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Selective Partition of Lipopeptides from Fermentation Broth: A Green and Sustainable Approach

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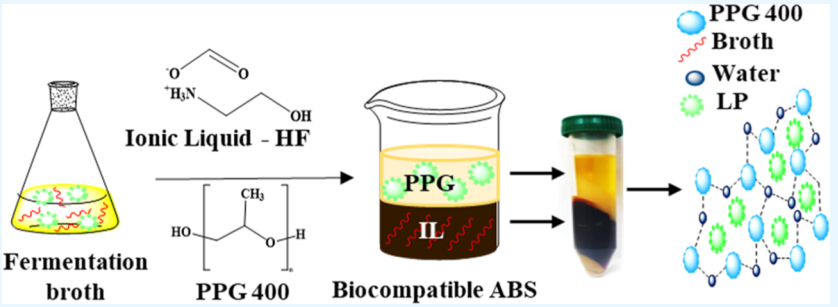

ABSTRACT: Lipopeptide (LP) biosurfactants from microbes have the potential to gradually replace chemical synthetic surfactants and fit the contemporary green and sustainable industrial production concept. However, their active participation is comparatively low in the global market pertaining to their low yield in microbial broth and costly downstream processes arising due to tedious isolation and purification methods. Herein, an efficient extraction method is developed that utilizes an aqueous biphasic system (ABS) comprising ionic liquids and polypropylene glycol 400 (PPG) to selectively extract a mixture of cyclic lipopeptides, namely, surfactin and fengycin from the culture broth of *Bacillus amyloliquefaciens* 5NPA-1, isolated from the halophyte *Salicornia brachiata* Roxb. Out of four different ABSs, the ABS composed of 2-hydroxyethyl ammonium formate and PPG displayed a maximum extraction efficiency of 82.30%. PPG-rich phase containing lipopeptides exhibited excellent antimicrobial and mosquito larvicidal properties with no toxic effect on plants. The developed method is simple, novel and accelerates the application of cyclic lipopeptides produced by the microbial source.

INTRODUCTION

Lipopeptide biosurfactants are a family of amphiphilic secondary metabolites having a plethora of unique biochemical properties, for instance, strong antagonistic effects on fungi, bacteria, and viruses. Lipopeptides show hemolytic properties and are associated with several physicochemical properties such as low toxicity, biodegradability, environmental compatibility, and resistance to extreme environments (temperature, pH, and salinity). Lipopeptides find applications in the agriculture sector, food industry, chemical industry, environmental engineering, oil recovery, cosmetics, and pharmaceuticals.[1,2] The bacterial genus *Bacillus* is one of the most prolific producers of cyclic lipopeptides (LPs), namely, iturins, fengycins, and surfactins.[2] Structurally, LPs encompass a short hydrophilic peptide head and a long-chain hydrophobic fatty acid tail, with the peptide moiety often being cyclic and either having a neutral or negative charge. The surfactins consist of a polypeptide chain of 7 amino acid residues cross-linked with β-hydroxy fatty acid chains containing 13 to 15 carbon atoms through ester bonds to form polar compounds with a lactone ring structure. Similarly, fengycins have a polypeptide chain of 10 amino acid residues cross-linked with saturated or unsaturated fatty acid chains containing 14 to 18 β-hydroxyl groups.[3,4] The microbial lipopeptides have the potential to gradually replace synthetic chemical surfactants and fit the contemporary green and sustainable industrial production concept. Despite their advantages over synthetic surfactants, the global market of biosurfactants is stunted, pertaining to low productivity from microorganisms and the heavy cost attached to downstream processes.[5] The various methods involved in the extraction of microbial cyclic lipopeptides from the *Bacillus* sp. are acid precipitation, solvent extraction,[6] ultrafiltration,[4,7] flocculation,[8] membrane adsorption,[9] macroporous resins,[10] etc. The inadequacies such as the involvement of multiple steps, prolonged time duration, specific instrumentation, and use of acids and solvents that

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are associated with the mentioned extraction methods make the process unsafe and expensive. Hence, there is a clear requirement for an easily scalable extraction process that may be simple, confined to limited steps, cost-effective, and less time-consuming.

