan et al., 2014). There are report on the algicidal (Li et al., 2016), antifungal (Selvakumar et al., 2009) and antibacterial (Shanthakumar et al., 2015) effect of *Exiguobacterium* species, however mechanism of antibacterial effect of *Exiguobacterium* pigment on food-borne pathogens was not reported. In this study pigment producing *Exiguobacterium* sp GM010 was isolated, and characterized. The antibacterial properties of pigment against the food-borne pathogens, mode of action, and non-toxic effect suggested the potential applications in food industries.

MATERIALS AND METHODS

Food-Borne Pathogens

Food-borne pathogens *Escherichia coli* EFR02, *Staphylococcus aureus* FRI722, *Pseudomonas aeruginosa* ATCC 15442, *Bacillus subtilis* subsp *spizizenii* ATCC 06633, *B. cereus* ATCC 11778, *Klebsiella* sp, *Listeria monocytogenes* Scott A, and *Micrococcus luteus* ATCC 9341 were obtained from Food Safety and Analytical Quality Control Laboratory, CSIR-Central Food Technological Research Institute, Mysore, Karnataka, India. Stock cultures of pathogens were maintained on nutrient agar at 4°C and sub-cultured in nutrient broth at 37°C, before activity assay.

Isolation and Identification

Sediment samples collected from Tamil Nadu coastal regions were used for isolation of bacteria. The isolated chromosomal DNA (Marmur, 1961) was amplified using universal primers 27F (50-AGA GTT TGA TCC TGG CTC AG-30) and 1492R (50-GGT TAC CTT GTT ACG ACT T-30) (Lane, 1991). The 16S rRNA gene sequence was compared using the NCBI BLAST for similarity with the reference bacterial species in GenBank database. Multiple alignments of the sequences using the Clustal W program and phylogenetic tree construction using treeing algorithms were performed in MEGA X software package.

Antibacterial Activity and Mode of Action

The strain GM010 was inoculated into 100 ml of zobell marine broth (ZMB) in 250 ml conical flask and incubated for 3 days at 30°C, 150 rpm. After incubation, the pigment was extracted by solvent-solvent partition by adding equal volume of ethyl acetate to the culture broth. Ethyl acetate fraction was separated and concentrated. The stock of pigment was prepared by dissolving in DMSO (50 mg/ml) for antibacterial assay (Dhale et al., 2007). The overnight grown food-borne pathogen (Table 1) bacterial cultures (200 µl) were spread on nutrient agar. The 6 mm disks impregnated with the 200 µg of GM010 pigment were placed and incubated at 37°C for 24–48 h. The activity was determined by measuring the inhibition zones. The disks with DMSO and standard antibiotics were used as negative and positive control, respectively.

Confocal Laser Scanning Microscopy (CLSM)

The mode of inhibition of food-borne pathogens, was determined using LIVE/DEAD BacLight™ Bacterial Viability Kit (Invitrogen, Molecular probes Inc) according to manufacturer's protocols. One ml of food-borne pathogen cells were treated with 200 µg of GM010 pigment in centrifuge tubes. After removing growth medium, the cells were washed with 0.85% saline and mixture of SYTO9 and propidium iodide (PI) was added to the treated cell. This was incubated in dark at room temperature for 20–25 min in dark. After incubation, the images were acquired in Zeiss LSM 700 CSLM to analyze samples. Images were acquired with 512 × 512 resolutions in at least three different fields. Zeiss ZEN software was used to acquire images. The laser was used at 488 nm for excitation and the emission was observed at 528 nm (SYTO9) and 645 nm (PI).

Scanning Electron Microscopic (SEM)

The morphological changes of food-borne pathogen cells were analyzed after treatment with GM010 pigment under SEM (Asensio et al., 2005). Briefly, 1 ml of bacterial culture was added to centrifuge tube containing 200 µg of pigment. This was centrifuged after incubation at 37°C for 6 h, the pellets were rinsed with phosphate buffer and fixed with 3% glutaraldehyde

