Extraction, antibiofilm activity and characterization of biosurfactant produced by *Limosilactobacillus reuteri* IDCC 3701

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ABSTRACT

Aims: This study aimed to extract and characterize a biosurfactant produced by *Limosilactobacillus reuteri* IDCC 3701, assess its emulsifying activity against crude oil, and investigate its antibiofilm properties against major foodborne pathogens.

Methodology: The biosurfactant, named I-BS, was extracted from the cell-free supernatant of *Limosilactobacillus reuteri* IDCC 3701 using acid precipitation. The emulsification index, emulsifying activity, oil spreading test, drop collapse test, and microplate distortion assay were performed to evaluate the surfactant properties of I-BS. The antibiofilm activity of I-BS against foodborne pathogens was assessed using the Calgary Biofilm Device. Finally, the cell-free supernatant of Limosilactobacillus reuteri IDCC 3701 was subjected to GC-MS analysis.

Results: I-BS demonstrated an emulsification index of 49.4% and emulsifying activity of 400.67, indicating its potential as an effective emulsifier for oils. Positive results were observed in the oil spreading test and microplate distortion assay, confirming its surfactant properties. Additionally, I-BS exhibited significant antibiofilm activity against foodborne pathogens. GC-MS analysis of the I-BS structure revealed the presence of octanoic acid, a surfactant compound.

Conclusion: The biosurfactant I-BS, derived from *Limosilactobacillus reuteri* IDCC 3701, displayed promising emulsifying activity and demonstrated notable antibiofilm properties against foodborne pathogens. These findings suggest that I-BS holds potential as a lead compound for the development of novel anti-biofilm agents and additives in the food industry.

Keywords: biosurfactant · probiotics · foodborne pathogens · *Limosilactobacillus reuteri*

1. INTRODUCTION

Biosurfactants, which are naturally occurring surface-active compounds produced by microorganisms, have a wide range of industrial applications due to their versatility and environmentally friendly production [1]. These unique biomolecules exhibit remarkable surface-active properties, reducing surface tension and facilitating the dispersion or emulsification of hydrophobic substances in aqueous environments [2]. As a result, they present promising alternatives to synthetic surfactants, which often suffer from limited biodegradability and potential environmental toxicity.

In the field of microbiology and biotechnology, extensive research has been conducted on biosurfactants for their antimicrobial and antibiofilm activities against various foodborne pathogens [3]. Foodborne pathogens pose a significant risk to human health, causing a wide range of gastrointestinal infections [4]. Traditionally, chemical disinfectants and antibiotics are used for pathogen control, however they have limitations, including the development of antibiotic resistance and the potential for chemical residues in food products [5]. Therefore, due to the growing imperative for effective, safe, and sustainable antimicrobial strategies, exploring biosurfactants derived from beneficial bacteria presents a highly promising solution [6].

Limosilactobacillus reuteri (*L. reuteri*), formerly known as *Lactobacillus reuteri*, is a Gram-positive bacterium that naturally resides in the gastrointestinal tract of humans and animals [7]. When used as a probiotic, *L. reuteri* offers various health benefits, including the modulation of the host immune response and the inhibition of pathogens [8]. Furthermore, previous studies demonstrated that several strains of *L. reuteri* could inhibit the biofilm formation of pathogens and therefore could be a promising candidate for biosurfactant studies [9].

The objective of this research is to investigate the extraction, antibiofilm activity against foodborne pathogens, and characterization of the biosurfactant produced by *L. reuteri* IDCC 3701. By understanding the properties and mechanisms of this biosurfactant, valuable insights can be gained into its potential applications in the food industry. These applications include the prevention and control of biofilm formation on food contact surfaces and the development of novel food preservatives with improved safety and efficacy.

2. METHODOLOGY

2.1 Strains and growth conditions

Foodborne pathogens (*Listeria monocytogenes* NCCP 15743, *Salmonella* Typhimurium ATCC 15812, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* NCCP 01328, and *Escherichia coli* O157:H7 ATCC 43895) were obtained from the Korean National Culture Collection for Pathogens (NCCP) and American Type Culture Collection (ATCC). Each pathogen was cultured in 25 mL of tryptic soy broth (TSB) at 37 °C for 16 h. After centrifugation, the bacterial pellet was resuspended in phosphate buffered saline (PBS, pH 7.4). The concentrations of the bacterial suspensions were adjusted using optical density measurements at 600 nm. The probiotic strain, *L. reuteri* IDCC 3701, provided by Ildong Bioscience Co., was cultured in 25 mL of De Man, Rogosa and Sharpe (MRS) broth at 30 °C for 48 h. The bacterial pellet was resuspended, and the concentration was adjusted using optical density measurements at 600 nm.