Aqueous biphasic systems (ABSs) are composed of two aqueous-rich phases that are immiscible and based on polymer−polymer, salt−salt, or polymer−salt combinations. ABSs are alternatives to the traditional liquid−liquid extraction techniques which use vast amounts of volatile organic solvents.[11] ABSs are recognized as biocompatible media for the extraction and separation purposes of bioactive molecules due to their aqueous nature.[12] Several bioactive molecules lose their activity during the extraction process due to degradation in the presence of harsh organic solvents. ABSs are considered viable alternatives for the separation and purification of value-added biopharmaceuticals.[13−15] Ionic liquid-based aqueous biphasic systems (IL-ABSs) were reported to be prepared by the addition of inorganic salts to IL aqueous solutions that can facilitate separation applications.[16] The well-established advantages of IL-ABSs for the extraction of bioactive molecules have been reported previously for selective extraction of IgG, an antibody of rat blood serum using bio-IL-ABSs.[17] Furthermore, selective partitioning of a polypeptide, namely, ε-polylysine from the microbial culture broth, was also achieved using an ABS comprising polypropylene glycol 400 (PPG) and an IL with a maximum extraction efficiency of around 90%.[18]

Following the same trajectory, a successful attempt was made to extract cyclic lipopeptides from the microbial broth of the strain *Bacillus amyloliquefaciens* 5NPA-1 using the ABS made of PPG and an IL. However, it was observed that the cyclic lipopeptides were soluble in PPG rather than in the IL due to their amphiphilic nature. Four different ABSs containing various ILs were screened for the extraction process depending upon the extraction efficiency of lipopeptides. Cyclic lipopeptides from microorganisms such as *Bacillus* sp. and *Pseudomonas* sp. are known to exhibit remarkable antimicrobial properties[19] as well as inhibitory capacities against mosquitoes *Anopheles stephensi*, *Aedes aegypti*, and *Culex* sp. that are vectors for various deadly diseases.[20−23] Therefore, the extracted lipopeptides in the PPG layer were further assessed for their antimicrobial and mosquitocidal properties along with plant toxicity. This is so far the first report on the extraction of lipopeptides from microbial broth employing an ABS (Figure 1). Furthermore, lipopeptides could be partitioned in simple steps using the IL-ABSs in contrast to the several steps required in conventional extraction methods such as acid precipitation or chromatography, and the systems can be explored further from the scale-up point of view. Thus, a novel and sustainable process is reported for the selective extraction of cyclic lipopeptides (surfactins and fengycins) into the PPG phase of ABSs that can be directly utilized for various agricultural and mosquitocidal applications.

EXPERIMENTAL SECTION

Chemicals and Materials. Analytical-grade choline hydrogen carbonate, 2-hydroxyethylamine, and polypropylene glycol were purchased from Sigma-Aldrich, USA. Acetic acid and formic acid were purchased from Spectrochem Pvt. Ltd, Mumbai, India. Microbial medium components were purchased from HiMedia, India.

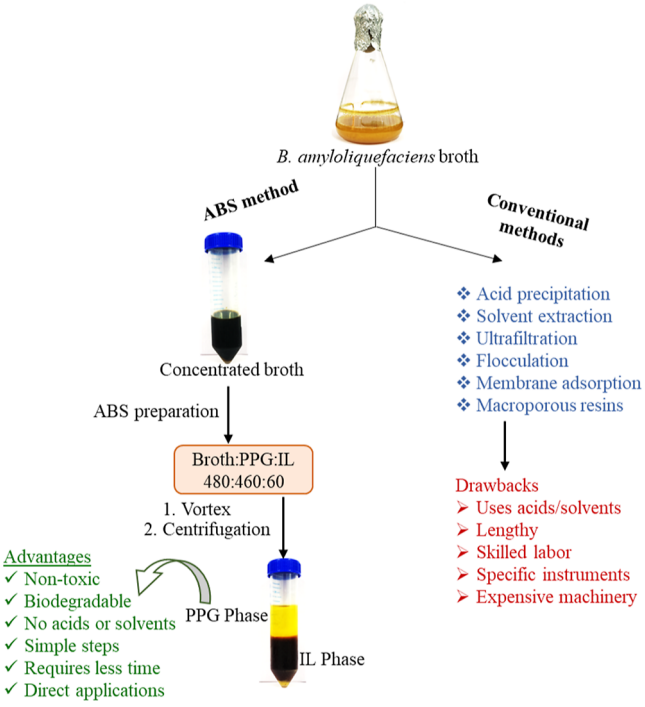

Figure 1. Process of separation using ABSs and its comparison with conventional methods.

