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Surfactant exopolysaccharide of *Ochrobactrum pseudintermedium* C1 has antibacterial potential: Its bio-medical applications *in vitro*

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ABSTRACT

Since the advent of biologics in human welfare various bio-molecules have been explored. Different bacterial exopolysaccharides have proved their worth in many industrial and commercial applications. In this perspective, while exploring a surfactant exopolysaccharide of *Ochrobactrum pseudintermedium* C1, it is strikingly observed that it possesses a potent antibacterial property which encourages its bio-medical applications. Following isolation and purification of the said exopolysaccharide, its structural configuration and functional attributes are studied by several analytical procedures involving FTIR, ^{13}C- NMR, CHN-analysis, estimation of zeta potential, XRD-study and digital tensiometry. When treated with pathological samples *in vitro*, it distinctly elicits its antibacterial property by exhibiting a characteristic zone of inhibition. Combined with a standard antibiotic (like ciprofloxacin), it enhances the action of antibiotic also. Mechanism of its antibacterial action is evaluated by crystal violet entrapment assay with UV–vis spectrophotometry, bacterial cell viability assay by trypan blue staining and SEM study. Results show that its basic surfactant property, anionic character, crystalline nature and scaffolding architecture are supposed to facilitate its antibacterial property which is manifested by its capability of disrupting bacterial cell envelope causing eventual cell death. In the current global scenario, an increasing threat of antibiotic resistance is prevailing due to their indiscriminate use. If used as an adjuvant with a judicious dose of antibiotic, this bio-molecule might play a significant role in bio-medicine to combat such threat.

1. Introduction

In order to survive in a competitive world, bacterial consortia adopt various defense mechanisms in adverse environments (Cavicchioli et al., 2019; Cramm et al., 2019) and adapt accordingly (Dutta and Dutta, 2016; Varma and Sharma, 2017). Such defense mechanisms are usually diverse and multifarious. Microbial adaptation is often an outcome of some intracellular orientation as a consequence of some extracellular changes (Bleuven and Landry, 2016). Besides extracellular machineries like capsular polysaccharide (Schmid et al., 2015), O-antigen and lipopolysaccharides (Lerouge and Vanderleyden, 2016), bacteria also produce several other protective polymers which are largely polysaccharide derivatives. They are generally synthesized intracellularly and translocated to the external medium by the orchestration of a band of proteins like Wzx/Wzy, synthase and sucrase (Schmid et al., 2015). One such metabolite produced during bacterial idiophase was coined as exopolysccharides (EPS) by Sutherland (1972). It generally forms an extracellular architectural framework called biofilm on the surface of a nutrient medium to meet both nutritional and oxygen demand of producer organism. Storage of nutrients by EPS and their gradual utilization in exigency displays a survival strategy.

Such a type of bacterial EPS, having a potent surfactant property, has been termed as surfactant EPS or SEPS (Sengupta et al., 2019). Surfactants are categorized as cationic, anionic, nonionic and zwitterionic surfactants (Sar et al., 2019; Mondal et al., 2016, 2015). These surfactants, when dissolved in aqueous medium, form different types of micellar aggregations above a certain concentration called critical micelle concentration (CMC). Such micelles can be of various shapes like normal spherical, vesicular, cylindrical and so on. (Saha et al., 2013a; Ghosh et al., 2014; Saha et al., 2013b). The interior hydrophobic part of a normal micelle is composed of hydrocarbon chains and is called palisade layer, whereas the surface of the polar heads face water. A concentric shell of hydrophilic head groups with $(1-\alpha)N$ counter ions, where α is the degree of ionization and N is the aggregation number or number of molecules in the micelle, and is called stern layer (Saha et al., 2014; Acharjee et al., 2019a, 2019b).

For the sake of competitive survival, a specific EPS produced by a specific bacterial strain might have toxic attribute against other species of microorganism. For instance, a thermo-stable surfactant EPS from *Paenibacillus macerans* shows remarkable antimicrobial activity not only

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against *Escherechia coli* and *Staphylococcus aureus* but also against fungal strains like *Fusarium oxysporum* and *Aspergillus fumigates* (Liang et al., 2014). Similarly bio-surfactants produced by *Lactobacillus jensenii*, *Lactobacillus rhamnosus* and *Bacillus subtilis* R15 show antibacterial action and antibiofilm formation against several multidrug resistant pathogens (Sambanthamoorthy et al., 2014; Fernandes et al., 2017). Unlike synthetic antibiotics which are targeted by specific bacterial antibiotic-resistant proteins, SEPS manifests its antimicrobial property with a wider spectrum in a non-specific manner (Vazquez-Rodriguez et al., 2018). Being anionic in character, SEPS generally destabilizes the cell membrane by binding with membrane cations resulting in significant alteration of bacterial cell permeability (Lang et al., 1989). A higher zeta potential of SEPS indicates better stability when applied as colloidal suspension or emulsion (Araújo et al., 2019) ensuring its sustained release resulting in effective bioavailability in biomedical context.

