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Amelioration of lipopeptide biosurfactants for enhanced antibacterial and biocompatibility through molecular antioxidant property by methoxy and carboxyl moieties

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ABSTRACT

Biosurfactants having surface-active biomolecules have been the cynosure in environment research due to their vast application. However, the lack of information about their low-cost production and detailed mechanistic biocompatibility limits the applicability. The study explores techniques for the production and design of low-cost, biodegradable, and non-toxic biosurfactants from *Brevibacterium casei* strain LS14 and excavates the mechanistic details of their biomedical properties like antibacterial effects and biocompatibility. Taguchi's design of experiment was used to optimize for enhancing biosurfactant production by optimal factor combinations like Waste glycerol (1%v/v), peptone (1%w/v), NaCl 0.4% (w/v), and pH 6. Under optimal conditions, the purified biosurfactant reduced the surface tension to 35 mN/m from 72.8 mN/m (MSM) and a critical micelle concentration of 25 mg/ml was achieved. Spectroscopic analyses of the purified biosurfactant using Nuclear Magnetic Resonance suggested it as a lipopeptide biosurfactant. The evaluation of mechanistic antibacterial, antiradical, antiproliferative, and cellular effects indicated the efficient antibacterial activity (against *Pseudomonas aeruginosa*) of biosurfactants due to free radical scavenging activity and oxidative stress. Moreover, the cellular cytotoxicity was estimated by MTT and other cellular assays revealing the phenomenon as the dose-dependent induction of apoptosis due to free radical scavenging with an LC50 of 55.6 ± 2.3 mg/ml.

1. Introduction

Biosurfactants are amphipathic surface-active compounds composed of structurally different biological macromolecules and functional groups produced by eukaryotic and prokaryotic microorganisms [1]. These are extracellular secondary metabolites, which plays an important part in survival of the microorganisms producing it, either by interfering in host microbe interaction or by acting as an antimicrobial agent [2]. These compounds are amphiphilic molecules with both hydrophilic and hydrophobic moieties found on the microbial surface as well as are secreted in the culture medium of the bacterial habitat [3]. Biosurfactants have triggered an extensive interest in recent decades possessing several advantages over their synthetic counterpart, including biocompatibility, ease of production, reduced toxicity, stability in extreme conditions, and higher biodegradability which has been demonstrated by their applications in different biomedical fields [4,5]. Biosurfactants produced by microorganisms are characterized as low molecular weight polymer (glycolipid, lipopeptide), which helps to reduce surface and interfacial tension. Additionally, they are also known as high molecular weight compound (polysaccharide, lipoproteins) which acts as an effective emulsifier [6,7]. They hold various functional properties like detergency, foaming, and emulsification making them a promising molecule for environmental remediation, industrial applications (including food, cosmetics, detergent), and pharmaceuticals industries applications such as an antimicrobial, antiviral, anticancer, antioxidant, anti-inflammatory, and drug delivery agent [8,9].

Despite of possessing several advantages by biological surfactants compared to chemical surfactants, the limitation lies in low production yield and high production and recovery cost. In this context, the use of industrial waste as a low-cost substrate for biosurfactant production has

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piqued researchers interest, owing to the 50% reduction in cost of substrate [10]. As a result, new strategies are currently being explored to improve the economy of biosurfactants production. Maximizing the biosurfactant production yield requires optimization of culture condition and medium. In this regard, the use of statistical models to standardize the culture condition and growth parameters can be a significant help to improve the production yield and reduce the recovery cost. The most challenging parameter while optimizing the media is the presence of interactive effect of the medium component. Different statistical model such as Artificial neural networks [11], Factorial design [12], Central composite design, Plackett-Burman design [13], Box-Behnken design [14], response surface methodology [15], and Taguchi designs [16] has been used for the optimization of culture condition and medium.

Taguchi's design of experiments has significant advantages over other statistical methods. Taguchi parameter designing are frequently used to ensure that all possible factor interaction in the experimental setup are taken into account [17]. The advantage of using Taguchi model is the use of an array system that can optimize different factors in an unbiased way with minimum experimental runs simultaneously. Based on these observations, this methodology has been used by many industries, including biotechnology, molecular biology, bioremediation and food processing [18].

Isolation and production of beneficial biosurfactants have been reported by using different microbial cultures; however, the quest of a low cost and high beneficial one with regard to eco-compatibility and biocompatibility has always been in quest. One of most recent microbe isolated from an ecologically unique and unexplored habitat, Loktak Lake in Imphal, India, is *Brevibacterium casei* strain LS14, which has gained attention because of its multiple beneficial applications. *Brevibacterium casei* is a Gram positive, non-pathogenic actinomycetial strain reported to harbor industrially important biosurfactant producing genes [19]. This study details about the use of *Brevibacterium. casei* LS14 for the production of biosurfactants [19] and explores the use of Taguchi optimization using low cost substrate to maximize the biosurfactant production by *B. casei* LS14, followed by purification and characterization of biosurfactant using NMR. The purified microbial biosurfactant was evaluated for their mechanistic antibacterial properties. Additionally, the biosurfactants was estimated of their antioxidant, anti-proliferative and cell migration potential for biomedical applications. The mechanistic details of the anti-proliferation effects were evaluated using different high end techniques. The results were speculated to propose the isolated novel biosurfactants for different environmental and biomedical applications.

2. Materials & methods

2.1. Materials

All the microbiological reagents and media used in this study were of pure analytical grade and purchased from HiMedia (HiMedia Labs, Mumbai, India) and Merck chemicals (Merck Millipore, Darmstadt, Germany). Crude oil was a generous gift from IOCL (Paradeep, Odisha, India). Waste glycerol used was a biodiesel co-product. The pure culture of *B.casei* LS14 were isolated and obtained under the previous project framework carried in laboratory [19].

2.2. Screening of production parameters at shake flask level

2.2.1. Inoculum preparation

The pure culture of *B. casei* LS14 were grown and maintained routinely in Luria Bertani (LB) media. Pre-culture of *B. casei* LS14 were grown in MSM media with 1% carbon and energy source (Glucose) incubating at 37 ℃ at 200 rpm for 72 hrs in shaking incubator.

Table 1
Factors and their levels used in Taguchi's orthogonal array design for surfactant production.

Factors	Level 1	Level 2
pH	6	7
Salt Conc.	0.4%	0.6%
Carbon source	Waste glycerol	Molasses
Nitrogen source	Peptone	Yeast

Table 2
Matrix layout for $L_8(2)^4$ Orthogonal array experimental design.

Trials	pH	NaCl Concentration	Carbon source	Nitrogen source
1	1 (pH-6)	1(0.4% w/v)	1 (Waste Glycerol)	1(Peptone)
2	1(pH-6)	1(0.4% w/v)	2 (Molasses)	2 (Yeast)
3	1(pH-6)	2(0.6% w/v)	1 (Waste Glycerol)	2 (Yeast)
4	1(pH-6)	2(0.6% w/v)	2 (Molasses)	1 (Peptone)
5	2(pH-7)	1(0.4% w/v)	1 (Waste Glycerol)	2 (Yeast)
6	2(pH-7)	1(0.4% w/v)	2 (Molasses)	1 (Peptone)
7	2(pH-7)	2(0.6% w/v)	1 (Waste Glycerol)	1 (Peptone)
8	2(pH-7)	2(0.6% w/v)	2 (Molasses)	2 (Yeast)

2.2.2. Evaluation of the impact of pH, carbon & nitrogen source, salt concentration (NaCl %w/v), on the production of biosurfactant