Production of Lipopeptides in the Culture Broth. The cyclic lipopeptides in this study were produced by the fermentation of a bioactive endophytic strain *B. amyloliquefaciens* 5NPA-1 isolated from the halophyte *Salicornia brachiata* Roxb.[19] The seed inoculum was prepared by transferring loopfull culture to 10 mL of a nutrient broth culture medium (3 g of beef extract, 1 g of yeast extract, 5 g of peptone, 10 g of glucose, pH 6.8−7.0; water 1 L) and incubating at 30 °C, 120 rpm for 24 h. 20 mL of this cell suspension was then used as the inoculum in the biosurfactant production medium at 5% (v/v) concentration in a 1000 mL Erlenmeyer flask containing 400 mL of the production medium and incubated for 5 days at 30 °C and 120 rpm. The production medium contained glucose 35 g, glutamic acid 8 g, yeast 4 g, KH_2PO_4 1.1 g, KCl 0.6 g, $MgSO_4{\cdot}7H_2O$ 0.6 g, and trace elements $FeSO_4{\cdot}7H_2O$ 0.6 mg, $MnSO_4{\cdot}4H_2O$ 10 mg, $CuSO_4{\cdot}6H_2O$ 0.2 mg with a final pH of 7.0 ± 0.2 and autoclaved at 115 °C, 15 lb pressure for 30 min. The culture was incubated for 5 days at 120 rpm and 30 °C. The culture broth was further concentrated under pressure using a rotary evaporator, which was then used for the extraction of cyclic lipopeptides (surfactins and fengycins).

Partitioning Lipopeptides Using an ABS. *Preparation of the ABS.* For partitioning of lipopeptides into the ABS, four different ILs, namely, 2-hydroxyethyl ammonium formate (HF), 2-hydroxyethyl ammonium acetate (HA), choline formate (CF), and choline acetate (CA), were synthesized in the presence of a nitrogen atmosphere, giving them inert conditions and were further subjected to high vacuum to remove traces of water. The samples of ILs were transferred to NMR tubes, and their ^{1}H NMR spectra were acquired to determine the purity of synthesized ILs. The synthesized ILs were utilized for the formation of ABSs as reported earlier.[18] Highly biodegradable and biocompatible PPG[24] with a molecular weight of 400 g mol^{-1} was used as a second phase-forming component. Ternary phase diagrams (Figure

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S1) for each IL + PPG 400 + water were initially determined to infer the mixture compositions required to form two-phase systems and their ability to form ABSs for the extraction, separation, and purification. The cloud-point titration of the ILs with respect to PPG 400 was carried out at RT under atmospheric pressure to determine the binodal curve of each ABS.[25]

Extraction of Lipopeptides. The cyclic lipopeptides were first extracted from 100 mL of culture broth using the conventional method of acid precipitation, and the concentration of lipopeptide was determined.[26] Using the information from the ternary phase diagrams, ABSs consisting of different weight compositions of ILs, PPG, and concentrated culture broth were prepared (Table S1).[18] Each ABS was mixed thoroughly on a vortex and further centrifuged for 15 min at 10,000 rpm for the separation into two phases, the PPG-rich phase (LP-PPG) and the IL-rich phase. These phases were used to ascertain the concentrations of cyclic lipopeptides in each phase using analytical reversed-phase ultrahigh-pressure liquid chromatography (Thermo Scientific Dionex UltiMate 3000 system comprising an LPG-3400SD pump, a WPS-3000 autosampler, and a DAD-3000 detector). The extraction efficiency percentage of cyclic lipopeptides (EE_{LP} %), which represents the selectivity of lipopeptides to a given phase, was determined by eq 1

$$EE_{LP}\% = W_{LP}^{IL}/(W_{LP}^{IL} + W_{LP}^{PPG}) \quad (1)$$

where W_{LP}^{IL} = total weight of lipopeptides in the IL-rich phase and W_{LP}^{PPG} = total weight of lipopeptides in the PPG-rich phase.