Globally, on the perspective of indiscriminate use of antibiotics, human population at large is suffering from undesirable side-effects, drug resistance and immune-suppression. According to FDA, almost all important bacterial infections in the US are becoming resistant to antibiotics, and thus it has been regarded as “one of the world’s most pressing public health problem” (Ventola, 2015). Many synthetic antimicrobials are remarkably bactericidal, but often interfere with host immune system (Anderson et al., 2010). With the advent of biologics and bio-pharmaceutics, biological drugs, owing to their immunological boosting, have become more popular and acceptable (Moscovici, 2015; Vultaggio et al., 2016). With a diverse applicative profile, bacterial EPS is currently of interest in biomedicine as an immune-modulator, an antioxidant, a recipe in vaccine formulation, an adjuvant, an antimicrobial agent, an ingredient in ointment formulation, and so on (Adebayo-Tayo et al., 2018; Liu et al., 2011). Biologically derived macromolecules, in combination with a judicious use of conventional antibiotics, might be considered as a rational and better therapeutic option to provide effective antimicrobial activity without compromising long term immunity. For instance, amalgamation of antibacterial pigment prodigiosin and a biosurfactant from *Serratia marcescens* proves to be an effective antibacterial agent (Steinfath et al., 2018). The biosurfactant sophorolipid acting as an adjuvant enhances antibiotic efficacy (Joshi-Navare and Prabhune, 2013). Similarly, two different bacterial biosurfactant metabolites like rhamnolipid from *Pseudomonas aeruginosa* and BS15 from *Bacillus stratosphericus* exert synergistic antimicrobial activity (Sana et al., 2017).

The present study evaluates *in vitro* a potent antibacterial activity of an SEPS isolated from *Ochrobactrum pseudintermedium* C1, a Gram negative, non-spore-bearing bacterial strain of Family Brucellaceae. The composition, texture, morphology and ionic charge of the SEPS have been studied by several procedures with relevance to its antibacterial property. It has also been applied in combination with a relatively low dose of antibiotic (ciprofloxacin) to explore whether it enhances the activity of the antibiotic.

2. Materials and methods

2.1. Collection and maintenance of bacterial strain

As a sequel of the prior study on *Ochrobactrum pseudintermedium* C1, showing its capability to produce surface-active EPS (Sengupta et al., 2019) the same strain (accession no. KJ094035 following 16S rRNA sequence in GenBank database) was collected from Pharmaceutical laboratory, Department of Chemical Technology, University of Calcutta. Its viability was maintained by sub-culturing in nutrient agar slants at 15 days interval and storing at 4 °C.

2.2. Preparation of culture media

The type of bacterial EPS produced largely depends on the substrate utilized. Among several substrates including starch, bagasse, molasses and fruit pulp extracts, 4% molasses extract was selected due to its high sugar content (US Department of Agriculture, Food Data Central, 2019). It was prepared by microwave for 2 min prior to bacterial inoculation. Bushnell Haas media (HiMedia Labs) was supplemented to it. As per optimization studies (Sengupta et al., 2019), the strain was incubated for 12 days at 34 °C in Scigenics Biotech Orbitetek LJE incubator and an optimum pH of 7.6 was maintained that facilitated adequate bacterial growth and maximum yield of EPS.

2.3. Extraction and purification of EPS

After incubation EPS formed a slimy matrix on the surface of culture media which was gently scooped and purified overnight with 95 % ethanol. The culture media was centrifuged for separation of bacterial cell mass and residual EPS was extracted, purified and dried.

2.4. Verification of surfactant property

Surface tension (ST) of aqueous suspension of extracted EPS was measured at 28 °C by digital tensiometer (Dataphysics DCAT 11, Germany) (Lunkenheimer and Wantke, 1981). Distilled water (ST = 71 mN/m) was regarded as reference. It was repeated thrice.

2.5. Estimation of Critical Micelle Concentration (CMC)

CMC was determined by measuring the surface tension of various concentrations of SEPS in HPLC water at 28 °C by DuNouy ring method using Test Master Tensiometer (Test Instruments Mfg. Co. Pvt. Ltd., India) and also with digital tensiometer (Dataphysics DCAT 11, Germany) (Lunkenheimer and Wantke, 1981). The concentrations of SEPS considered were from 0.1 g/l to 20 g/l. Respective STs (in mN/m) were plotted against the corresponding SEPS concentrations to determine the change in ST against SEPS concentrations and to find the CMC (Liang et al., 2014).

2.6. Analysis of chemical composition

Phenol-sulfuric acid test for total carbohydrate content was performed followed by UV–vis spectro-photometry at 490 nm (UV-1800 Spectrophotometer, Shimadzu Corp, Japan) (DuBois et al., 1956). Combustion rate of carbon, hydrogen and nitrogen of SEPS was analyzed by CHN analyzer (CHNS 932, Leco Corp, USA) considering acetophenone as reference. FTIR spectral scanning of SEPS considering KBr as standard was done at mid-range spectra from 4000 to 400 cm^{-1} (Perkin Elmer Spectrum Version 10.5.1) and was repeated thrice. Solid phase ^{13}C-NMR (DSX 300, Bruker, USA) of SEPS was performed to analyze its major structural constituents corroborating with prior ^{1}H-NMR study (Sengupta et al., 2019).

2.7. Determination of zeta potential

Dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments Ltd., UK) and determination of zeta potential of aqueous dispersion of SEPS was done with 4 mW helium-neon (He-Ne) laser beam at 633 nm with back scattering angle of 173°. The system temperature was 25 °C with count rate at 279.2 kcps. The study was repeated thrice.

2.8. Analysis of texture

To understand its texture, whether crystalline or amorphous, X-ray diffraction (XRD) study of SEPS was performed in X-Pert Pro X-ray Diffractometer (PW 3050/60, Philips, India) exposing to 45 kV generator voltage and 30 mA emission current with λ of 1.54 Å using CuK_{α} radiation. Then scanning electron microscopy of SEPS before its

desiccation was done. SEPS was fixed with 2% glutaraldehyde for 2 h followed by sequential dehydration by 20 %, 40 %, 70 % and 90 % v/v ethanol and was air dried by laminar air flow. The sample was sputter-coated with platinum and scanned (Zeiss Evo 18 Special Edition, Zeiss, Germany) at 15 kV.