2.2 Biosurfactant Extraction

The biosurfactant was extracted from the cell-free supernatant (CFS) portion of *L. reuteri* IDCC 3701 [10]. To produce CFS, *L. reuteri* was cultivated in MRS broth for 48 h at 30 °C under anaerobic conditions and CFS was separated by centrifugation at 8,000-×g for 40 min at 4 °C and filtered through a syringe filter with a pore size of 0.22-μm. To extract the biosurfactant, 20 ml of CFS was acidified to pH 1.8 with hydrochloric acid and incubated overnight at 4 °C. The precipitated biosurfactant was obtained by centrifugation (8,000 rpm, 10 min, 4 °C), washed in acidic water, and resuspended in PBS with the same initial volume [11]. The extracted biosurfactant was named as I-BS.

2.3 Surfactant Properties of I-BS

Emulsification Index (EI) was calculated based on the increase in the height of emulsion [12]. A mixture of 1 ml of I-BS, 4 ml of water, and 6 ml of vegetal oil was shaken vigorously for 2 minutes. After 24 hours, the height of the emulsion was measured, and the emulsification index was calculated using the equation 1:

$$EI = \frac{Height\ of\ emulsion\ layer}{Total\ height} \times 100$$

Equation 1. Calculation of emulsification index (%).

Emulsification activity was measured by mixing 4 ml of I-BS with 1 ml of vegetable oil, shaking vigorously for 2 minutes, and allowing to stand for 10 minutes before measuring turbidity at 540 nm. The absorbance before emulsification subtracted from the absorbance after emulsification was expressed as emulsifying activity [12].

Oil spreading test was performed in a Petri dish containing 50 ml of distilled water overlaid with 20 µl of vegetable oil. Then 10 µl of I- BS was added to the surface of the oil and the presence of clear zones were examined [13].

The drop collapse test was used to measure the decrease of the surface tension of the liquid. Briefly, 10 µl of I- BS was pipetted as a drop onto a parafilm and the spread of the drop was examined over 5 minutes [13]. A positive result was present if the droplet diameter was at least 1 mm larger than that of the negative control.

Microplate distortion assay was performed by adding 100 µl of I-BS into a microwell of a 96-microwell plate. The plate was viewed using background paper with a grid. When a biosurfactant is present, the concave surface distorts the image of the underlying grid. The optical distortion of the grid provides qualitative evidence for the presence of surfactant [14].

2.4 Antibiofilm Activity of I-BS

To investigate the effect of I-BS on biofilm inhibition, the Calgary Biofilm Device was used (Biofilm Formation Assay Kit, Dojindo, Japan). The methodology was performed in accordance with the technical manual, with slight modifications [15]. A 96-well plate was prepared, where 90 µl of sterile TSB, 10 µl of microbial cell suspension of each genus of foodborne pathogens, and 50 µl of I-BS were placed. A lid plate was positioned on top. The plates were incubated at 37 °C for 24 h under aerobic conditions to allow biofilm formation on the peg. Subsequently, the lid plate was immersed in 200 µl of sterile PBS to remove planktonic cells and was stained with 200 µl of crystal violet solution (CV) for 30 min at room temperature. The excess CV in the lid plate was removed by soaking it in sterile PBS. The plate was placed in a 96-well plate containing 200 µl of absolute ethanol and was incubated at room temperature for 15 minutes. Finally, the peg-lid plate was removed, and the absorbance of the 96-well plate with the dissolved CV was measured in a microplate reader at 590 nm. The results were compared with the negative control (sterile well) and the growth control (untreated biofilm).

2.5 Chemical Analysis by GC-MS

The method described by Burgut [16] was used to identify volatile biosurfactant compounds by gas chromatography-mass spectrometry analysis (GC-MS) in CFS. The analysis was performed using an Agilent 7890A gas chromatograph coupled directly to a mass spectrometer system. A nonpolar (5% - phenyl)-methylpolysiloxane column (30 m × 250 µm × 0.25 µm, Agilent 1901S-433HP-5MS) was used. Helium was the carrier gas, and the flow rate was 1.5 ml/min. The initial oven temperature was 50 °C and was maintained for 2 minutes until the final temperature reached 240 °C at a rate of 2°C/min. The injection volume was 1 µl of diluted sample in hexane. The total GC run time was 86 minutes. Detected peaks in GC-MS were matched with those in the commercial library of NIST/EPA/NIH. The relative percent amount of each compound was expressed by comparing its average peak area to the total area.