Characterization of Lipopeptides Using HPLC and ESI-MS. The qualitative analysis of the cell-free broth, PPG-rich phase, and IL-rich phase for detection of lipopeptides was achieved by analytical reversed-phase ultrahigh-pressure liquid chromatography (Thermo Scientific Dionex UltiMate 3000 system comprising LPG-3400SD pump, WPS-3000 autosampler, and DAD-3000 detector). For the detection of surfactins and fengycins, a chromatographic method with a total run time of 20 min was optimized using the previously isolated surfactins and fengycins in the laboratory. Detection was achieved with gradient elution using water (A) and acetonitrile (B) run from 25−45% B in 5 min, 45−60% B in 5 min, 60−95% B in 5 min, and 95% B for 5 min on a Luna C_8 column (Phenomenex C8, 5 μm, 250 × 4.6 mm ID) with an injection volume of 80 μL and a flow rate of 2 mL min^{-1} with UV detection at 214 nm.

ESI-MS of the LP-PPG phase was performed on an Agilent 6545 Q-TOF mass spectrometer (comprising an Agilent G7104A-1290 flexible pump and Agilent G7129B-1290 autosampler). The sample was prepared by adding the LP-PPG phase and methanol in the ratio of 1:1, and the solution was further filtered through a 0.22 μm sterile membrane filter. The spectral data was obtained through collision-induced fragmentation (source voltage at 100 V, N_2 gas at 10 L min^{-1} flow, and capillary temperature at 150 °C).

Antimicrobial Activity. The extracted LP-PPG phase was directly tested for antimicrobial activity against the human pathogens *Staphylococcus aureus* MCC 2043 and *Pseudomonas aeruginosa* MTCC 3541, the marine pathogen *Vibrio parahaemolyticus* MTCC 451, and the plant pathogens *Xanthomonas campestris* NCIM 5028 and *Fusarium oxysporum* NCIM 1008 and few other fungi isolated from the garden soil. For the antimicrobial investigation, a well diffusion assay was performed according to the CLSI guidelines. In brief, Muller-Hinton agar plates were used, on which 100 μL of log phase culture ($O.D._{600nm}$ 0.1) of pathogenic strain was spread using a sterile cotton swab. Furthermore, a heat-sterile metallic well borer was used to bore wells where 100 μL of the LP-PPG phase (100% concentration) was loaded. Only the PPG phase obtained via the ABS and streptomycin antibiotic (against bacterial pathogens) was kept as negative and positive controls, respectively. The plates were further incubated at 30 °C overnight, and afterward, a zone of inhibition was observed.

Plant Toxicity Experiment. Germination studies were performed using green grams (*Vigna radiata L.*). Initially, seeds were surface-sterilized three times using a 2% sodium hypochlorite solution and sterile water. A factorial completely randomized design was used in the present study. The experiment consisted of treating seeds of green grams with different concentrations of the extracted LP-PPG phase diluted in the range of 1 to 20%. About five seeds were incubated in each Petri plate, and each treatment had at least three replicates. To assess the germination percentage, the shoot length and root length of the seeds were measured, which were incubated in Petri plates containing moistened germination paper at 25 °C. The observations were recorded each day for 7 days, and at the end of the incubation period, all the above parameters were either measured or calculated. The germination percentage was calculated as the ratio of the number of seeds that germinated at the end of the incubation period to the total number of incubated seeds. The data recorded from the experiment were analyzed statistically using the MSTAT-C statistical software (MSTAT-C 1991, Michigan State University, East Lansing, MI). The significant differences between treatment means were analyzed with least significant difference at $p < 0.05$.