2.9. Estimation of Minimum Inhibitory Concentration (MIC)

Fresh cell suspensions with 0.5 optical density (OD) at 600 nm and CFU around1 $\times 10^5$ /ml of *Staphylococcus aureus* (ATCC25923) and *Escherichia coli* (K8813), both strains having been obtained from MTCC Chandigarh, were considered for MIC assay (Andrews, 2001). SEPS concentrations ranging from 50 to 2000 μg/ml were prepared in Muller Hinton broth at pH 7.4, and then added to bacterial strains and incubated at 37 °C for 12 h. Ciprofloxacin (Albert Davis Ltd, India) was considered as standard. OD was measured at 600 nm before and after treatment with each SEPS concentration and MIC was determined.

2.10. Assessment of anti-bacterial activity with time

Based on MIC, time dependent antibacterial activity of SEPS was done (Sendi and Ruppen, 2015). Incubation periods for untreated and SEPS treated bacterial cultures in nutrient broth were successively 0, 3, 6, 9, 15 and 24 h followed by individual plating in nutrient agar and overnight incubation at 37 °C. Colonies were counted in a digital colony counter.

2.11. Determination of zone of inhibition

Aqueous SEPS suspensions were prepared by overnight stirring in magnetic stirrer (Remi 5MLH magnetic stirrer) at 800 rpm. Antibiotic disc diffusion assay was performed separately with sterile filter paper discs soaked in (a) SEPS (as per MIC), (b) low dose (10 μg/ml) ciprofloxacin, (c) high dose (20 μg/ml) ciprofloxacin and (d) combination of SEPS and low dose (10 μg/ml) ciprofloxacin, to determine the zone of inhibition in each situation against *E. coli* and *S. aureus* (Hudzicki, 2009).

2.12. Understanding mechanism of antibacterial action

2.12.1. Crystal violet entrapment assay

Crystal violet (CV) was selected for assessment of cell permeability of SEPS (Sana et al., 2017).

To understand the extent of such permeation of CV, initially the absorbance of CV at 590 nm in UV–vis spectrophotometer (UV-1800, Shimadzu Corp, Japan) was measured and considered as reference. Then sequentially samples of bacterial culture only, bacterial culture treated with SEPS, bacterial culture treated with ciprofloxacin, and bacterial culture treated with both SEPS and ciprofloxacin were studied. In each case 2 ml of the sample was taken and centrifuged (Tarson MC 01 Microcentrifuge) at 5000 rpm for 10 min. Precipitated cell pellets were treated with equal volume of CV and centrifuged at 10,000 rpm for 15 min. Supernatants were collected and absorbance was measured at 590 nm in the UV–vis spectrophotometer.

2.12.2. Trypan blue assay

For estimation of cell viability, trypan blue exclusion assay was performed with haemocytometer. Equal vol of 0.4 % of the dye was added to 50 μl of SEPS treated bacterial cell suspension (Louis and Siegel, 2011). 20 μl of mixture was mounted on haemocytometer and observed under light microscope. The percentage of viable cells was calculated as [1 − number of blue cells / total number of cells] X 100 (Strober, 2001).

2.12.3. Scanning electron microscopy (SEM) of bacterial cells

Overnight grown cultures of *E. coli* and *S. aureus* were treated with SEPS and incubated for 5 h at 37 °C. The cultures were washed twice and fixed with 3% glutaraldehyde for 2 h followed by sequential dehydration by 20 %, 40 %, 70 % and 90 % v/v ethanol and air dried. Bacterial samples were sputter-coated with platinum under vacuum and scanned in Zeiss Evo 18 Special Edition, Zeiss, Germany at 15 kV for SEM images.

2.12.4. Collection of pathological samples and biomedical applications in vitro

For biomedical application, pathological samples of urine containing *E. coli* and wound swab containing *S. aureus*, both having CFU as high as 100,000/ml and being sensitive to ciprofloxacin, were collected from Vidyasagar State General Hospital, Behala, Kolkata and were diluted until an OD of 0.5. The samples were then subjected to treatment by (a) SEPS (750 μg/ml), (b) low dose (10 μg/ml) ciprofloxacin, (c) high dose (20 μg/ml) ciprofloxacin and (d) combination of SEPS and low dose (10 μg/ml) ciprofloxacin. Zone of inhibition in each case was measured and compared.

3. Results and discussion

3.1. Chemical composition

Percentage of elemental carbon, hydrogen and nitrogen concentrations in SEPS obtained after CHN analysis was C 30.22 %, H 4.75 % and N 5.88 % respectively. This would go in favor of glycoprotein nature of SEPS (Upreti et al., 2003).

The FTIR findings (Fig. 1A) of this macromolecule suggested a polysaccharide backbone with constitutional carbohydrate moieties being linked by α-glycosidic bonds. The finding, as shown in Table 1, indicated presence of intramolecular hydrogen or hydroxyl bonding from the intense broad band observed at 3318.18 cm^{-1}. The peak at *circa* 2900 cm^{-1} suggested presence of C—H stretching by constituent aliphatic hydrocarbons (Parikh and Madamwar, 2006). Presence of C═O bond stretches of polysaccharide moiety was quite evident from peak at 1651.96cm^{-1} (Cao et al., 2017). Peak at 1537.33 cm^{-1} suggested presence of carboxyl or carboxylate group. Distinct peak at 1060.11 cm^{-1} in fingerprint region (1200 to 950 cm^{-1}) justified polysaccharide backbone of EPS (Chen et al., 2015). Peak around 830 cm^{-1} suggested presence of α-glycosidic linkage. Absence of such peaks at 900 or 890 cm^{-1} defied presence of β-glycosidic linkage in SEPS moiety (Ye et al., 2009). However, such FTIR peaks of SEPS simulated those of chitosan which is also a linear polysaccharide biomolecule composed of glucosamine units (Ramkumar and Sundaram, 2016a, b).