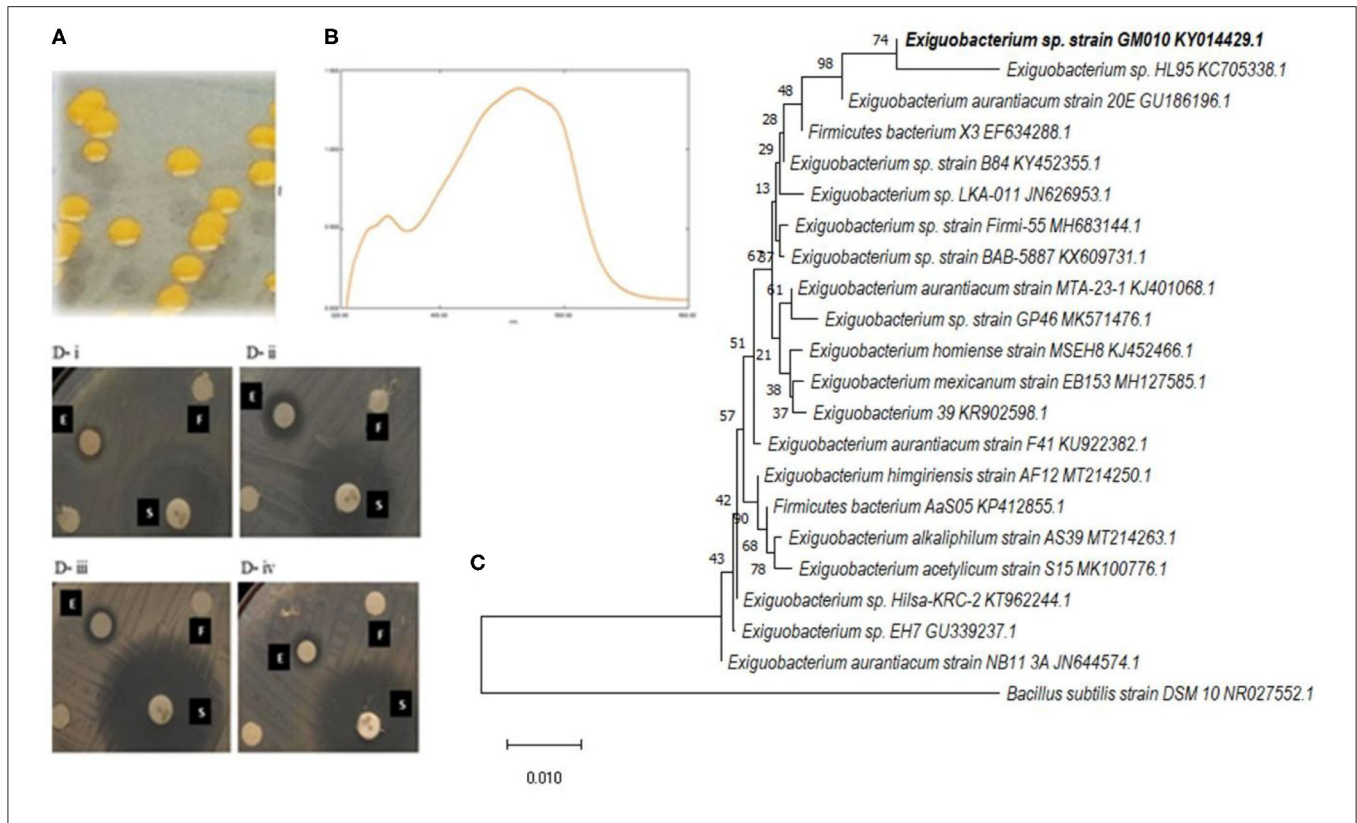


FIGURE 1 | The pigmentation was observed on zobell marine agar by strain GM010 (A) and the extract of strain GM010 showing absorption peak at 465 nm confirmed the pigment production (B). Based on 16S rRNA gene sequence, neighbor-joining phylogenetic tree showing the relationship of strain GM010 with related species of *Exiguobacterium* (C). Numbers at the nodes indicate percentage bootstrap values (500 replicates). Disc diffusion assay (D) showing inhibitory action of *Exiguobacterium* sp GM010 (E) against food-borne pathogens *B. cereus* ATCC 11778 (i), *S. aureus* FRI722 (ii), *Klebsiella* sp (iii) and *E. coli* EFR02 (iv). The DMSO (F) and standard antibiotic (S) were used as negative control and positive control, respectively.

TABLE 2 | Minimal inhibitory concentration and minimal bactericidal concentration of pigment of *Exiguobacterium* GM010 against food-borne pathogens.

Strain/ Antibiotics (µg/ml)	Gram +ve								Gram -ve							
	<i>B. cereus</i> ATCC 11778		<i>B. subtilis</i> ATCC 06633		<i>S. aureus</i> FRI722		<i>M. luteus</i> ATCC 9341		<i>L. monocytogenes</i> Scott A		<i>E. coli</i> EFR02		<i>P. aeruginosa</i> ATCC 15442		<i>Klebsiella</i> sp	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Exiguobacterium</i> sp GM010	125	250	125	250	125	250	62.5	125	62.5	125	250	500	125	250	125	250
AMP	10	20	10	20	10	20	5	10	5	10	10	20	5	10	10	20
TET	5	10	5	10	5	10	10	20	10	20	5	10	10	20	5	10

AMP, Ampicillin; TET, Tetracycline are standard broad-spectrum antibiotics.

for overnight in refrigerator (4°C). After dehydration with series of ethanol, sample were examined under SEM (LEO 435VP, Japan). Tetracycline was used as positive control.