Optimization of process parameters for biosurfactant production were studied in 50 ml MSM media [$(NH_4)_2HPO_4$, 0.5 gL^{-1}; $MgSO_4$0.7 H_2O, 0.2 g L^{-1}; K_2HPO_4, 0.1 g L^{-1}; $FeSO_4$0.7 H_2O, 0.01 g L^{-1}; $Ca(NO_3)_2$, 0.01 g L^{-1}] with 1% glucose as sole carbon source and inoculated with 1% v/v (approx. 2 $\times 10^8$ cells per ml) of *B. casei* LS14. The effect of initial pH was investigated by adjusting the pH of the growth medium with 0.1 N NaOH and 0.1 N HCL to various pH values ranging from 5 to 9, and then determine the optimum pH condition. The impact of carbon source on biosurfactant production was studied using five different carbon sources namely molasses, kerosene, purified glycerol, waste glycerol, and D-glucose. All these carbon sources were used in 1% w/v ratio for uniformity. Similarly, five nitrogen sources namely NH_4Cl, $NaNO_3$, urea, peptone, and yeast extract with 1% (w/v) each were investigated for their effect on biosurfactant production. The effect of salinity on biosurfactant production were studied by adding six different NaCl concentration such as; 2 g L^{-1}, 4 g L^{-1}, 6 g L^{-1}, 8 g L^{-1} and 10 g L^{-1}. As a control, a culture flask was used with no adjusted pH, NaCl, carbon and nitrogen source. The samples were incubated at 37 ℃ (200 rpm) and collected every 24-hour interval up to 120 hrs.

Then the cells were separated from each flask by centrifugation at 10,000 rpm for 20 mins at 4 ℃ and was monitored for emulsification index (E_{24}), oil displacement, bacterial load (CFU/ml) and biomass (g/L). All the experiments were performed in triplicate.

2.2.3. Optimization of screened medium components for enhanced biosurfactant production from B. casei strain LS14 by Taguchi design of experiment

2.2.3.1. Design of Experiment (DOE). Taguchi Orthogonal array methodology was used to find the best factor combination of media component variables to maximize the biosurfactant production. For each of four factors two best parameters were selected after the optimization of process parameters at the shake flask levels. Then an orthogonal array for experimental runs was designed to ensure a robust combination. Four factors with their two levels were shown in Table 1. Standard $L_8(2)^4$ was used to decide the best combination of four factors for enhancing the production of biosurfactants. Here 'L' denotes the Latin square model, subscript 8 represents the total number of experimental

runs, 2 represents the number of four factors and superscript 4 depicts the number of factors. The orthogonal matrix layout with their two levels of the four factors considered with eight runs is depicted in Table 2.

2.2.3.2. Analysis of Taguchi Orthogonal array methodology. All eight experimental runs in Taguchi Experimental design were analyzed by estimating the Signal-to-Noise ratio (S/N) ratio. This (S/N) ratio helps in measuring the robustness of factors involved which reduce the process or product variability. In the context of this particular work, the (S/N) ratio was determined using the following formula, taking the "Larger is better" objective;

$$\frac{S}{N} = -10 \times \log\left(\sum\left(1/Y^2\right)/n\right)$$

Here 'Y' is the signal (here the emulsification index (E_{24}),), "n" is the number of repetitions in each experimental run.

This method aids in estimating the relative significance and percentage contribution of various parameters (pH, NaCl concentration, carbon, and nitrogen source) in maximizing product yield. The percentage contribution of four factors considered in the media optimization was also calculated by analysis of variance (ANOVA). By combining the four factors with their respective levels that had the highest main effect values, the optimal condition for the biosurfactant production (emulsification index (E_{24})) was determined. The Taguchi orthogonal array was designed and analyzed using Minitab 17 (Minitab Inc., USA) software.

2.2.3.3. Determination of optimum level and validation of Taguchi design of experiment using unpaired t-test. The optimum factor (pH, NaCl, carbon and nitrogen source) combinations obtained after the recommendation of software, were validated by different assays such as: oil displacement characteristics, emulsification index (E_{24}), and surface tension measurement with the optimum conditions recommended by the software model. To determine the model's statistical significance, an unpaired t-test was performed using Graphpad Prism version 7.

2.3. Extraction and purification of biosurfactant

Followed to optimization study, biosurfactant extraction was carried out using the protocol of [20]. The isolate *B. casei* LS14 was inoculated in optimized production media and incubated at 37 ℃ for 72 h in orbital shaker (200 rpm). The culture supernatant was acidified (pH 2) and precipitated using concentrated HCl and incubating it overnight at 4 °C. Then the precipitate was extracted with an equivalent volume of chloroform: methanol (2:1) mix. Following separation, the organic phase was collected and using rotary evaporator the solvent was evaporated at 45 °C, leaving behind the viscous brown color product, which was reconstituted in methanol for further analysis.

2.3.1. Purification of biosurfactant compound

2.3.1.1. Adsorption chromatography and Size exclusion chromatography. The biosurfactants was purified using Adsorption chromatography and Size exclusion chromatography with the method as detailed in supplementary files.

2.3.1.2. Biosurfactant Analysis by TLC (Thin Layer Chromatography). Thin layer chromatography (TLC) can be applied to detect the presence of compounds such as lipids and peptides. 10 μl of the purified biosurfactant from Sephadex G-25 column was put onto TLC plate (Merck DC, Silica gel 60) at the point of origin near the bottom of the plate. Once dried the plate was developed with different solvent system. The solvent system, mobile phase chloroform: methanol: water (65: 25: 10, v/v) resulted in better development. After that, the silica plate was developed with 0.2% ninhydrin solution in absolute alcohol followed by heating at 110 ℃ to detect the peptides and amino groups [21] and with the fumes of iodine in a chamber to detect the lipids [22]. Commercially available lipopeptide Surfactin (Sigma-Aldrich, United States) was used as a reference standard. The retention factor (Rf) values on TLC plate were calculated using formula.

$$\mathrm{Rf} = \frac{\text{Distance travelled by solute(cm)}}{\text{Distance travelled by solvent(cm)}}$$

2.4. Chemical characterization of the biosurfactant using NMR

The structural elucidation of the purified biosurfactant was performed using nuclear magnetic resonance. Liquid-state NMR spectroscopy can be considered as an efficient tool for the characterization of biosurfactant. All ^{13}C NMR and ^{1}H NMR was obtained in a AV400-Bruker 400 MHz High Resolution Multinuclear FT NMR spectrometer. The liquid-state ^{13}C and ^{1}H NMR spectra were registered in a AV400-Bruker 400 MHz with deuterated chloroform as a solvent. The acquisition time for ^{13}C NMR was 6 ms and 12.41 ms for ^{1}H NMR.

2.5. Physiochemical properties of biosurfactants

2.5.1. Oil displacement test

Oil displacement assay was conducted using the protocol described by [23] as mentioned in supplementary files.

2.5.2. Emulsification index

The seed culture of the bacterial isolates was prepared as discussed in the section number 2.2.1 "Inoculum preparation". In a 500 ml conical flask, actively growing bacterial strains (5 ml) were inoculated into MSM media (100 ml) and incubated for 120 hrs at 37 ℃ and 200 rpm. The cell free supernatant was then obtained by centrifugation at 10,000 rpm for 20 min at 4 ℃ to remove the cell biomass. The emulsification index was measured by adding 2 ml of cell free supernatant to equal amount of diesel, kerosene and sunflower oil respectively, followed by 2 min' vortex. Then the mixture was left undisturbed at room temperature for 24 h and $E\%_{24}$ for each substrate was calculated using the equation:

$$\mathrm{EI}\%24 = \frac{\text{Height of emulsion formed(mm)}}{\text{Toltal height of the solution(mm)}} \times 100$$

Similarly, Emulsification index for Taguchi optimized parameter and purified biosurfactant was performed followed by incubation of 72 hrs (37 ℃, 200 rpm).