2.6 Statistical Analysis

All the experiments were conducted in triplicates. The data were shown as the mean ± standard deviation. Statistical analysis of the data was performed using GraphPad Prism Version 8.3.0 (GraphPad Co., San Diego, CA, USA). The means were compared using the student's paired t-test for two-group comparisons and one-way analysis of variance (ANOVA) for multi-group comparisons. A *p*-value of less than 0.05 indicated statistical significance.

3. RESULTS AND DISCUSSION

3.1 Extraction and Characterization of I-BS

In this study, the biosurfactant was extracted from the CFS of *L. reuteri* IDCC 3701 using a two-step process. The first step involved acidization of the medium to achieve the biosurfactant's isoelectric point, resulting in reduced solubility and enabling its extraction through centrifugation. Subsequently, the biosurfactant (designated as I-BS) was thoroughly characterized in terms of its overall properties and emulsion behavior.

The obtained results revealed significant differences in the emulsion index and emulsifying activity values of the biosurfactant compared to the negative control. The biosurfactants exhibited notable emulsifying activity with an emulsion index of 49.4% and an emulsifying activity value of 400.67. These findings suggest that I-BS has the ability to form stable emulsions and can be effectively employed as an emulsifier for oils. Furthermore, positive outcomes were obtained from the oil spreading test and microtiter plate test, indicating the potential applications of the biosurfactants. However, the drop collapse test yielded a negative result, suggesting that the biosurfactants may not efficiently reduce the surface tension of the liquid. A summary of the results is presented in Table 1.

Table 1. Characterization of the I-BS regarding emulsification index, emulsification activity, oil spreading test, microtiter plate test and drop collapse test. All the experiments were conducted in triplicates. The data were shown as the mean ± standard deviation. A *p*-value of less than 0.05 indicated statistical significance between treatments.

Assay	Biosurfactant	Negative Control
Emulsification Index	49±2a	39±0.7b
Emulsification Activity	400.67±10.15a	0.33±0.05b
Oil Spreading	Positive	Negative
Drop Collapse	Negative	Negative
Microtiter Plate Distortion	Positive	Negative

Comparatively, the observed emulsion index of 49% in this study was lower than previous findings. For instance, Saravanan and Vijayakumar [17] reported an emulsion index of 65.5% for the bacterium *Pseudomonas aeruginosa* PB3A, while Menezes Bento, de Oliveira Camargo [18] found a consortium of isolates from a Long Beach soil sample with an emulsion index of 64%. These higher emulsion index values suggest that the biosurfactants produced by those organisms possessed stronger emulsifying properties.

It is important to note that biosurfactant molecules can serve various roles in bacteria, including pathogenesis, as highlighted by Phale, Malhotra [19]. However, *L. reuteri* IDCC 3701, the strain used in this study, is a non-pathogenic probiotic isolated from breast milk [20]. Previous study performed a comprehensive genomic, phenotypic, and toxicity analysis, including an acute oral toxicity test, and concluded that *L. reuteri* IDCC 3701 is safe for human consumption as a probiotic [20].

In addition, the majority of studies focused on biosurfactants primarily extract them from pathogenic strains, while neglecting safety assessments. This lack of evaluation inhibits our ability to determine whether these biosurfactants can be safely employed in practical applications for humans and food. Complicating matters further is the utilization of the hemolysis test, commonly employed in the preliminary screening of microorganisms for biosurfactant production [21]. This test is also utilized in the safety evaluation of probiotics, where a negative result is required to ensure their safety [22]. Such conflicting analyses make it challenging to identify a probiotic strain that is both safe and suitable for subsequent analysis of biosurfactant production.

In this study, the seemingly conflicting results of a negative hemolysis test but positive biosurfactant production by *L. reuteri* IDCC 3701 can be explained by findings from Schulz, Passeri [23]. Their research showcased the capability of non-hemolytic strains to produce biosurfactants, and some biosurfactants were shown to have no hemolytic activity at all. This sheds light on the possibility that *L. reuteri* IDCC 3701 may indeed be a non-hemolytic strain that produces biosurfactants, aligning with the observed outcomes.