UV-Visible and FT-IR Spectroscopy

The UV-visible spectrum was recorded (UV-Visible 2450, Shimadzu Spectrophotometer, Japan) in the range of 400–700 nm to determine the pigment characteristic. The FT-IR spectrum was recorded using a fourier transform infrared (FTIR) spectrophotometer (Bruker IFS 25 model, Bruker,

Germany) in the 4,000–400 cm⁻¹ range in transmission mode. The characteristic functional groups of GM010 pigment were identified.

Determination of MIC and MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of pigment was determined by broth microdilution method (Pandit et al., 2018). All tests were performed in nutrient broth. Overnight bacterial culture broth of each strain were prepared and the final concentration in each

well was adjusted to 2×10^4 cfu/ml. A serial doubling dilution of the pigment was prepared in a 96-well microtiter plate over the range 15.625–2000 $\mu\text{g/ml}$. The plates were incubated at 37°C for 24 h. To determine MBC, the pigment treated broth from each well was inoculated on nutrient agar and incubated for 24 h at 37°C . Ampicillin and Tetracycline were used as positive control over the range of 1.25–160 $\mu\text{g/ml}$. The controls were maintained without pigment to demonstrate growth of bacterial pathogen.

Toxicity Assay

Toxicity assay was performed using *Artemia franciscana* nauplii (Pandit et al., 2019). About 100 μl of growth medium containing ~26 nauplii were used for bioassay in microtiter plates. The GM010 pigment (100 and 1,000 $\mu\text{g ml}^{-1}$) was added to microtiter plates and incubated at $27 \pm 2^\circ\text{C}$ for 36 h. Artificial sea water was used as negative control and potassium cyanide was used as positive control. The viability of cysts was monitored at different time intervals of 12, 24, 36 h independently (Babu et al., 2015). Under 10x magnification, percentage mortality was calculated using the formula.

$$\text{Mortality rate (\%)} = \frac{\text{Death nauplii} \times 100}{\text{Total nauplii}}$$

Statistical Analysis

Data were expressed as the mean \pm standard deviation of triplicate measurements. Results were processed by 1-way analysis of variance (ANOVA). A Duncan multiple range test was used to determine significant differences. Differences at $P < 0.05$ were considered as significant.

RESULTS

Isolation of Active Strain

The heterotrophic marine bacteria were isolated on five different culture media from the samples collected from different ecological niche. Based on morphological characteristics 43 strains were selected to isolate antagonistic strains. Since the selection of antagonistic strains was usually affected by the species and number of indicator microorganisms (Shnit-Orland and Kushmaro, 2009), all 43 strains were screened against both Gram positive and Gram negative food-borne pathogens (Data not shown). Among these, pigment of strain GM010 inhibited both Gram-positive and Gram-negative bacteria compared to broad spectrum antibiotics. The antimicrobial activities showing the zone of inhibitions were summarized in **Table 1**.

Identification and Characterization

The isolate GM010 produced yellow to orange pigmentation on ZMA (**Figure 1A**). The physiological and biochemical characteristics of the strain GM010 were presented in **Table S1**. UV-vis spectrum of GM010 pigment showed absorption peak at 460–470 nm indicating orange pigment characteristic (**Figure 1B**). The 16S rRNA sequence analysis revealed that, the bacterium belongs to the phylum Firmicutes and *Bacillaceae* family. The strain GM010 was closely related to *Exiguobacterium* sp HL95 (**Figure 1C**). Therefore, the strain GM010 was a species