2.5.3. Surface tension measurement (ST)

The cell free broth obtained after 72 hrs of incubation were tested for surface tension reduction using Du Nouy ring tensiometer [24]. The surface tension of the surface active biosurfactant in the cell free supernatant was quantified at room temperature. To ensure the precision of the measurement, instrument calibration was done using distilled water, and surface tension of MSM was measured before each sample.

2.5.4. Determination of the Critical Micelle Concentration (CMC)

The CMC was determined by plotting surface tension as a function of the concentration of the biosurfactant. It was determined as the concentration of the biosurfactant required to form micelles. The surface tension was measured using a Du Nouy ring tensiometer [25].

2.6. DPPH assay

The anti-radical or hydrogen donating potential of the silica purified biosurfactants isolated from *B. casei* strain LS14 was estimated by using stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) [26]. DPPH stock solution of 200 mM was prepared in 95% (v/v) methanol. Different

concentration (1, 3, 5, 7, 10, 15, 20, 25 mg/ml) of silica gel-purified biosurfactant from *B. casei* strain LS14 (40 μl) was mixed with 120 μl of DPPH to measure the anti-radical activity. L-ascorbic acid in the same concentration as the biosurfactant was used as a reference standard [27]. After 30 min of incubation in dark, the absorbance was taken at 517 nm. The experiment was performed in triplicate and percent inhibition was calculated using the following equation

$$\text{Percentage of Inhibition}(\%) = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample})}{\text{Absorbance of Control}} \times 100$$

2.7. Antibacterial assessment

The purified surface active compound from *B. casei* strain LS14 was tested for antimicrobial activity using standard antimicrobial method against *Pseudomonas aeruginosa* ATCC 15442. The preparation of the inoculum for the bacteria was done as detailed in supplementary information. Qualitative agar diffusion Assay and Broth microdilution assay was performed for the initial confirmation of the antibacterial activity [28]. The detailed methodology is mentioned in supplementary information.

2.7.1. Dead-live assay

Dead-live assay was performed using flow cytometry and fluorescent microscopy [29]. The bacterial cells were stained with 2.5 μM Syto 9 and 15 μM Propidium Iodide (PI). Syto 9 was used to stain live bacteria with intact membrane perceived as green fluorescence and PI was used for staining bacteria with damaged membrane and was perceived as red fluorescence. The imaging was performed in EVOS Fluorescent microscope (ThermoScientific, USA). *Pseudomonas* cells were treated with different concentration (5,10,15,20 mg/ml) of biosurfactants. The images were taken at 4 h after incubation of bacteria. Untreated cells were taken as control. For flow cytometry analysis, similar set of experiment was performed and the cells were detected using BL1 and BL2 filters of Attune nextgen flow cytometer (ThermoScientific, USA). The data were processed using FCS express7 (Denovo, USA).

2.7.2. ROS analysis

The effect of biosurfactants on ROS induction in bacterial cells was analyzed using flow cytometry [30]. The cells were treated with different concentration (5,10,15,20 mg/ml) of biosurfactants and incubated for 4 h. After incubation, the cells were washed and stained with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 20 min in dark. The stained cells were then detected in BL1 filter of Attune Nextgen flow cytometer (ThermoScientific, USA). The data was processed using FCSexpress7.

2.8. Cell culture and cytotoxicity analysis

HEK293 cells were cultured in RPMI 1640 medium supplemented with penicillin-streptomycin (100 U/ml) and 10% fetal bovine serum (FBS) and then incubated in a 37 ℃ incubator at 5% CO_2 and 95% humidity.

2.8.1. Cell proliferation assay

The proliferative effect of the purified biosurfactant was evaluated using an MTT assay [31]. Briefly, HEK293 cells were seeded in flat 96 well plates until a density of 10^3 cells/ml was achieved. The cells were exposed to various doses of biosurfactant (2 mg/ml, 5 mg/ml, 7 mg/ml, 10 mg/ml, 12 mg/ml, 15 mg/ml) for 24 h, 48 h, and 72 h. Media containing only cells (without biosurfactant) were taken as a control for better comparison. After the treatment, the media was replaced with for (3-(4,5-Dimethylthiazol-2-yl)− 2,5-Diphenyltetrazolium Bromide) MTT dye (0.5 mg/ml) followed by 4 h of incubation. Subsequently, the formazan crystals formed were dissolved and the absorbance was measured at 570 nm with a microplate reader. The following formula was used to calculate the percentage of alive cells:

$$\text{Percentage of alive cells}(\%) = \frac{\text{Absorbance of bio surfactant treated cells}}{\text{Absorbance of control cells}} \times 100$$

2.8.2. Effect of biosurfactant on the migration of HEK293 cells

The effect of silica purified biosurfactant on migration of Human Embryonic Kidney (HEK-293) cells were investigated. HEK293 cells were seeded in six-well plates and cultured according to the standard procedure till 80% of confluence. A sterile 10 μl plastic pipette tip was then used to make a scratch at the center of each well. The cells were washed thrice with sterile PBS to clear cellular debris before being treated with various doses of silica purified biosurfactants (2, 5, 10, and 20 mg/ml) for 6 h, 12 h, 24 h, and 36 h. The area of cell migration was measured by Image J software and it was expressed as the percentage of gap closure.

2.8.3. ROS analysis

Reactive oxygen species(ROS) estimation in HEK293 cells treated with biosurfactants was analyzed qualitatively and quantitatively using flow cytometry by the detection of green signal of 2′− 7′-Dichlorodihydrofluorescein (DCF) in BL1 filter (530/30) of Flow cytometer (Attune nextgen, ThermoScientific, USA) [32]. The untreated and treated cell were trypsinized after treatment and were washed with PBS before staining with H_2DCFDA. The data were processed using FCSexpress7 (Denovo, USA).

2.8.4. Apoptosis analysis (AnnexinV-FITC/PI assay)

The estimation of cell death in Untreated and treated HEK293 cells was done using AnnexinV-FITC/PI assay [32]. HEK 293 cells were treated with different concentration (2, 5, 10, and 20 mg/ml) of biosurfactants for 24 h. Annexin V-FITC detection Kit (Imgenex, India) was used to measure Phosphatidylserine exposure. The staining of cells was performed with FITC conjugated Annexin V according to manufacturer's protocol and analyzed by flow cytometry (Attune Nextgen flow cytometer, ThermoScientific, USA). All the experiments were performed in triplicate. The data were processed using FCSexpress7 (Denovo, USA).

2.9. In Silico analysis

The mechanistic analysis of antibacterial activity and biocompatibility activity was done by *in silico* approach through molecular docking [33]. The approach was done to understand the molecular interaction of carboxyl and methoxy radicals present in biosurfactants with membrane proteins of *P. aeruginosa* and metabolic proteins like Sod1 and P53 of human kidney cells [34]. The study was done using Autodock 4.2 (Morris and Huey, 2009) with Carboxyl and methoxy radicals as ligands and sod1, tp53 as receptor proteins. The structure of carboxyl and methoxy was prepared with the help of Chimera and their geometry was optimized by Gaussian 03 program. The program was also used to minimize energy in the proteins. The parameters were set for Autodock 4.2. by setting the grid dimensions to 40 × 40×40, with a spacing of 1 Å for all the protein receptors. The population size was taken as 150 and the maximum number of evaluations were set to 2500,000. A maximal generation were used for docking runs with the help of Genetic algorithm. The post-docking analysis was done with the help of Discovery Studio Visualizer.

Table 3
Matrix layout of the $L_8(2)^4$ Taguchi orthogonal array depicting four factors with their two levels and their corresponding emulsification index and biomass in terms of response values and S/N ratios.