Among the various biological roles they fulfill, biosurfactants act as versatile molecules that facilitate interactions between microorganisms and their environment, playing crucial roles in the biological functions of microbial cells [24]. These compounds effectively reduce surface tension at liquid interfaces, thereby enhancing the solubility and availability of hydrophobic substances like organic pollutants or insoluble nutrients, making them more accessible for microbial uptake [25]. In addition to this, biosurfactants also contribute significantly to biofilm formation, cell motility, and adhesion processes, enabling microorganisms to colonize various ecological niches [26]. Their unique ability to modulate surface properties and promote cellular interactions highlights the profound significance of biosurfactants in microbial physiology and the overall functioning of ecosystems.

3.2 Antibiofilm Activity of I-BS

The antibiofilm properties of I-BS were investigated against significant foodborne pathogens. To evaluate the inhibition of biofilm formation, the biofilm formation percentages of the treatment group were compared with those of the positive control group. Results showed that I-BS had an inhibition percentage of 6.7% against *L. monocytogenes*, 8.1% against *S*. Typhimurium, 34.1% against *B. cereus*, 20.5% against *E. coli*, and 21% against *S. aureus*. These findings highlight the potential of I-BS as an effective agent to combat biofilm formation in various foodborne pathogens.

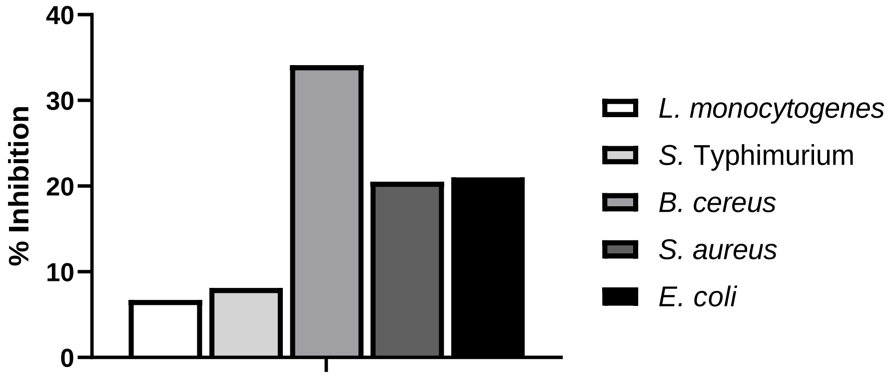

Fig 1. Biofilm inhibition of the biosurfactant extracted from *L. reuteri* IDCC 3701 against major foodborne pathogens.

The biofilm inhibition percentages of I-BS were compared to those reported in reference studies, providing insights into the effectiveness of I-BS as a potential antibiofilm agent. For example, Padmavathi and Pandian [27] supports the notion that biosurfactants can effectively inhibit biofilm formation. In that study, biosurfactant extracted from coral associated bacteria showed significant biofilm inhibiting activity against *P. aeruginosa* ATCC10145, with inhibition percentages ranging from 79% to 89%. A similar trend was observed in study by Jose, Krishnankutty [28], where the biosurfactant BSB1 exhibited considerable biofilm inhibition against *S. aureus* MTCC 1430. At a concentration of 1 mg/ml, BSB1 achieved a biofilm inhibition percentage of 41.79%, which increased to 79.22% at a concentration of 2 mg/ml.

Comparing these reference studies to the results obtained in the present study, it can be observed that I-BS exhibited moderate to low biofilm inhibition percentages when compared to the highly effective biosurfactants described in the references. The inhibition percentages achieved by I-BS were generally lower than those reported for coral associated bacteria and BSB1. This difference might be attributed to variations in biosurfactant composition, structure, and concentration, as well as the specific bacterial strains and conditions used in each study.

It is worth noting that the reference studies employed different methodologies for evaluating biofilm inhibition, such as direct observation, optical density measurement, or quantification of biofilm formation percentages. Furthermore, as mentioned earlier, most of these studies often fail to include safety assessments of used strains. These variations in experimental approaches can also contribute to differences in reported inhibition percentages.

In summary, the unique properties of biosurfactants enable them to modify surface tension and the physicochemical characteristics of the environment, thereby disrupting microbial adhesion and impeding the formation of strong biofilm structures [29, 30]. Furthermore, certain biosurfactants exhibit antimicrobial properties that directly target the viability and growth of microorganisms associated with biofilms, providing an additional barrier against biofilm development [31].