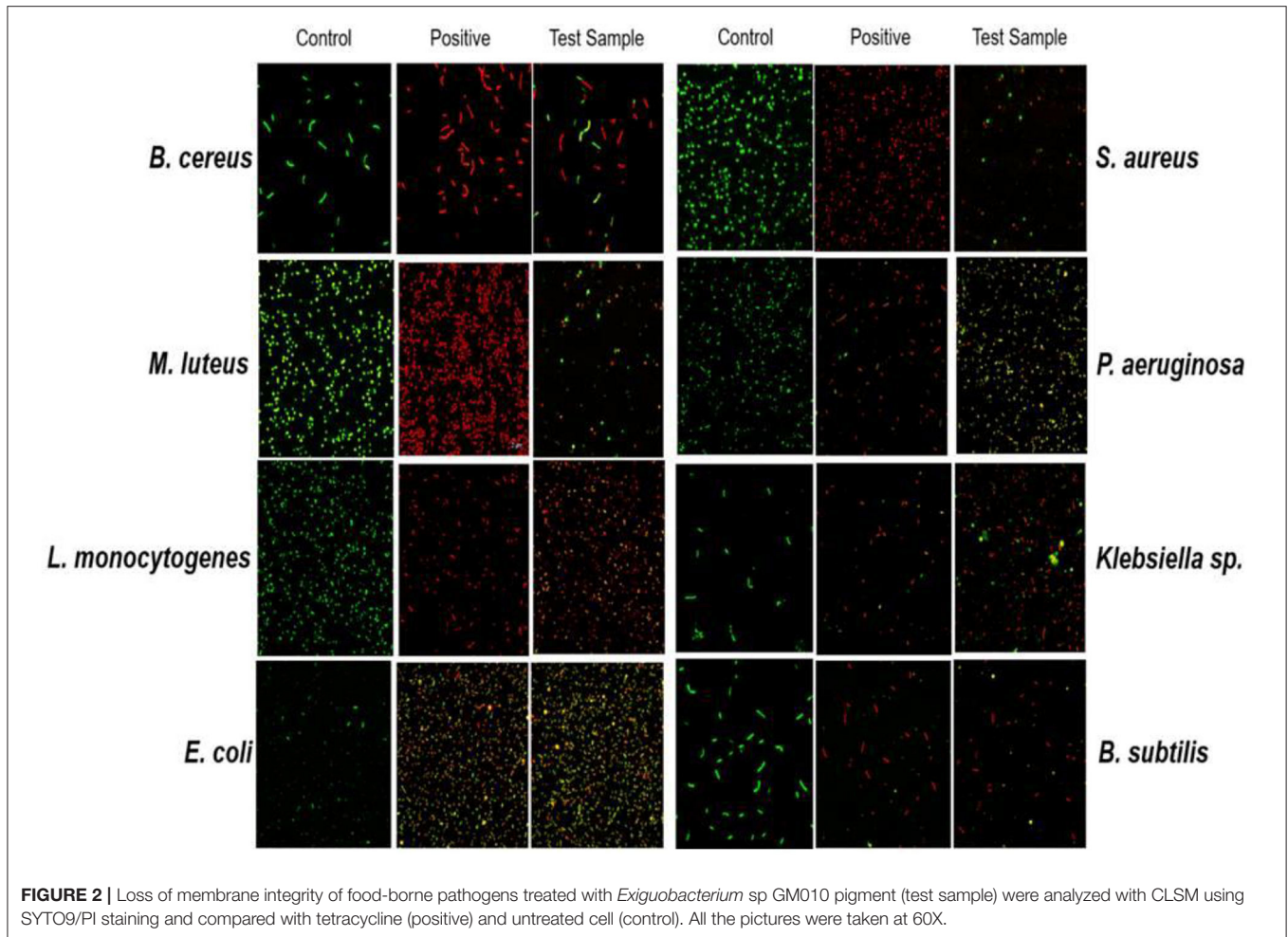
of genus *Exiguobacterium* for which *Exiguobacterium* sp GM010 was proposed. The sequences were submitted in GenBank with accession number KY014429 (<https://www.ncbi.nlm.nih.gov/genbank/>).

Further to consider the applications of the pigment, the antibacterial functional properties were evaluated (**Figure 1D**). The zone of inhibition were observed in the range of 6.6–14.3 mm. The *Exiguobacterium* sp GM010 pigment has strongly inhibited *L. monocytogenes* Scott A (14.33 mm) followed by *M. luteus* ATCC 9341 (10.83 mm) compared to other food borne pathogens. Whereas, zone of inhibition for *E. coli* EFR02 observed was 6.66 mm. Further, the MIC values of *Exiguobacterium* sp GM010 pigment against the food-borne pathogens were found to be in the range of 62.5–500 $\mu\text{g/ml}$. The pigment was tested against both Gram positive and negative bacteria for antibacterial activity with a broth dilution microtiter system. The pigment inhibited *L. monocytogenes* Scott A and *M. luteus* ATCC 9341 at minimum concentration of 62.5 $\mu\text{g/ml}$ and *Klebsiella* sp followed by *P. aeruginosa* ATCC 15442 was inhibited at minimum concentration of 250 $\mu\text{g/ml}$. Whereas, *E. coli* EFR02 was inhibited at 500 $\mu\text{g/ml}$ concentration of pigment (**Table 2**). Even though these results revealed that *Exiguobacterium* sp GM010 pigment can inhibit both gram-positive and gram-negative food-borne pathogens, the MIC values were greatly varied. MIC determines antimicrobial susceptibility and sensitivity by measuring inhibition of bacterial growth. Whereas, MBC is the lowest concentration of test sample that results in a 99.9% reduction in the initial microbial density. MBCs values were generally within a two-fold dilution of the MIC value.