^{13}C-NMR findings (Fig. 1B) revealed presence of mostly saturated and partly unsaturated hydrocarbons. Table 2 showed the suggested function groups according to the ^{13}C-NMR peaks. Presence of carboxyl and amino groups were evident. Chemical analysis by ^{13}C-NMR in accordance with previously performed ^{1}H-NMR analysis (Sengupta et al., 2019) speculated presence of certain functional groups that formed an integral part of the SEPS moiety. For instance in ^{13}C-NMR spectra sharp signals at 170 − 180 ppm range suggested presence of carboxyl group. Peak at approximately 105 ppm suggested presence of unsaturated hydrocarbons ($R_2C{=}CH_2$). However multiple signal peaks from 60 to 80 ppm predicted presence of functional groups like C—OH, C—OR and/or C—NH. Further signal peak at 30 ppm was suggestive of saturated hydrocarbons (alkanes). Finally peaks at 20 ppm would suggest presence of multiple C—C—C bonds (Drouillard et al., 2015). On comparing the SEPS with standard EPS like dextran, xanthan, curdlan and cellulose, it was found that the isolated SEPS shared significant similarity with dextran (~82 %) (Shukla et al., 2011) and cellulose (>50 %) (Adebayo-Tayo et al., 2017) in contrast to xanthan (Nur Hazirah et al., 2016) or curdlan (Kalyanasundaram et al., 2012).

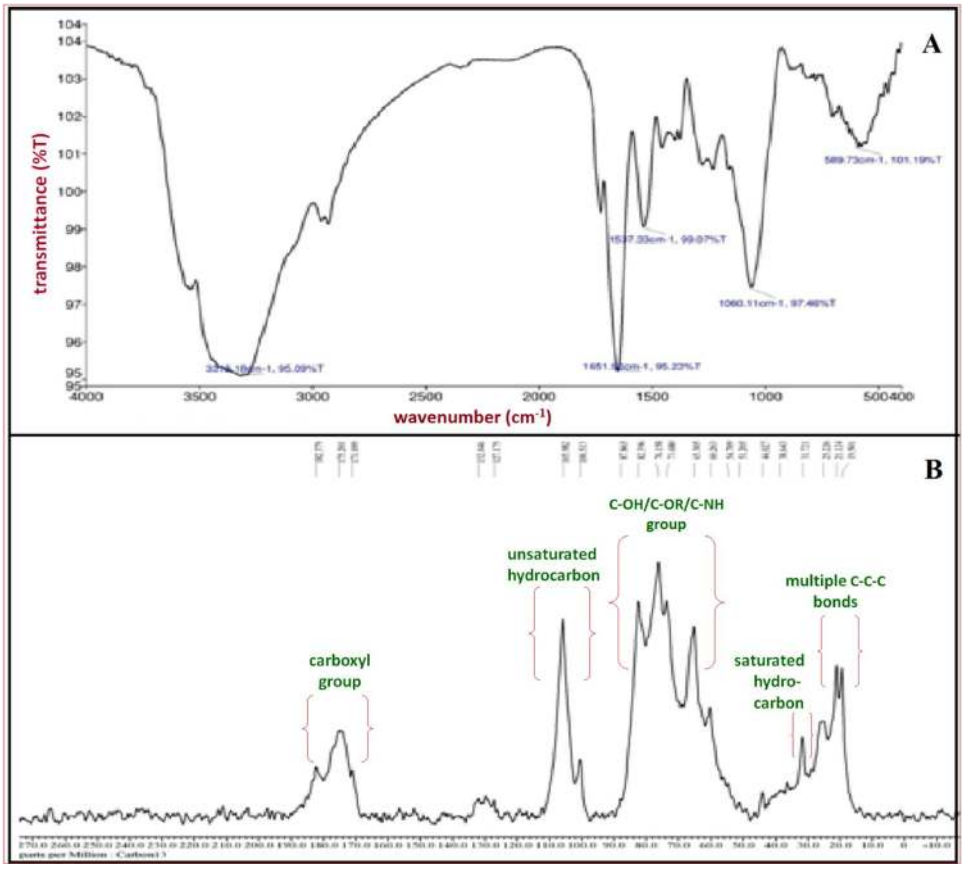

Fig. 1. (A) FTIR and (B) ^{13}C-NMR spectra of SEPS extracted from *Ochrobactrum pseudintermedium* C1 after purification.

Table 1
FTIR spectral analysis of SEPS isolated from *Ochrobactrum* sp.

FTIR spectral analysis	
Transmittance (cm^{-1})	Functional groups suggested
3318.18	Intra-molecular hydrogen bond or Hydroxyl group
~2900	C—H stretching by aliphatic hydrocarbon
1651.96	C═O bond stretches of polysaccharide moiety
1537.33	Carboxyl or carboxylate group
1060.11	Polysaccharide backbone
~830	α-glycosidic linkage

Table 2
^{13}C NMR spectral analysis of SEPS isolated from *Ochrobactrum* sp.