Mosquito Larvicidal Bioassay. The larvicidal activity was carried out against the laboratory-reared third instar larvae of *Culex quinquefasciatus* according to the WHO standard protocol (WHO 2005). Four replicates of 25 healthy third instar larvae were exposed to each dose in a 500 mL capacity plastic bowl containing 250 mL of sterile distilled water. Four concurrent controls were also maintained with each set of experiments. The number of dead larvae was counted after 24 and 48 h of exposure. The dose mortality response data were subjected to probit analysis for calculating LC_{50} and LC_{90} values and associated 95% fiducial limits, that is, upper and lower confidence limits using SPSS software.

RESULTS AND DISCUSSION

5NPA-1 is an endophytic strain isolated from the halophyte *S. brachiata* and was identified as *B. amyloliquefaciens* via 16s rRNA sequencing. The strain was reported to inhibit the growth of pathogens *S. aureus* and *X. campestris* due to the production of cyclic lipopeptides.[19] The culture broth was harvested at the end of 5 days, and then, the broth was concentrated using a rotary evaporator. The conventional acid precipitation method yielded 45 mg of the acid precipitate from 100 mL of the fermented broth of *B. amyloliquefaciens* 5NPA-1, and the obtained acid precipitate was found to contain 78% cyclic lipopeptides. The purity of the synthesized ILs was determined based on their NMR spectra as no additional peaks for impurity were observed (Figures S2−S9). The concentrated culture broth was extracted using different ABSs, and the final separation was done by centrifugation, resulting in the upper LP-PPG phase and the lower IL-rich phase.

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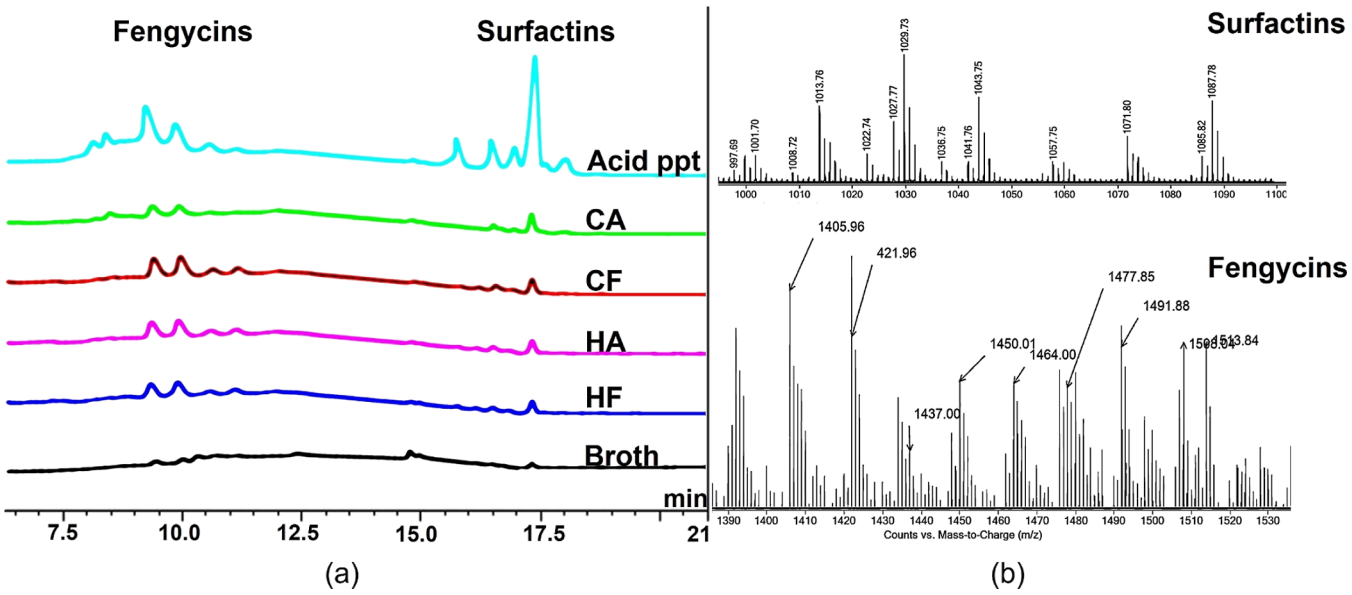

Figure 2. (a) Chromatographic overlay of lipopeptides partitioned in the LP-PPG phase of four different ABSs compared to *B. amyloliquefaciens* 5NPA-1 broth and lipopeptides obtained through the acid precipitation method. (b) ESI-MS confirming the presence of lipopeptides in the PPG-rich phase.