Mode of Antibacterial Action

The CLSM studies revealed mode of antibacterial action of *Exiguobacterium* sp GM010 pigment on food-borne pathogens. The SYTO9 and PI staining indicated loss of membrane integrity of food-borne pathogens treated with *Exiguobacterium* sp GM010 pigment and compared with tetracycline treatment. The appearance of red and yellow fluorescence indicated dead cells. Whereas, the live cells appeared green (control) did not receive any treatment (**Figure 2**). These results confirmed that, *Exiguobacterium* sp GM010 pigment disrupted the cell membrane of food-borne pathogens leading to bactericidal action. Further SEM observations revealed morphological deformation of food-borne pathogen cells, while untreated (control) cells were uniform in shape (**Figure 3**). The distorted structure as indicated by low electron density regions, ruptured cell wall, and cavity formation in the bacterial cells. Similar effects were also observed in tetracycline treatment (**Figure 3**).

The results of FT-IR spectrum of *Exiguobacterium* sp GM010 pigment revealed absorption bands characteristic for the functional groups of the components (**Figure 4**). Infrared spectrum showed characteristic functional groups that mainly included hydroxyl, carbonyl, and carboxylic groups. The strong broad band at $3,459 \text{ cm}^{-1}$ was assigned to the presence of OH stretching in hydrogen bonds and N-H vibration. Absorption peaks between 2999 and 2915 cm^{-1} correlated to stretching frequencies of aliphatic C-H groups. This spectrum showed an



absorption band at $1,666\text{ cm}^{-1}$ that was indicative for stretching frequencies of an C=O group. The band at about $1,437\text{ cm}^{-1}$ was indicative for asymmetric vibrations of the carboxylic group. Peaks at $1,000\text{--}1,200\text{ cm}^{-1}$ correlated to C-O-C linkages of sugar components remaining in the extract.

Toxicity Effect

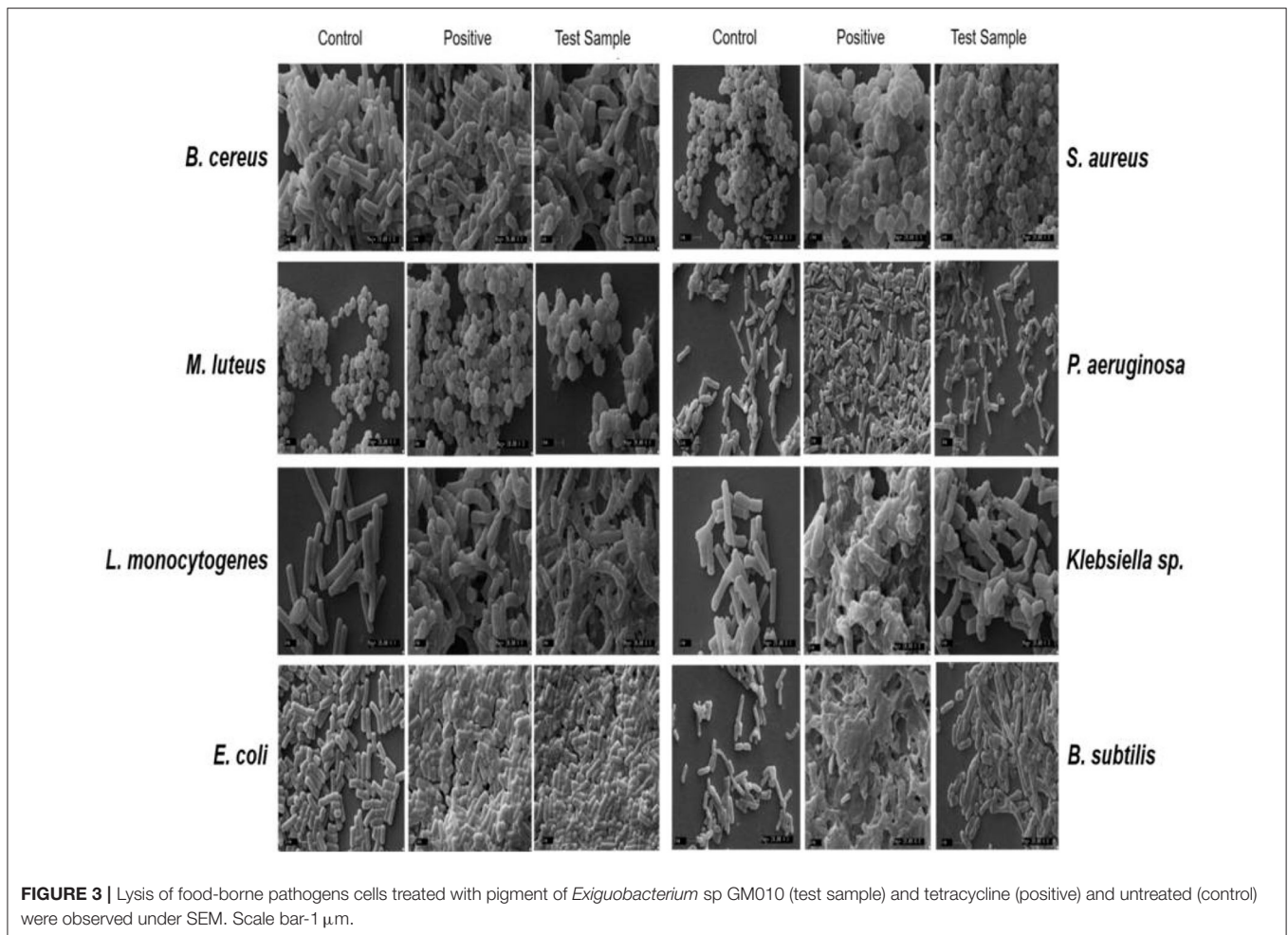
Toxicity of *Exiguobacterium* sp GM010 pigment was analyzed by estimating the mortality rate of *A. franciscana*, a relatively rapid way to detect toxic compounds (Meyer et al., 1982). The mortality rates at $100\text{ }\mu\text{g ml}^{-1}$ of pigment were not significantly different at 12 h intervals of time. However, at $1,000\text{ }\mu\text{g ml}^{-1}$ the *Exiguobacterium* sp GM010 has shown $38.33 \pm 1.44\%$ mortality at 36 h of treatment (Figure 5). The mortality rate (%) was compared with the Clarkson's toxicity assessment (Clarkson et al., 2004) and it was confirmed the non-toxic effect of *Exiguobacterium* sp GM010 pigment.