^{13}C NMR spectral analysis	
Peaks (ppm)	Functional groups suggested
170-180	Carboxylic acid
~105	Unsaturated hydrocarbons ($R_2C{=}CH_2$)
60 to 80	C—OH, C—OR and/or C—NH
30	Alkanes
20	Multiple C—C—C bonds

3.2. Solubility

Essentially being polysaccharide in nature the SEPS was not readily soluble in HPLC grade water but exhibited a hygroscopic tendency. However upon sonication by Biobase UC-20ST ultrasonic cleaner sonicator and/or stirring by Remi 5MLH magnetic stirrer at 500 − 800 rpm the aqueous SEPS suspension exhibited considerable solubility which was efficiently utilizable for the antibacterial study. A better solubility of SEPS, following rapid vortexing and standing, was however observed in case of chloroform but was not considered for the antimicrobial study due to its inherent toxicity.

3.3. Surface activity and CMC

Purified bacterial EPS showed appreciable mean ST reduction of media to as low as 28.274 mN/m (ST of distilled water being 71 mN/m) which conferred with prior studies on its inherent nature (Sengupta et al., 2019). CMC was identified from the changes in surface tension with respect to gradual increase in the surfactant concentration. Upon increasing the concentration of SEPS the ST gradually decreased from 71 to 29 ± 0.5 mN/m and remained constant after an SEPS concentration of 4.8 g/l which indicated the CMC (Fig. 2).

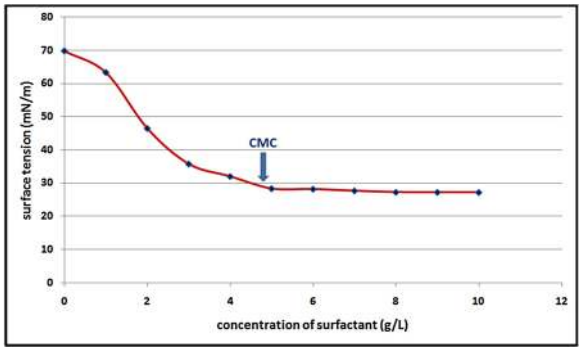

Fig. 2. Reduction of surface tension by SEPS at different concentrations.

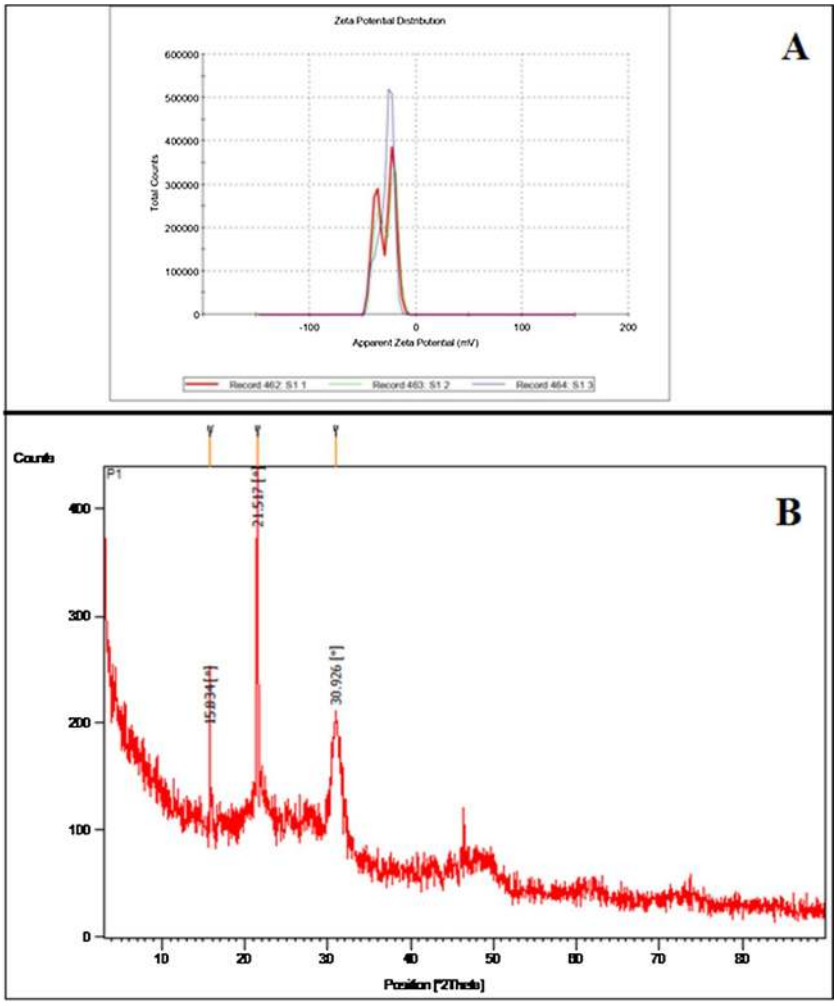

Fig. 3. (A) Zeta potential and (B) XRD spectra analyzing the inherent charge and surface morphology of SEPS respectively.

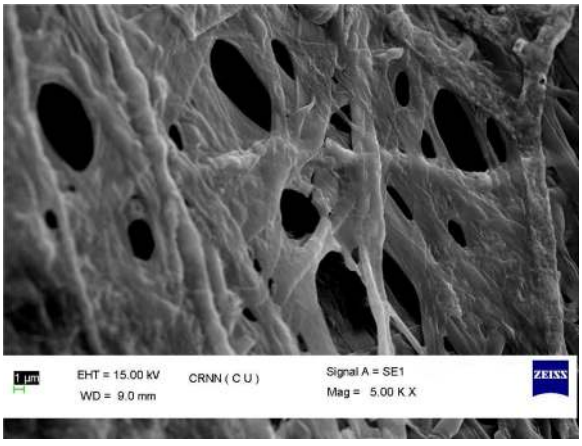

Fig. 4. SEM images of purified SEPS (before desiccation) displaying scaffold-like porous matrix (5K magnification).