The chromatographic analysis of the separated phases revealed the presence of cyclic lipopeptides in the LP-PPG phase (Figure 2a). However, in some studies, extraction of proteins and polypeptides using the ABS made of the IL and PPG was reported to happen in the IL layer instead of the PPG phase.[18,27] Extraction of lipopeptides in PPG can be ascertained to the surfactant behavior of both the entities and that they may follow the basic rule of "like dissolves like". Based on the optimized chromatographic method prepared using standard cyclic lipopeptides, fengycins were observed to be eluted at the retention time between 7 and 10 min and surfactins eluted between 16 and 18 min. The ESI-MS of the LP-PPG phase further confirmed the presence of different surfactins with masses *m/z* 1008.72 and 1029.73 $(M + Na)^+$ for C_{13}, 1022.74 and 1043.75 $(M + Na)^+$ for C_{14}, 1036.75 and 1057.75 $(M + Na)^+$ for C_{15}, 1087.78 $(M + Na)^+$ for C_{18} surfactin. Fengycins with masses $(M + H)^+$ 1437.00 (C_{14} A), 1450.01(C_{15} A), 1464.00 (C_{16} A), 1477.85 (C_{15} B), 1491.88 (C_{16} B), and 1508.04 (C_{17} B) were also detected (Figure 2b).[19,28] The peak area covered by the cyclic lipopeptides in the chromatogram of each phase was calculated and compared with each other as well as with the culture broth to determine the extraction efficiency of ABS. Although the maximum concentration of lipopeptides was noticed to be partitioned in the PPG phase of CF-ABS, a comparatively high amount of lipopeptides was also phased out in the IL, ultimately reducing the extraction efficacy of the ABS made using CF. However, the ABS prepared using PPG and HF was observed to be most suitable with an extraction efficiency of 82.30% (Table 1).

Furthermore, the ABS made using choline-based ILs exhibited a low extraction efficiency for lipopeptides when compared with that made using hydroxyethyl ammonium-based ILs. A comparison between the area under curves from the HPLC chromatograms of the cyclic lipopeptides obtained from the acid precipitation method and the PPG layer of HF revealed that the concentration of cyclic lipopeptides in the PPG layer is 0.57 mg mL^{-1}.

Table 1. Extraction Efficiency of Different ABSs[a]

ABS	area under the curve for the LP-PPG phase	area under the curve for the IL-rich phase	extraction efficiency (%)
PPG-HF	3237.316	086.971	82.30
PPG-HA	2135.805	199.653	72.78
PPG-CF	3595.766	433.358	50.93
PPG-CA	2764.842	428.527	44.60

[a]Where PPG: polypropylene glycol 400, HF: 2-hydroxyethyl ammonium formate, HA: 2- hydroxyethyl ammonium acetate, CF: choline formate, CA: choline acetate.

The antimicrobial potential of the LP-PPG phase (LP-PPG) containing cyclic lipopeptides was determined using a well diffusion assay against multiple pathogens. The LP-PPG phase demonstrated the capability to inhibit human pathogens *S. aureus* MCC 2043 and *P. aeruginosa* MTCC 3541, an aquatic pathogen *V. parahaemolyticus* MTCC 451, and phytopathogens *X. campestris* NCIM 5028 and *F. oxysporum* NCIM 1008 along with some fungal strains isolated from the garden soil (Figure 3). High inhibition zones against soil-derived fungi and phytopathogens indicated the possible application of the LP-PPG phase in the agricultural sector as biocontrol formulations. *Bacillus* lipopeptides are known to exhibit excellent antibacterial and antifungal properties against various pathogens,[1] and similar bioactivities were replicated by the LP-PPG phase in the present work.