DISCUSSION

Marine bacteria *Streptomyces*, *Pseudomonas*, *Pseudoalteromonas*, *Bacillus*, *Vibrio*, and *Cytophaga* isolated marine environment have shown various biological activities (Azamjon et al., 2011).

The majority of marine microorganisms are not easily culturable in the laboratory (Valliappan et al., 2014), due to dynamics of nutrient conditions. To isolate maximum number of marine bacteria, five different culture media were used for isolation of bacteria showing broad spectrum antibacterial activity. Further the NaCl concentration of the isolation media was increased to ensure the isolated bacteria were truly associated with marine niche.

Generally the Gram-positive bacteria are more sensitive to antibiotics compared to Gram-negative bacteria (Nikaido, 1996) and using more Gram positive indicator bacteria may lead to false positive results of more antibiotic-producing bacteria. Hence, the isolated marine bacteria were screened equally against both Gram positive and Gram negative food-borne pathogens (Table 1), to isolate potential marine bacterium (Figure 1D). Among the marine bacteria screened, a bacterium inhibited both Gram positive and Gram negative food-borne pathogens was identified based on morphological, biochemical, and molecular analysis (Figure 1). These results revealed, the isolated bacterium is belongs to the genus *Exiguobacterium*. Even though there are reports on *Exiguobacterium* species showing the algicidal (Li et al., 2016), antifungal (Selvakumar et al., 2009) and antibacterial (Shanthakumar et al., 2015) effect, there are no report on the



antibacterial action of *Exiguobacterium* sp GM010 pigment and the mechanism of antibacterial action.

The *Exiguobacterium* sp GM010 pigment has shown antibacterial action against both Gram positive and negative food-borne pathogens. The cellular and membrane integrity is considered important to distinguish between viable and dead bacterial cells for physiological activities (Stiefel et al., 2015). Viable cells have intact membranes and cannot be penetrated by some staining compounds, whereas dead cells are considered to have disrupted or damaged membranes (Stiefel et al., 2015). The CLSM studies suggested that pigment of *Exiguobacterium* sp GM010 damaged the cell membrane (Figure 2) of food-borne pathogens. The SEM image analysis of the food-borne pathogens treated with pigment have shown shrunken shape compared to the untreated. This is due to the cell membrane damage caused the release of cytoplasmic content and lysis of cell as observed in the figure 3.

Several studies have reported that, functional group of the bioactive compounds can damage the membrane integrity of food-borne pathogens. The hydroxyl group present in the substances of *Vibrio* sp (Horta et al., 2014) and *Pseudoalteromonas phenolica* (Isnansetyo and Kamei, 2003) exhibited antibacterial action against *B. subtilis* and *S. aureus*.

The characteristic functional groups of the algicidal substance produced by the *Exiguobacterium* sp. h10 mainly included carbonyl, amino, and hydroxyl groups (Li et al., 2016). Similarly 3,6,18-trione, 9,10-dihydro-12-hydroxyl-2methyl-5-(phenyl methyl) (5- α , 10- α)-dihydroergotamine and dipropyl-S-propyl ester molecules exhibit antibacterial action against clinical pathogens (Shanthakumar et al., 2015). The hydroxyl group of compounds and the presence of a system of delocalized electrons are important for the antimicrobial activity (Ultee et al., 2002). The hydroxyl groups destabilizes the cytoplasmic membrane and acts as a proton exchanger, thereby reducing the pH gradient across the cytoplasmic membrane. The resulting collapse of the proton motive force and depletion of the ATP pool (Ultee et al., 2002) eventually lead to cell death. This was evidenced by the FTIR spectrum, showing characteristic functional groups that mainly included hydroxyl, carbonyl, and carboxylic groups in *Exiguobacterium* sp GM010 pigment (Figure 4). These functional group cause a system of delocalized electrons leading to destabilization of membrane and decrease in the membrane potential that resulted in bactericidal action of *Exiguobacterium* sp GM010 pigment.