3.4. Determination of charge

SEPS exhibited an inherent negative charge with an average zeta potential of -27.87 mV shown in triple sample runs as evident from Fig. 3A with an average electrophoretic mobility of -2.182 µm cm/Vs. The anionic nature of SEPS also favored its antimicrobial propensity. A negative charge of SEPS might trigger its binding to membrane cations like Na^+, K^+ and Ca^{2+} which are involved in cell membrane stabilization through respective gated channels (Furini and Domene, 2018). The SEPS binding might interfere with bacterial normal exchange of ions across cell membrane and destabilize the membrane potential. This might lead to an imbalance in bacterial intracellular turgor pressure leading to formation of cracks and pores in the cell envelope eventually resulting in extrusion of intracellular matrix and lysis of affected bacterial cells.

3.5. Analysis of texture and morphology

The morphology and topography of a bacterial EPS depends not only on methods of its extraction, preparation and purification, but also on diversity in its physico-chemical properties (Kanamarlapudi and Muddada, 2017). The XRD pattern of SEPS exhibited a moderately crystalline nature which was evident from intermittent sharp peaks exhibited in diffraction pattern (Fig. 3B). Three clearly resolved sharp peaks were observed at 2θ positions of 15.8345°, 21.5167° and 30.9260°. A fourth peak, comparatively short yet sharp was also evident at *circa* 46°. Considering all the four peaks conferring to crystalline nature of SEPS, a percentage crystallinity of about 41.3 % was calculated [% crystallinity = (total area of crystalline peaks / total area of all peaks) x 100]. XRD pattern of SEPS simulated that of bacterial cellulose (Fang and Catchmark, 2014; Jia et al., 2017). This crystalline nature corroborated with SEM findings of dehydrated SEPS of the same bacterial strain which upon dehydration resembled a scaly or flake-like structure. Its swelling and hygroscopic tendency (Wex et al., 2007) showed a distinct simile with bacterial cellulose (Park et al., 2009).

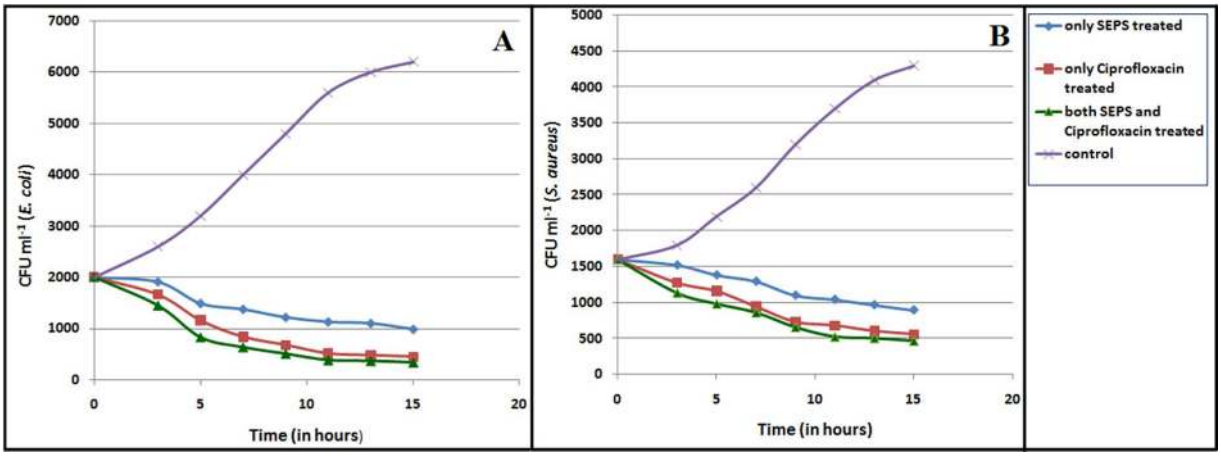

Fig. 5. Time kill kinetics assay of SEPS against (A) *E.coli* and (B) *S. aureus*.