The phytotoxicity of the LP-PPG solution was analyzed using germination studies of green gram (*V. radiata L.*) variety P9072 procured from the Indian Agricultural Research Institute, Karnal, Delhi, India. Germination behavior was not affected by the treatment of seeds with the LP-PPG phase. Furthermore, no toxic behavior was observed in the seeds treated with the LP-PPG phase in comparison to the control plants when tested at different concentrations (Figure 4). All the physiological parameters were found statistically at par with the control. The germination study results further support the implementation of the LP-PPG phase for sustainable

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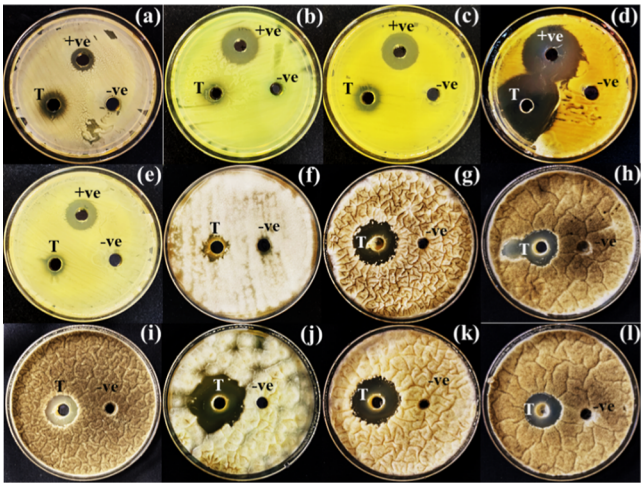

Figure 3. Antimicrobial activity of the LP-PPG phase (T) against multiple bacterial and fungal pathogens (a) *S. aureus* MTCC 2043, (b) *P. aeruginosa* MTCC 3541, (c) *M. smegmatis* MTCC 6, (d) *X. campestris* NCIM 5028, (e) *V. parahaemolyticus* MTCC 451, (f) *F. oxysporum* NCIM 1008, (g) GS1, (h) GS2, (i) GS3, (j) GS5, (k) GS6, and (l) GS 9. Streptomycin was used as the positive control (+ve) in the case of bacterial pathogens, and only PPG without any lipopeptides was used as the negative control (−ve).

agricultural practices, minimizing the use of chemicals. Nevertheless, greenhouse experiments will be required to completely sequester the in vivo biocontrol potential.

Biosurfactants from *Bacillus and Pseudomonas* bacteria are reported for their mosquitocidal activities against vectors *Anopheles, Culex,* and *Aedes* at their different life cycle stages.[20−23] Upon investigation, the LP-PPG layer exhibited significant mosquitocidal activity against *C. quinquefasciatus* with LC_{50} and LC_{90} values at 2.003 ppm and 13.525 ppm, respectively, after 24 h of post-treatment (Table 2). Mortality of the larvae was observed at different concentrations of the LP-PPG phase and also documented for 48 h (Tables S2 and S3).

The process of extraction of cyclic lipopeptides mainly surfactins and fengycins using the ABS is a fresh concept in the field. Low production and high downstreaming cost along with a lengthy process create room for the development of new protocols that accelerates the isolation of these biosurfactants. The mentioned ABS method does not use any harmful solvents or acids, is less laborious, has simple steps, easy on the pocket, and can be considered sustainable when compared to the other existing methods. Furthermore, the preparation of a direct antimicrobial formulation of these cyclic lipopeptides from the PPG-rich phase for application in fields without purification can significantly reduce the downstream processing cost. Conversely, in the case of other methods, the extracted lipopeptides need to be purified for various applications. However, in the case of the ABS method, the further extraction of cyclic lipopeptides from the PPG-rich phase was tried with acetone, but the obtained efficiency was not very significant. This factor limits the extraction of lipopeptides from PPG and restricts its use as the LP-PPG formulation.