Trials	pH	NaCl conc. (%w/v)	Carbon source	Nitrogen source	Response (Emulsification index)	S/N ratio
1	6	0.4	Waste Glycerol	Peptone	70	36.9020
2	6	0.4	Molasses	Yeast	21	26.4444
3	6	0.6	Waste Glycerol	Yeast	58	35.2686
4	6	0.6	Molasses	Peptone	40	32.0412
5	7	0.4	Waste Glycerol	Yeast	30	29.5424
6	7	0.4	Molasses	Peptone	38	31.5957
7	7	0.6	Waste Glycerol	Peptone	34	30.6296
8	7	0.6	Molasses	Yeast	15	23.5218

3. Results and discussion

3.1. Screening of production parameters

3.1.1. Evaluation of the impact of pH, carbon & nitrogen source, salt concentration

3.1.1.1. Effect of pH. The bacterial strain *B. casei* strain LS-14 used in this study was isolated from a lake ecosystem, which would possess a pH gradient across its environment. It supported the growth in all pH ranges except pH 5 (Fig. S1A), whereas maximum biomass (3.65 g L^{-1}) obtained was in pH 6 (Fig. S1B, **F**). Maximum bio-surfactant production activity such as emulsification index and oil displacement was obtained at pH 6 and 7 (Fig. S1C, D, G, and H). Among optimized parameters, medium pH is a key factor for bacterial growth and metabolism. The pH regulates the ion balances in the cell, thereby supporting an active metabolic functions to take place and the optimum biosurfactant production has been reported to be near neutral pH [35,36].

3.1.1.2. Effect of salt concentration (NaCl % w/v). The effect of addition of different concentrations of salt (NaCl) in growth of bacteria and biosurfactant production was studied using *B. casei* LS-14. As shown in Fig. S2, the osmotic pressure of the culture solution was found to be risen dramatically with the increase in concentration of salt (NaCl) in the culture medium [37]. Maximum biosurfactant production in terms of emulsification index (40.81% and 41.83%) and oil displacement (1.44 cm and 1.53 cm) at 72 h (Fig. S2C, D, G, and H) was observed with 4 g L-1 and 6 g L^{-1} of NaCl respectively. It has been said that in general, the microorganisms thrive in isotonic solutions. The results showed that, high salinity (> 6 g L^{-1}) had caused a slow growth of the bacteria, hence stating that a mild saline environment would be of great help in biosurfactant production. The result obtained was consistent with previous reports [38,39] where salinity range of 4–8% NaCl was identified as limit for the stability of biosurfactant.

3.1.1.3. Effect of auxiliary carbon source. The Carbon sources present in culture medium are involved in regulation of variety of metabolic pathways which supports the formation of precursors for biosurfactant production. The carbon flow, for example are regulated by both lipogenic pathway (lipid moiety) and glycolytic pathway (hydrophilic moiety), both being constrained by microbial metabolism [40]. The effect of various carbon sources was studied for growth and production of biosurfactant by *B. casei* LS-14. The results showed higher growth (74.66 $\times 10^6$ CFU ml^{-1}) and biomass production (5.96 gL^{-1}) upon addition of molasses (1%) to the media and it remained for a longer time up to 120 hrs (Fig. S3A-B). Highest biosurfactant production activity in terms of emulsification index (49.65%) and oil displacement (4.1 cm) at 72 hrs was obtained upon addition of waste glycerol (Fig. S3C, D, G, H). The results obtained were in accordance with the studies done by [41], where higher biosurfactant were produced when glycerol was used as a substrate compared to molasses. This can be reasoned to the lipogenic pathway and gluconeogenesis directed by microbes in the presence of water insoluble substrate [42]. Next to waste glycerol, molasses had shown a comparable biosurfactant production supporting the growth as well (Fig. S3A-D).

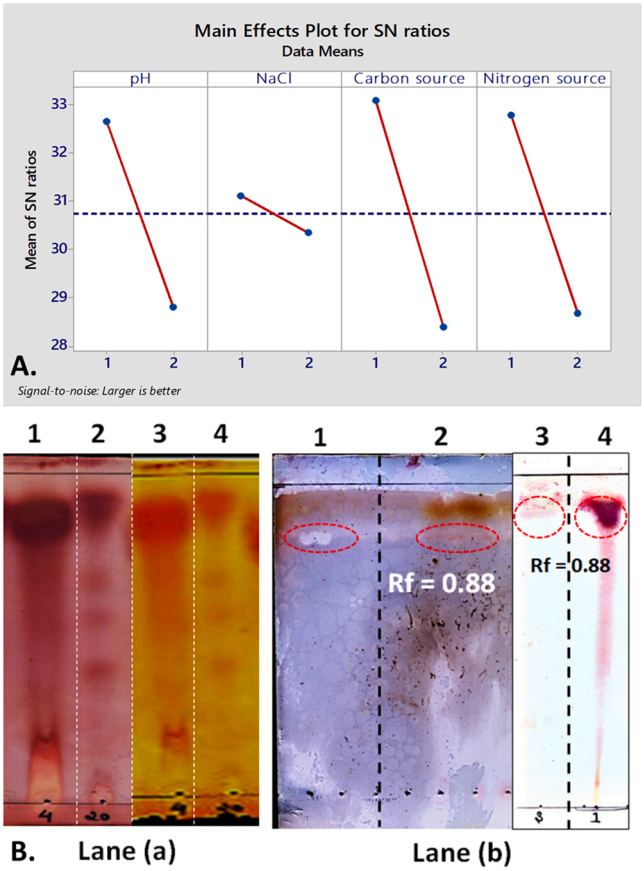

Fig. 1. (A) The mean of S/N ratios were calculated and presented against two levels of four parameters (pH, NaCl, carbon and nitrogen source). The X-axis here depicts the factor levels, while the Y-axis depicts the mean value of S/N ratios. (B) Thin layer chromatography of silica gel purified and crude biosurfactant fraction on silica gel GF254 precoated plates. The plates were developed with a solvent system of chloroform: methanol: water (65:25:4). Lane (a)1 and (a)2, purified and crude biosurfactant fractions developed with 0.2% ninhydrin solution and Lane (a) 3 and (a)4, purified and crude biosurfactant fraction respectively developed with iodine vapor. (b) Lane (b)1 and (b)2, Commercial surfactin (Sigma-Aldrich, United States) and purified LS14 fraction developed with iodine vapor with an Rf value of 0.88 and Lane (b)3 and (b)4 Commercial surfactin (Sigma-Aldrich, United States) and purified LS14 fraction developed with 0.2% ninhydrin solution with an Rf value of 0.88.