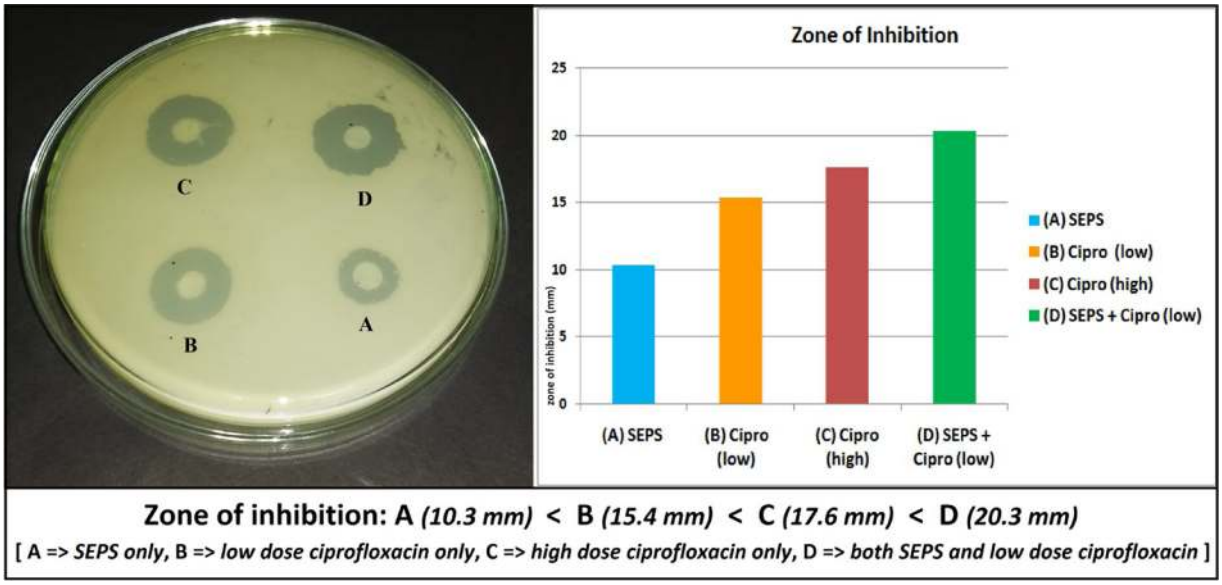

Fig. 6. Zone of inhibition exhibited by (A) SEPS, (B) low dose ciprofloxacin, (C) high dose ciprofloxacin and (D) combined SEPS and low dose ciprofloxacin.

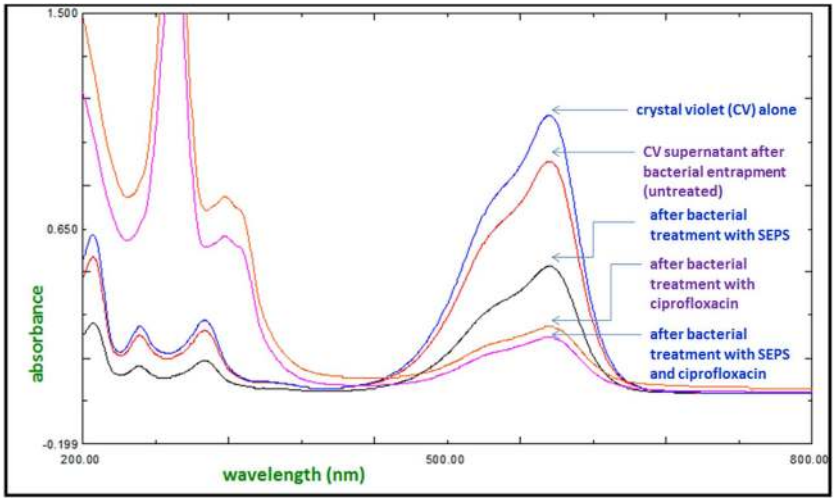

Fig. 7. Crystal violet (CV) entrapment assay: UV–vis spectra showing declining trend of CV absorbance in different samples.

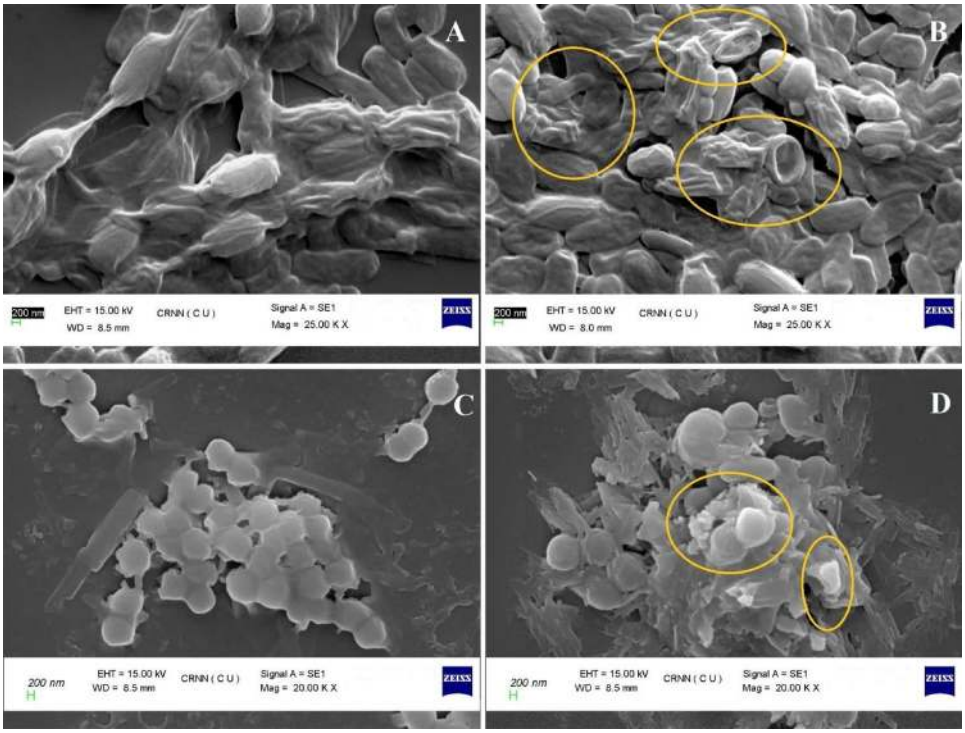

Fig. 8. SEM images of (A) dead and shrivelled *E. coli* treated with ciprofloxacin alone; (B) more affected (*marked*) by ciprofloxacin with SEPS; (C) dead *S. aureus* with disruption of coccoid morphology by ciprofloxacin alone; (D) more affected (*marked*) by ciprofloxacin with SEPS.