CONCLUSIONS

Microbial lipopeptides because of their bioactive properties have applications in multiple areas, but their isolation processes limit their use. To tackle this problem, an attempt has been made to develop a novel, facile, safe, and sustainable process for extracting cyclic lipopeptides surfactins and fengycins

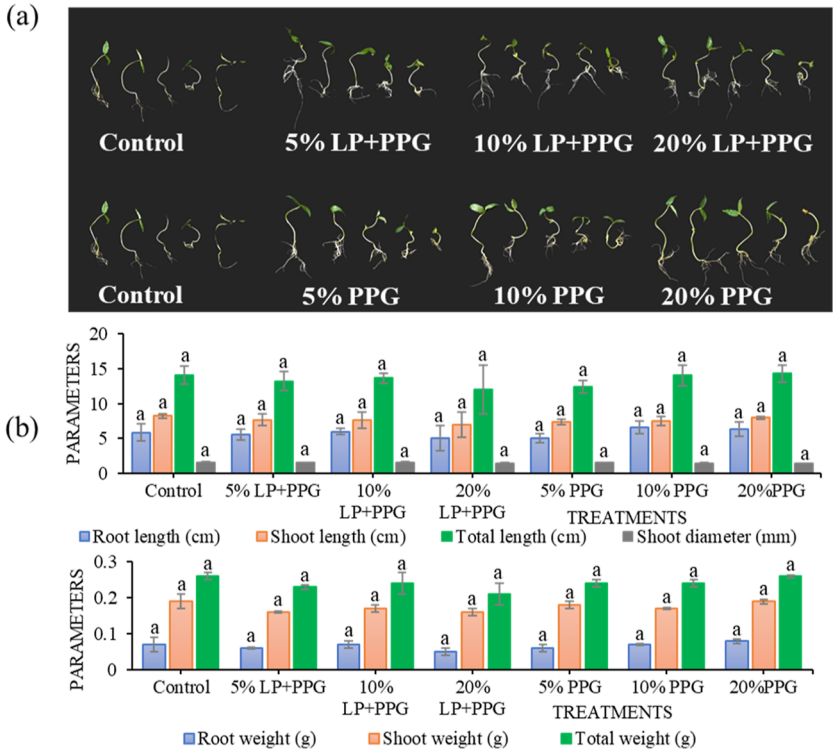

Figure 4. (a) Germination experiment to check the toxicity of the LP-PPG phase to the seeds of *Vigna radiata*. (b) Graphical representation of the effect of the LP-PPG phase on the germination of seeds.

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Table 2. Mosquito Larvicidal Activity of the LP-PPG Phase against the Third Instar Larvae of *Culex quinquefasciatus*[a]

exposure period	LC_{50} (ppm) (LCL-UCL)	LC_{90} (ppm) (LCL-UCL)	regression equation	slope (±SE)	X^2
24 h	2.003 (1.093−3.672)	13.525 (7.379−24.788)	$Y = 1.556x + 4.534$	1.556 ± 0.134	0.518
48 h	1.224 (0.632−2.374)	9.90 (5.106−19.194)	$Y = 1.412x + 4.875$	1.412 ± 0.147	0.956

[a]LC_{50} = lethal concentration killing 50% of the exposed larvae, LC_{90} = lethal concentration killing 90% of the exposed larvae, LCL = Lower confidence limit, UCL = Upper confidence limit, SE = Standard error, and X^2 = Chi-square.

directly from the fermentation broth of *Bacillus* sp. In contrast to conventional acid precipitation and membrane extraction methods, the developed procedure does not require heavy machinery, hazardous chemicals like strong acids or solvents, or specific expertise. Moreover, the lipopeptides extracted in PPG were found to be nontoxic to the plants and have direct antimicrobial and mosquitocidal applications and thus can work as a promising biocontrol alternative. There are possibilities of PPG supplementing the biological properties of the lipopeptides and hindering its toxic effects on plants. This technique will be a promising alternative to chemical fertilizers and pesticides, although further studies are required to prove these properties of the PPG and lipopeptide mixture. To conclude, the developed process requires no complex technology, and the production can be easily upscaled to meet the demand of an agriculture-based nation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c05587.

Synthesis and characterization of ILs, compositions of different ABSs, mortality data from larvicidal assay, ABS phase diagram, spectral data, and chromatogram (PDF)

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Notes

The authors declare no competing financial interest.

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