Toxicity of GM010 was evaluated by estimating the mortality rate of *A. franciscana*. This assay has been considered as efficient

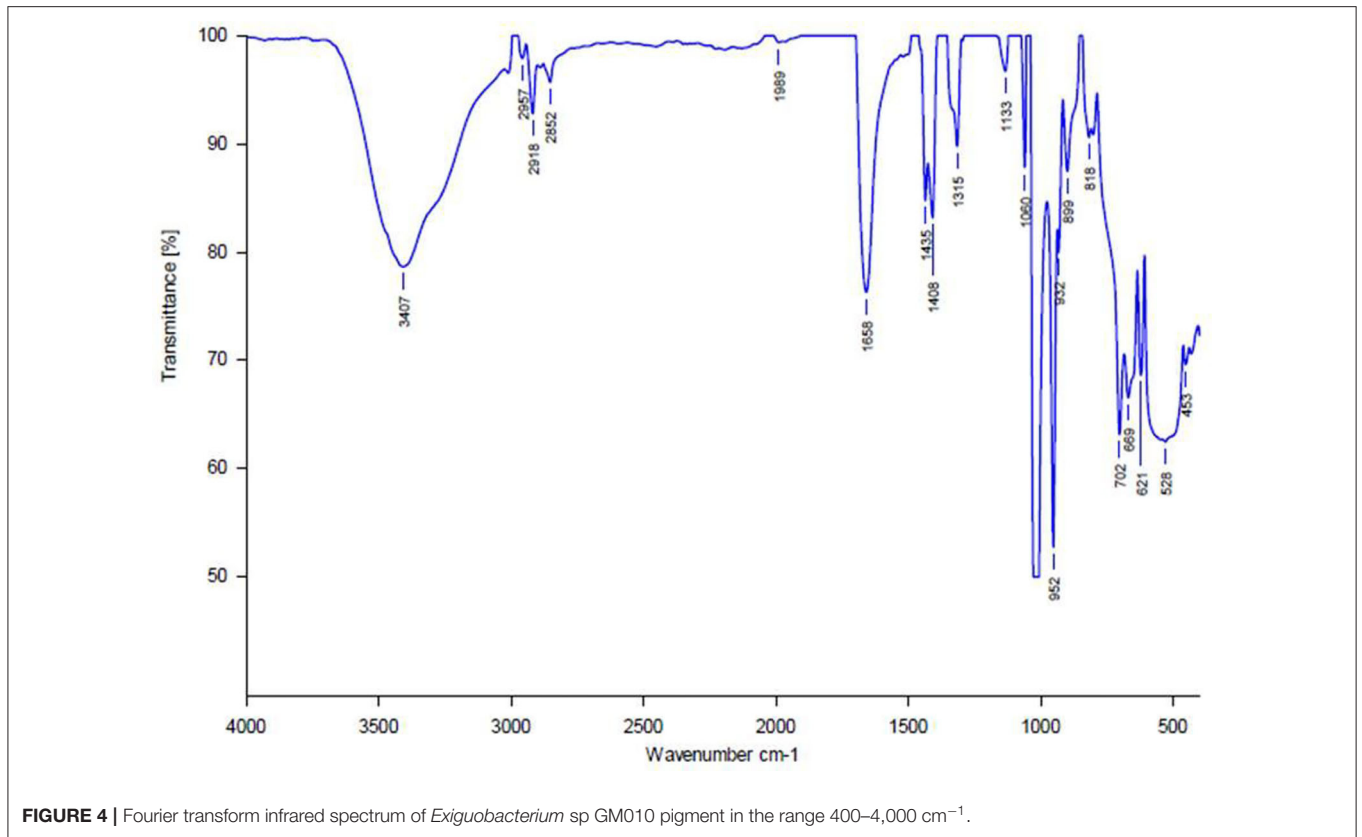


FIGURE 4 | Fourier transform infrared spectrum of *Exiguobacterium* sp GM010 pigment in the range 400–4,000 cm^{-1} .