The role of biofilms in contributing to foodborne illness outbreaks and posing significant challenges in the food industry cannot be underestimated [32, 33]. Approximately 60% of these outbreaks have been attributed to biofilms, emphasizing the critical need to develop effective strategies for their prevention and control [32].

The development of disinfectants that specifically target biofilms holds great promise for enhancing food safety and improving production efficiency in the food industry. However, further research is needed to gain a better understanding of the underlying mechanisms of action of I-BS and its potential application in real-world food processing environments. Additionally, comprehensive studies evaluating the safety and efficacy of I-BS on different types of biofilms and surfaces are necessary to establish its suitability for widespread implementation.

3.3 Structural Characterization of Biosurfactant (GC-MS)

During the screening process for compounds present in the CFS associated with the biosurfactants, a significant peak corresponding to octanoic acid (Figure 2) was observed. Octanoic acid, also known as caprylic acid, is commonly used as a surfactant in the lubricant industry for soaps and detergents [34]. It is also recognized for its antimicrobial and pesticidal properties in food processing facilities such as wineries and breweries.

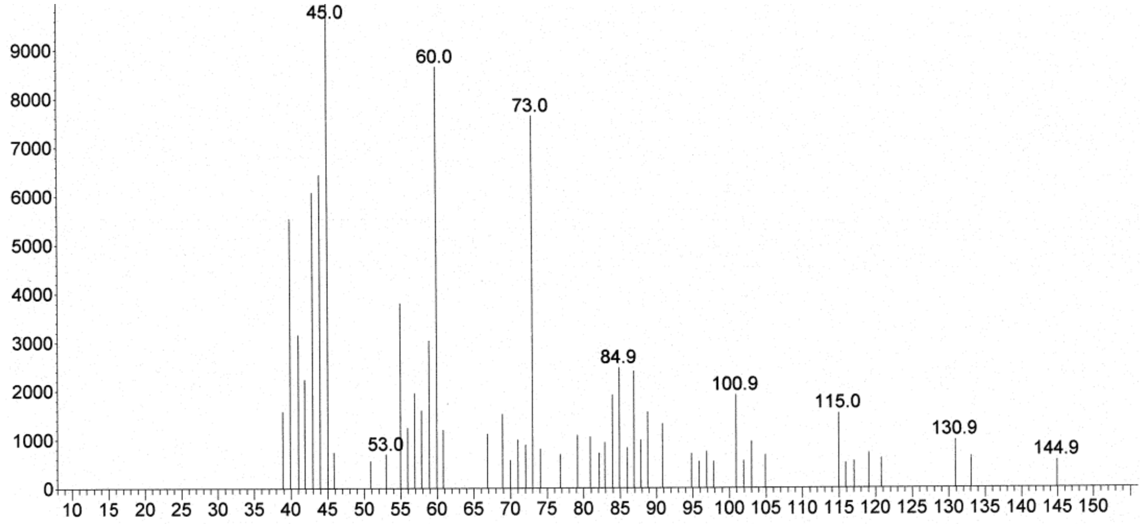

Fig. 2. Peaks obtained for octanoic acid during GC-MS analysis of cell-free supernatant of *L. reuteri* IDCC 3701.

Similar to our findings, Puntus, Sakharovsky [35] reported the presence of octanoic acid in the biosurfactant extracted from *Burkholderia*. In other studies, Sharma Sharma, Saharan [6] and Ibrahim, Ijah [36] identified a similar compound, octadecanoic acid (stearic acid), as the main fatty acid in biosurfactants extracted from *Lactobacillus helveticus* and *Serratia marcescens*, respectively (Table 2). Hexadecanoic acid and lipoteichoic acid were also identified as components of the biosurfactants produced by *Rhodococcus* sp. and *Lactobacillus casei* subsp. *rhamnosus*, respectively [37, 38].

Table 2. Surfactant extracted in other studies.