Here SEM micrographs at different magnifications clearly revealed porous, inter-connected web-like architecture of SEPS matrix (Fig. 4) which was quite analogous to cell adhesion molecule (CAM) of eukaryotic cells (Kerr, 1999). Porosity of SEPS was found to be heterogeneous and non-uniform. Also, texture appeared to be quite slimy which was a characteristic model pertaining to functional attribute of an EPS. SEPS fragments resembled a scaffold-like structure (Gorgieva and Trček, 2019), intermittently flaccid or shrunken in nature, interrupted by pores. It could be easily mistaken with fungal hyphae by its close structural resemblance. However, unlike hyphae SEPS threads were non-uniform in diameter and differed in pattern and texture.

3.6. Evaluation of antimicrobial potency

SEPS showed an MIC of approx 550 µg/ml against *E. coli* and approx 680 µg/ml against *S. aureus* with a peak bactericidal activity after 5 h of treatment at culture concentrations of O.D. 0.5 in Mueller Hinton broth at 600 nm.

According to time dependent antibacterial assay, the onset of action of SEPS alone against *E. coli* was at around 4 h, the peak action reached at 5 h and duration of such significant antibacterial activity lasted for about 8 h. Thereafter no further change in antibacterial activity was noticed (Fig. 5A). Almost similar findings were observed in case of *S. aureus* (Fig. 5B). However, onset of antibacterial activity was observed as early as around 1 hr in case of combined SEPS and low dose (10 µg/ml) ciprofloxacin treatment and the duration of action lasted for even 12 h in case of both the microorganisms.

3.7. Efficiency in biomedical application in vitro

When applied in case of cultures obtained from pathological samples of urine and wound swab it was observed that there was an enhanced zone of inhibition in case of combination of SEPS and low dose ciprofloxacin compared to that found in case of high dose ciprofloxacin alone. SEPS alone in a dose of 750 µg/ml exhibited a smaller zone of inhibition of 10.3 mm. With only low dose (10 µg/ml) ciprofloxacin, it was 15.4 mm and with high dose (20 µg/ml) it was 17.6 mm. But, with a combination of SEPS (750 µg/ml) and low dose (10 µg/ml) ciprofloxacin it was found to be as much as 20.3 mm (Fig. 6) which indicated the adjuvant action of SEPS with ciprofloxacin.

3.8. Assessment of antibacterial action

Staining with trypan blue after SEPS treatment of bacterial culture exhibited an overall bacterial cell mortality of approximately 88 %.

Considering the absorbance of CV itself as reference, sequential entrapment of CV by untreated bacterial culture, by culture treated with SEPS alone, by culture treated with ciprofloxacin alone and by culture treated with a combination of both SEPS and ciprofloxacin displayed contrasting antimicrobial potencies. The supernatants collected after CV entrapment showed gradual decline in absorbance at 590 nm in UV–vis spectra in the aforesaid order (Fig. 7). The combination of SEPS and ciprofloxacin elicited the most antibacterial efficacy which is evident from the maximum decline in absorbance with respect to the reference.

SEM images also clearly showed antimicrobial action of SEPS against *E. coli* (Fig. 8A, B) and *S. aureus* (Fig. 8C, D). SEPS efficiently impaired integrity of bacterial cell envelope by creating pores. Disruption of cell envelope was found to be more intense in affected *E. coli* cells compared to *S. aureus* which might be due to thicker peptidoglycan layer in the latter. There were excoriations and deflations in affected *E. coli* cells whereas affected *S. aureus* cells showed disruption with fragmentation of coccoid morphology.

Being a member of fluoroquinolone group, ciprofloxacin targets bacterial topoisomerase and gyrase inhibiting bacterial DNA replication (LeBel, 1988). Therefore the mechanism of antibacterial action of SEPS and ciprofloxacin are categorically different. But when treated in combination with ciprofloxacin, SEPS probably played its role as an adjuvant facilitating better penetration of ciprofloxacin to the cell interior by promoting formation of pores and excoriations on cell envelope. It was evident from the fact that the net additive role of SEPS with a low dose ciprofloxacin happened to be more effective than a high dose ciprofloxacin when used alone.

4. Conclusion

Since the inception of indiscriminate use of antibiotics and other antimicrobial drugs, drug resistance has been outgrowing and interfering with long term immunity (Shapiro, 2002). Of late many pathogenic microorganisms are exhibiting multi-drug resistance which is becoming a global threat (Nikaido, 2009). In the circumstances, combining a biologically derived potent antibacterial compound like SEPS with a relatively safe dose of antibiotic might overcome these challenges and evolve as an immune-protective and safe mode of antibacterial therapy for human benefit.

The interesting aspect of this study is that a mere combination of a bacterial metabolite having some anti-bacterial potential with a standard antibiotic not only accentuates the action of the latter, but also successfully reduces the dose of the antibiotic to attain a greater antibacterial efficacy than that elicited by the antibiotic alone even in a higher dose. This would cut short the risk of adverse effects of high dose antibiotics and development of antibiotic resistance. Another interesting observation is that this synergistic action of both has emerged through two distinctly different mechanisms of antibacterial action - SEPS by way of disrupting the bacterial cell envelop and ciprofloxacin by way of inhibiting bacterial DNA replication, thereby developing a strong co-ordination between them. The concept of the present study might therefore be a silver lining in futuristic applications.

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Ethical approval

The present study was not applied on any animal or human subject.

CRediT authorship contribution statement

Dipanjan Sengupta: Conceptualization, Methodology, Investigation, Formal analysis, Software, Data curation, Writing - original draft. **Sriparna Datta:** Supervision, Visualization, Validation, Resources, Project administration, Writing - review & editing, Funding acquisition. **Dipa Biswas:** Resources, Visualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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