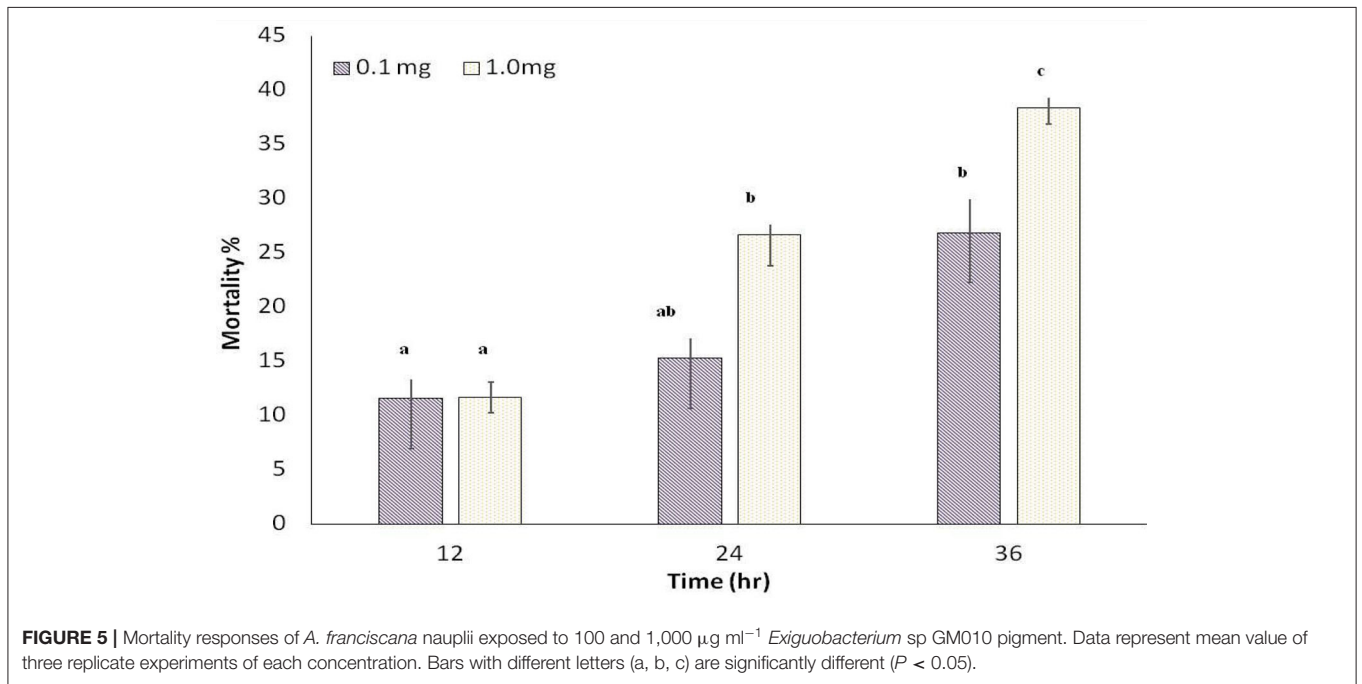


FIGURE 5 | Mortality responses of *A. franciscana* nauplii exposed to 100 and 1,000 $\mu\text{g ml}^{-1}$ *Exiguobacterium* sp GM010 pigment. Data represent mean value of three replicate experiments of each concentration. Bars with different letters (a, b, c) are significantly different ($P < 0.05$).

inexpensive and a relatively rapid way to detect toxic compounds tool for toxicity assay and requiring only less quantity of sample (<20 mg) (Meyer et al., 1982). At 1,000 $\mu\text{g ml}^{-1}$ the GM010 has shown $38.33 \pm 1.44\%$ mortality at 36 h of treatment (Figure 5).

Accordingly, extracts with LC_{50} above 1,000 $\mu\text{g ml}^{-1}$ are non-toxic, LC_{50} of 500–1,000 $\mu\text{g ml}^{-1}$ are low toxic, extracts with LC_{50} of 100–500 $\mu\text{g ml}^{-1}$ are medium toxic, while extracts with LC_{50} of 0–100 $\mu\text{g ml}^{-1}$ are highly toxic (Clarkson et al., 2004).

The pigment of *Exiguobacterium* sp GM010 was considered as non-toxic, since the 50% mortality of *A. franciscana* napuli was not observed at 1,000 $\mu\text{g ml}^{-1}$ concentration.

CONCLUSIONS

The results of *Exiguobacterium* sp GM010 pigment showing bactericidal activity against food-borne pathogens and non-toxicity toward *A. franciscana* suggested the application in food preservation and safety efficacy, respectively. Even though the pigment is non-toxic to *A. franciscana* napuli, additional data on preclinical studies are necessary in order to confirm that pigment is free of cytotoxic effects at different doses of treatment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

MD conceptualized, designed the experiment, and reviewed the paper. K-PM executed the experiments, data analysis, and wrote

the paper. SP executed the toxicity assay. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2020.00142/full#supplementary-material>

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