3.1.1.4. Effect of auxiliary nitrogen source. Nitrogen source is another necessary component for the enhanced microbial metabolism and biosurfactant production. The effect of different nitrogen sources was studied for growth and production of biosurfactant by *B. casei* LS-14. it

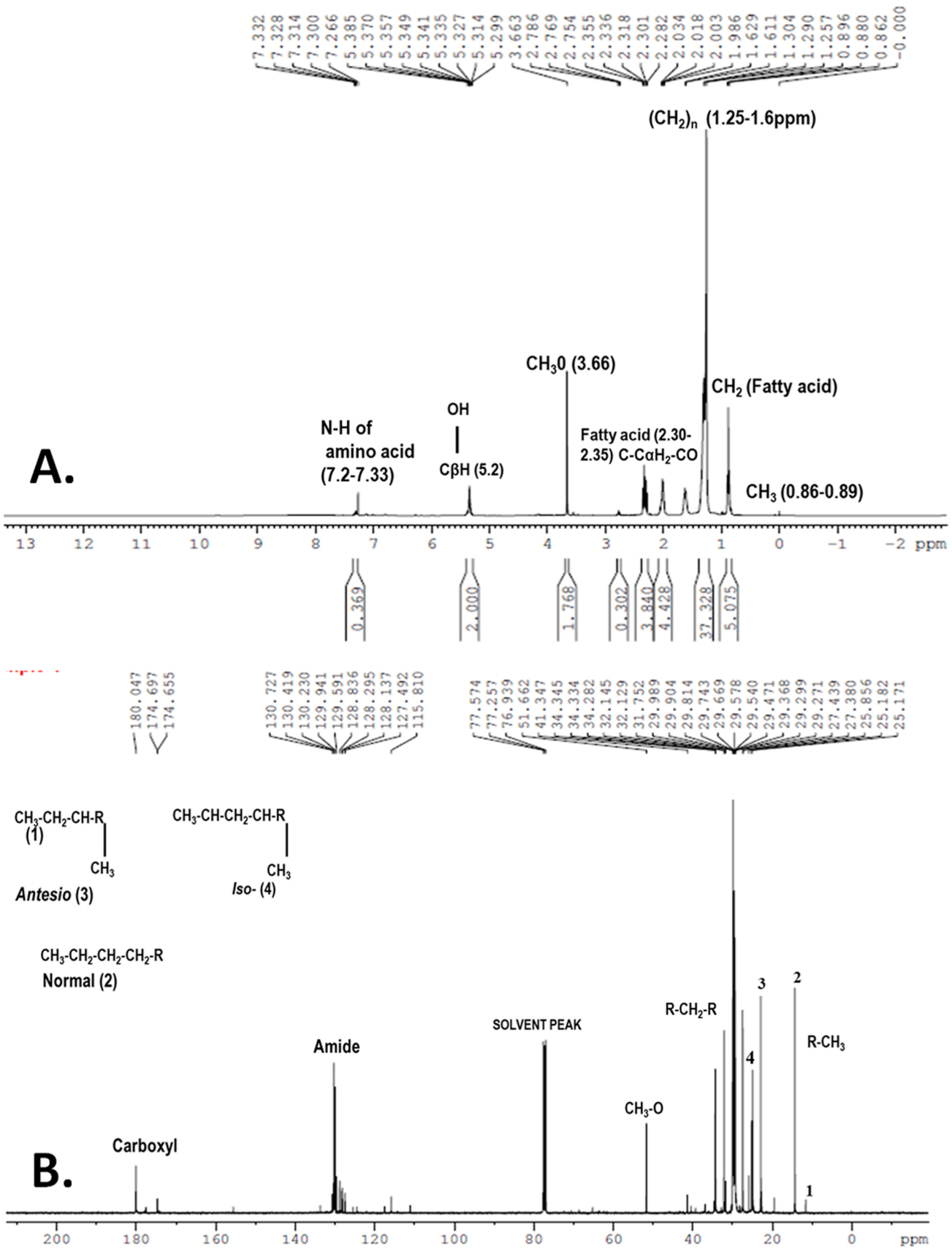

Fig. 2. ^{1}H NMR (a) and ^{13}C NMR (b) spectra of LS14. The liquid-state ^{13}C and ^{1}H NMR spectra were registered in a AV400- Bruker 400 MHz with deuterated chloroform as a solvent. The acquisition time for ^{13}C NMR was 6 ms and 12.41 ms for ^{1}H NMR.

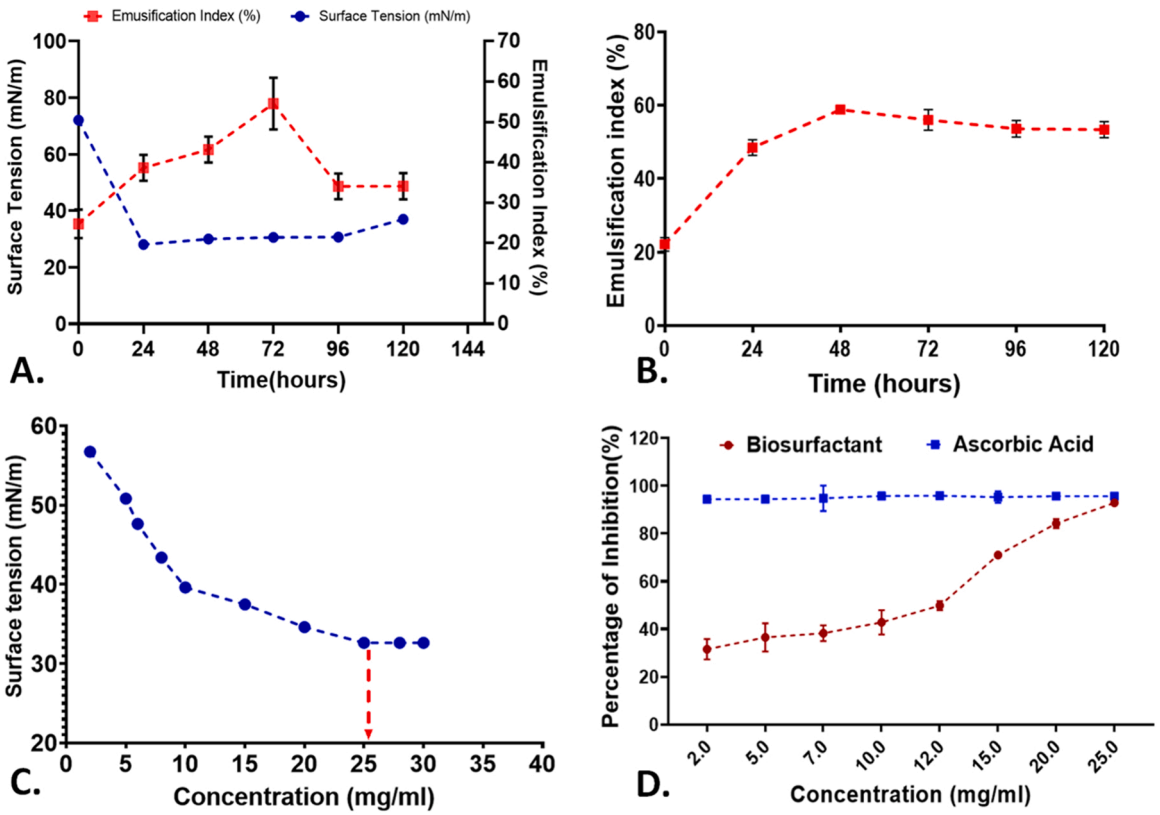

Fig. 3. (A) Surface tension and emulsification index of biosurfactant from *Brevibacterium casei* strain LS14 grown at 37 ℃, 180 rpm, 1% inoculum (v/v) plotted as a time function. (B) emulsification index of biosurfactant (C) Minimum surface tension and CMC of the biosurfactant was evaluated. Arrow illustrate CMC value of purified biosurfactant produced by *Brevibacterium casei* strain LS14. (D) Anti-radical activity of silica purified bio surfactants isolated from *Brevibacterium casei* LS14 assessed by DPPH assay. Ascorbic acid was taken as standard drug for comparison.

showed organic nitrogen source to be more conductive for biosurfactant production and microbial growth. It showed highest growth and biomass formation upon addition of 1% (w/v) of yeast extract (56.66 $\times 10^6$ CFU ml^{-1} and 2.16 gL^{-1}) and 1% (w/v) of peptone (58 $\times 10^6$ cfu ml-1 and 3 gL-1) at 72 h (Fig. S4A & B). Highest biosurfactant production activity (emulsification index: 36.11% and oil displacement: 3.97 cm) at 72 h was obtained upon addition of 1% (w/v) peptone (Fig. S4C, D, G, H). Next to peptone, yeast extract had shown a comparable biosurfactant production supporting the growth as well (Fig. S4A-D). Similarly, both peptone and yeast extract had shown the highest oil displacement characteristics (Fig. S4D). The result obtained were in accordance with the study done by [43]. These results demonstrated organic nitrogen sources to be more conductive for *B. casei* LS-14 growth and biosurfactant production compared to inorganic nitrogen sources. Organic nitrogen sources are reported to have some content of carbon component as well, which significantly supports the cell growth and formation of polysaccharides when compared to inorganic nitrogen sources [44]. In addition, the higher nitrogen content in inorganic nitrogen compared to organic sources are reported as a rate limiting step in biosurfactant synthesis [45].