Bacteria	Surfactant Compound	Reference
L. reuteri	Octanoic Acid	This study
Burkholderia	Octanoic Acid	Puntus, Sakharovsky [35]
Serratia marcescens	Octadecanoic acid	Ibrahim, Ijah [36]
Lactobacillus helveticus	Octadecanoic acid	Sharma, Saharan [6]
Lactobacillus jensenii	Methypentadecanoic acid	Morais, Cordeiro [39]
L. casei subsp*., rhamnosus*	Lipoteichoic Acid	Velraeds, Mei [37]
Rhodococcus sp.	Hexadecanoic acid	Peng, Wang [38]
L. delbrueckii	Glycolipid	Thavasi, Jayalakshmi [40]

The presence of octanoic acid in our study suggests that it may be the primary fatty acid component of a glycolipid biosurfactant. Glycolipid biosurfactants consist of a fatty acid combined with a carbohydrate component and belong to a group of compounds that vary in the composition of their lipid and carbohydrate constituents. These glycolipid biosurfactants exhibit various functional properties such as emulsification, foaming, wetting, anti-adhesive, and anti-biofilm formation, as well as biological properties including antibacterial activity. Consequently, they have potential applications in the food and food-related industries as food additives and preservatives [41].

Other studies focusing on biosurfactants have also investigated their related properties and aimed to identify their main components. Morais, Cordeiro [39] characterized biosurfactants produced by *Lactobacillus jensenii* P6A and *Lactobacillus gasseri* P65 and found that they exhibited antibiofilm and antimicrobial activities against *E. coli*, *Candida albicans*, *Staphylococcus saprophyticus*, *Enterobacter aerogenes*, and *Klebsiella pneumonia*. Gas chromatography-mass spectrometry analysis revealed a major peak corresponding to 14-methypentadecanoic acid in *L. jensenii* P6A, which was the predominant fatty acid component of the biomolecule, and eicosanoic acid in *L. gasseri* P65. In another study, biosurfactants extracted from *L. gasseri* BC9 through cell wall-bound biosurfactants in phosphate-buffered saline exhibited activity against biofilms of methicillin-resistant *S. aureus*, although the specific main component of the biosurfactant was not specified [42].

The molecular structure of biosurfactants contributes to their functional diversity and specificity. Biosurfactants can self-assemble and form different micellar structures, such as spherical, rod-like, and wormlike micelles, depending on their molecular composition [43]. This feature is particularly beneficial for applications in the food, cosmetic, and pharmaceutical industries, as well as in the detoxification of pollutants and the demulsification of industrial emulsions [2, 43]. Even small differences in the molecular structure of biosurfactant congeners can result in significant variations in functionality. For example, different forms of sophorolipids, with lactonic or acid nature, exhibit distinct antimicrobial properties [44]. This diversity in functionality allows for targeted applications and provides a competitive advantage over synthetic surfactants.

Moreover, the composition of biosurfactants makes them more biocompatible and biodegradable compared to synthetic counterparts. Biosurfactants are inherently biodegradable, and they can enhance the biodegradation of pollutants by solubilizing them and promoting the growth of indigenous microorganisms [45]. This property has been demonstrated in various scenarios, including the biodegradation of oil in sand and seawater samples and the enhanced biodegradation of motor oil from contaminated soils [46]. The biocompatibility and digestibility of biosurfactants, mainly comprising glycolipidic and lipoprotein structures, make them valuable compounds for use in the pharmaceutical, food, and cosmetic industries [40].

4. CONCLUSION

In conclusion, this study highlights the potential of the biosurfactant I-BS, extracted from *L. reuteri* IDCC 3701, as an effective biofilm control and emulsifier agent. Overall, I-BS demonstrated strong emulsifying properties and showed significant inhibitory effects on biofilms formed by foodborne pathogens. Octanoic acid was identified as the main compound responsible for its biofilm-inhibitory activity. These findings have important implications for industries facing biofilm-related challenges, such as food processing and healthcare, as I-BS offers a natural and eco-friendly alternative to conventional chemical agents.

Further research is needed to explore the underlying mechanisms of I-BS's biofilm-inhibitory activity and optimize its application conditions. Additionally, the safety, stability, and potential synergistic effects of I-BS with other antimicrobial agents should be thoroughly evaluated. Overall, this study suggests that biosurfactants like I-BS hold great promise for enhancing food safety and addressing biofilm-related issues in various industries, offering sustainable solutions that benefit both human health and the environment.

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COMPETING INTERESTS

The author declares that there are no competing interests or conflicts of interest to disclose regarding the research work submitted for publication. We have no financial or non-financial interests that could influence the findings or interpretation of the study.

AUTHORS' CONTRIBUTIONS

The author Rafael Levandowski designed the study, performed the experiments and statistical analysis, wrote the protocol, and wrote the manuscript. All authors read and approved the final manuscript.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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