3.2. Taguchi design of experiment

The optimum values of four screening parameters such as pH (6 and 7), NaCl concentration (4% and 6% w/v), carbon source (Waste glycerol and molasses), nitrogen source (Peptone and Yeast) were determined using Taguchi L8(2)4 design (Table 2). The optimum values for these four factors were determined by running eight experimental runs [46]. For all the eight runs, the samples were collected at 72 h, by centrifugation at 10,000 rpm for 20 min at 4 ℃ and was monitored for emulsification index (E24). The results showed that the emulsification index were maximum in experimental run 1 at 72 h, pH 6, 4% (w/v) NaCl, Waste glycerol (1%v/v), peptone (1%w/v) (Table 3). Similarly, emulsification index was low in experimental run 8 at pH 7, NaCl (6% w/v), molasses (1% w/v), yeast (1% w/v). The means of S/N ratios were calculated and presented against all four factors with their respective two levels, revealing the most optimized process parameters which are level 1 for pH (pH-6), level 1 for NaCl concentration (4% w/v), level 1 for carbon source (Waste glycerol), and level 1 for nitrogen source (peptone) (Fig. 1A).

The most competent factor contributing to the enhanced biosurfactant production (in terms of emulsification index) was determined using one-way ANOVA, which revealed that carbon source contributes maximum i.e. 33.02%, followed by nitrogen source (25.26%), pH (22.20%), and NaCl concentration (0.86%).

3.2.1. Determination of optimum level (for four factors) and validation of Taguchi design of experiment using unpaired t-test

Biosurfactant production in terms of increase in oil displacement, emulsification index, and reduction in surface tension was compared to Taguchi optimum parameters (pH 6, 0.4% (w/v) NaCl, Waste glycerol (1%v/v), peptone (1%w/v)) and un-optimized condition (absence of ancillary carbon source, nitrogen source, and NaCl with unadjusted pH ~6.8. Taguchi optimal factor combinations for biosurfactant production showed approximately six-fold, and three-fold increase in oil displacement, and emulsification index respectively as compared to the experimental factors at 72 h. It further decreased the surface tension of the biosurfactant by 1.22 times, when optimized factors were added to the culture media (Table S1). The unpaired t-test (Table S2) for biosurfactant production has a significant p- value of 0.0009 for oil displacement assay, 0.0005 for emulsification index assay and 0.0022 for surface tension measurement and $p \leq 0.05$ was statistically

significant.

3.3. Purification of the biosurfactant

The crude biosurfactant obtained by chemical isolation method (acidification of cell free broth) were extracted with chloroform and methanol to get a partially purified biosurfactant. A concentration of 500 mg/100 ml of biosurfactant was found to be produced by *B. casei* LS14. The solvent extracted biosurfactant was then subjected to silica gel column chromatography (60–120 mesh) with a gradient of methanol: water (65–100, v/v), the biosurfactant was resolved into 8 fractions. All the fractions were checked for oil displacement activity and for their emulsification activity; fractions showing positive oil displacement activity were subjected to Sephadex G-25 column and eluted with methanol. Emulsification activity for all the fractions was evaluated against crude oil.

3.4. Chemical nature of the biosurfactant

The Active fractions were further checked for its purity using TLC (Thin layer chromatography). The biosurfactant fractions were seen as a single spot on TLC with an Rf value of 0.88 (Fig. 1B). The fractions showed positive reaction with Ninhydrin reagent and Iodine vapour indicating the presence of peptide and lipid moiety.

3.5. Nuclear magnetic resonance of biosurfactant from B. casei LS-14

The ^{1}H NMR spectra for the purified biosurfactant at 500 MHz revealed the identity of biosurfactant (Fig. 2A) as a lipopeptide due to the presence of a long aliphatic chain in the range of (CH_2 1.6–1.25 ppm), a signal in the range of 2.03–2.28 ppm showing the presence of alpha carbon protons attached to carbonyl group, a peptide backbone (N-H at 7.2–7.33 ppm). The presence of carbonyl group at 2.03–2.28 ppm and the amide group in the range of 7.2–7.33 ppm confirms the conjugation of amino and carboxyl groups. The intense singlet at 3.66 ppm is similar to the ^{1}H NMR spectrum of monoesters lipopeptide [47] suggesting the presence of methoxy group on Glu or Asp amino residues. Presence of an ester carbonyl group at 5.2 ppm, indicated a lactone ring in the structure of biosurfactant [48,49].

The ^{13}C NMR showed the lipid signals present as a mixture of three different configurations (Fig. 2B), normal, anteiso, and isobranched with different CH_3 position being consistent with chemical shift at 11.5, 14.3, 19.4, and 22,8 ppm and CH_2 from 24.9 to 34.3 ppm respectively[50]. A chemical shift at 51.6 ppm in the ^{13}C NMR indicates a methoxy group attached to a Glu or Asp residue of the biosurfactant and an ester and carboxylic group at 178 ppm [49,51].

Liu et al. demonstrated the production and characterization of a C15-surfactin-O-methyl ester by a lipopeptide producing strain *B. subtilis* HS0 121, reported the same resonance [50]. Similar results were confirmed for surfactin produced by *Bacillus licheniforms* with two free carboxyl groups from Glu and Asp [52].

3.6. Physiochemical properties of the biosurfactant

Tensioactive properties of biosurfactant depends on their potential to lower the ST and CMC value *Pseudomonas aeruginosa*, one of the most studied microorganism for biosurfactant production has been reported to reduce the surface tension to around 28mN/m whereas biosurfactant from yeast are reported to reduce the surface tension to around 35mN/m [53]. This study showed the silica purified biosurfactant from *B. casei* LS14 effectively reduced the ST from 72.8 to 28 mN/m (Fig. 3A). Significant bio-emulsification activity started after 24 h of incubation and attended maximum value (EI% 24 58.8) after 48 h of incubation. In case of purified biosurfactant, the emulsification activity was found to be increased in first 48 h and was constant in further time period (Fig. 3B). Interestingly, the lowest surface tension value during the growth of *B. casei* LS14 was achieved after 24 h of growth, after that a slight increase was observed, with a higher increase at 120 h. Moreover, the emulsifying activity, the maximum value was achieved at 72 h, and an abrupt decrease is observed at 96 h, although the surface tension remains almost constant between 72 h and 96 h. The zigzag behaviors of the parameters can be attributed to crude nature of the biosurfactant which led it to show the instability [54]. The result was in justification with previous report [55], that defines the emulsification activity to be depended upon the affinity of biosurfactant or bioemulsifier with hydrocarbon substrate. The activity involves direct interaction with hydrocarbon, rather effecting on the surface tension of the medium. Hence, it can be deduced that the decrease in surface tension (43 mN/m to 28 mN/m) is independent of emulsification activity. CMC value was observed at 25 mg/L at 25 ℃, i.e., no further reduction of ST was observed on further increase of the concentration of biosurfactant (Fig. 3C). The CMC value of biosurfactant from strain LS14 was significantly lower than similar type of lipopeptide biosurfactants reported in previous studies [56]. The fatty acid portion of a lipopeptide is crucial in determining its surface active characteristics, and the number of carbon atoms in a fatty acid chain is inversely proportional to the CMC [57].

3.7. Antiradical activity of biosurfactant isolated from strain B. casei LS14

Anti-radical activity of the *B. casei* LS14 isolated biosurfactant has been investigated by DPPH assay. The DPPH method is based on the scavenging of the stable DPPH radical with an unpaired lone electron at one nitrogen atom by antiradical agent (biosurfactant). The results showed that the maximum scavenging effect of the biosurfactant on the free radical (DPPH) was at a concentration of 25 mg/ml (92.93% inhibition) (Fig. 3D). The result can be attributed to the presence of carboxy and methoxy group present in biosurfactant which can donate hydrogen to scavenge the free radical. Previous reports have shown the efficacy of different biosurfactant and liposomes having antioxidant capacity [58,59]. Our result was in line with the mechanistic interpretation of the antioxidant capacity of purified biosurfactant. Similarly,

Literatures have reported the use of synthetic antioxidants like propyl gallate (PG), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butyl hydroquinone (TBHQ) etc. in the pharmaceutical and the food industries [60] because of their high antioxidant efficacy. However, the potential toxicity associated to these synthetic compounds has been concerned as a matter of public health risk [61]. Hence, deciphering the sources of natural antioxidants like biosurfactant from microbial sources provides a potent solution of the issue.

3.8. Antimicrobial activity of B. casei LS14 biosurfactant

A detailed study was performed to evaluate the antimicrobial activity of the *B. casei* LS14 biosurfactant and estimate the mechanistic action of the surfactant for antimicrobial effects. Primary evaluation of antimicrobial activity of the purified biosurfactant was performed against Gram negative *Pseudomonas aeruginosa*. The agar disk diffusion assay showed zone of inhibition (Fig. S5) confirming antimicrobial action of the purified biosurfactant.

For better understanding of the antimicrobial action quantitatively, microdilution broth assay was performed to calculate the minimum concentration required to inhibit the microbial growth. The purified biosurfactant in various concentration showed antimicrobial activity against *Pseudomonas aeruginosa*. The MIC50 of the purified biosurfactant against the test strains was found to be 7.5 mg/ml (Table S3). Previous reports have suggested the antimicrobial activity of biosurfactant like rhamnolipid against *Trichoderma viride*, *Bacillus* sp., *Pseudomonas* sp., *Cellulomonas flavigena*, *Rhodococcus erythropolis* with a conclusion that biosurfactant were more potent against Gram positive bacterial strains [62]. Similarly, biosurfactant derived from *Bacillus subtilis* C19 has been

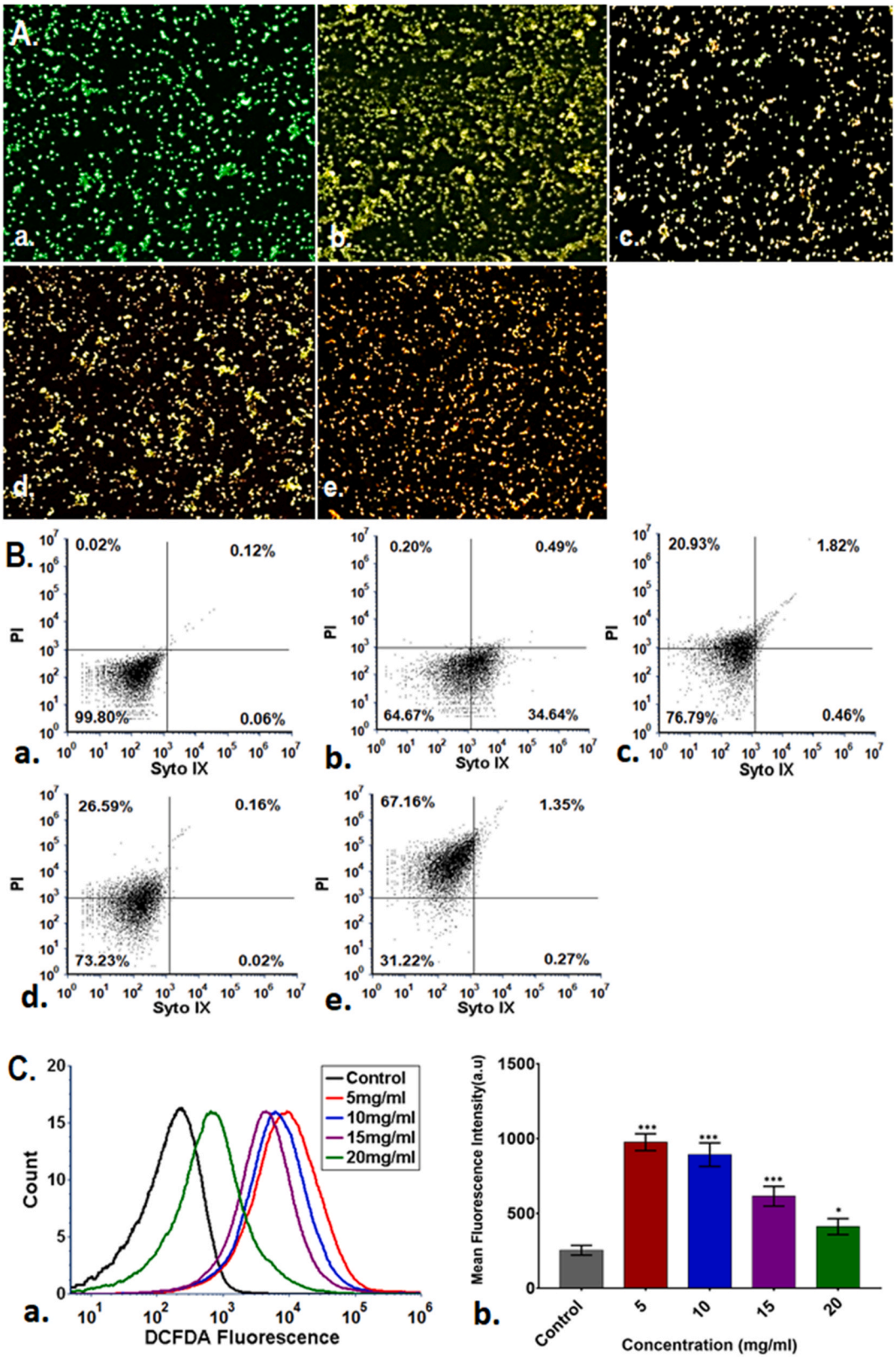

Fig. 4. Antibacterial effect of the *B. casei* strain LS14 biosurfactant: (A) Live-dead analysis of the *Pseudomonas aeruginosa* treated with different concentration (a) Control (b) 5 mg/ml (c) 10 mg/ml (c) 15 mg/ml (d) 20 mg/ml of biosurfactant by fluorescent microscopy. (B) Live-dead analysis of the *Pseudomonas aeruginosa* treated with different concentration (a) Control (b) 5 mg/ml (c) 10 mg/ml (c) 15 mg/ml (d) 20 mg/ml of biosurfactant by flow cytometry. The untreated and treated bacterial strains were stained with Syto IX and Propidium iodide (PI). (C) ROS analysis by flow cytometry in *Pseudomonas aeruginosa* treated with different concentration of biosurfactant; (a) Histogram presentation (b) mean fluorescent intensity. The bacterial strain was stained with DCFDA. The values show Mean ± SD. All the experimental analysis was done in triplicate and thrice independently. The values represent the mean ± SD of three independent experiments. *P > 0.5, * *P > 0.01, and * **P > 0.001 denote the compared significant change at each exposed concentration as obtained from post hoc analysis after one-way ANOVA.

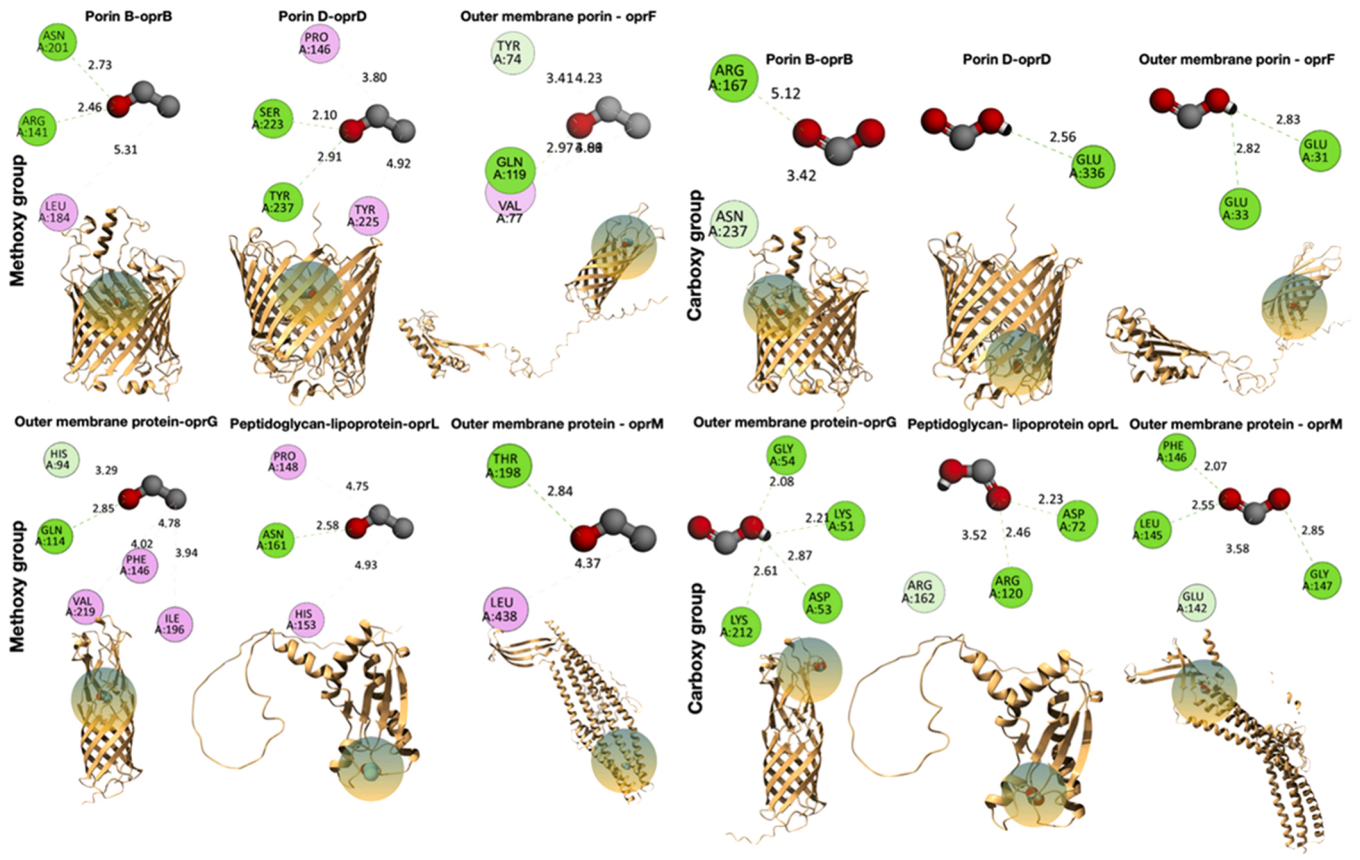

Fig. 5. *In silico* molecular docking analysis of interaction of carboxyl and Methoxy moieties of biosurfactant with outer membrane protein of *Pseudomonas aeruginosa.*

shown to have antimicrobial activity against human pathogens like *Staphylococcus aureus*, *Escherichia coli* [63]. Some other biosurfactant obtained from other bacterial strains has also shown the similar antimicrobial activity in recent studies [64,65]. The results found in our study were in concomitant with the previous reports and inspired towards the excavation of the mechanism behind the antimicrobial behavior of the biosurfactant.

Antimicrobial activity of biosurfactant was proposed by interaction with biological membrane systems. Hence, the antimicrobial action of *B. casei* LS14 biosurfactant against *Pseudomonas aeruginosa* was estimated by the dead-live assay using fluorescent microscopy and flow cytometry. As shown in Fig. 4A, the red fluorescence obtained by the staining of bacteria using Propidium Iodide (PI) was perceived at treatment of higher concentration of *B. casei* LS14 biosurfactant (20 mg/ml) while, at very low concentration (5 mg/ml) perceivance of green fluorescence (Syto IX staining) was prominent. The bacterial strain treated with 10 and 15 mg/ml showed high intensity of yellow fluorescence indicating a combined effect of Syto IX and PI staining. PI has been reported to stain cells with damaged membrane. The result indicated the dose- dependent antibacterial effect of surfactant as a consequence of membrane damage. The result was further verified and estimated quantitatively through flow cytometry. As shown in Fig. 4B, at lower concentration (5 mg/ml), 34.64% of cells were found to be stained with Syto IX which was decreased to 0.46%, 0.02%, and 0.27% in case of higher concentration treatment of 10, 15 and 20 mg/ml respectively. Meanwhile, the percentage of cells with PI stain was found to be increased from 0.02% to 0.2%, 20.93%, 26.59%, and 67.16% indicating the increasing percentage of dead cells. The results were in line with the fluorescent microscopy results and verified the dose-dependent membrane damage effect of surfactants on *Pseudomonas aeruginosa*.

Further, to excavate the mechanism of antibacterial effect, reactive oxygen species (ROS) was analyzed in surfactant-treated *Pseudomonas aeruginosa* cells. The fluorescent intensity of DCFDA was found to be decreased with increase in concentration depicting the scavenging of ROS radicals by the surfactants (Fig. 4C, D). The results can be attributed to the donation of hydrogen molecule by the present methoxy and carboxyl group in the surfactants as depicted by the NMR analysis. Moreover, the imbalance of ROS in bacterial cells can also be reasoned to the interaction of surfactant molecules with ROS metabolizing proteins present inside the cells. The hypothesis of molecular membrane damage was checked using *in silico* approach. As shown in Fig. 5, the outer membrane protein of *Pseudomonas aeruginosa*, OprB, OprD, OprF, OprG, OprL, OprM was found to interact with carboxyl radical via amino acids like Arg, Glu, Lys, Asp through hydrogen bond at different bond-length and bond- energies (Fig. S6). Similarly, the methoxy group was found to interact through Asn, Arg, Ser, Tyr, His, Pro and Leu. These interactions can be reasoned for destabilization of bacterial membrane leading to their damage.

The results proved the antibacterial action of biosurfactant and depicted the molecular action through membrane damage and ROS activity.

3.9. Cytotoxicity activity of *B. casei LS14* biosurfactant

3.9.1. Effect of biosurfactant on cell proliferation

Biosurfactants are secondary metabolites produced by different microorganisms and are known to offer biocompatibility and low toxicity, however some of the microbial metabolites can often possess negative effects on host organisms triggering several epidemic diseases together with cytotoxic and neurotoxic effect [66]. Therefore, it is important to evaluate the toxicity of these biomolecules before proposing their industrial applications. Although there are various reports on the potential of biosurfactants, their application as anti-proliferative agents are still

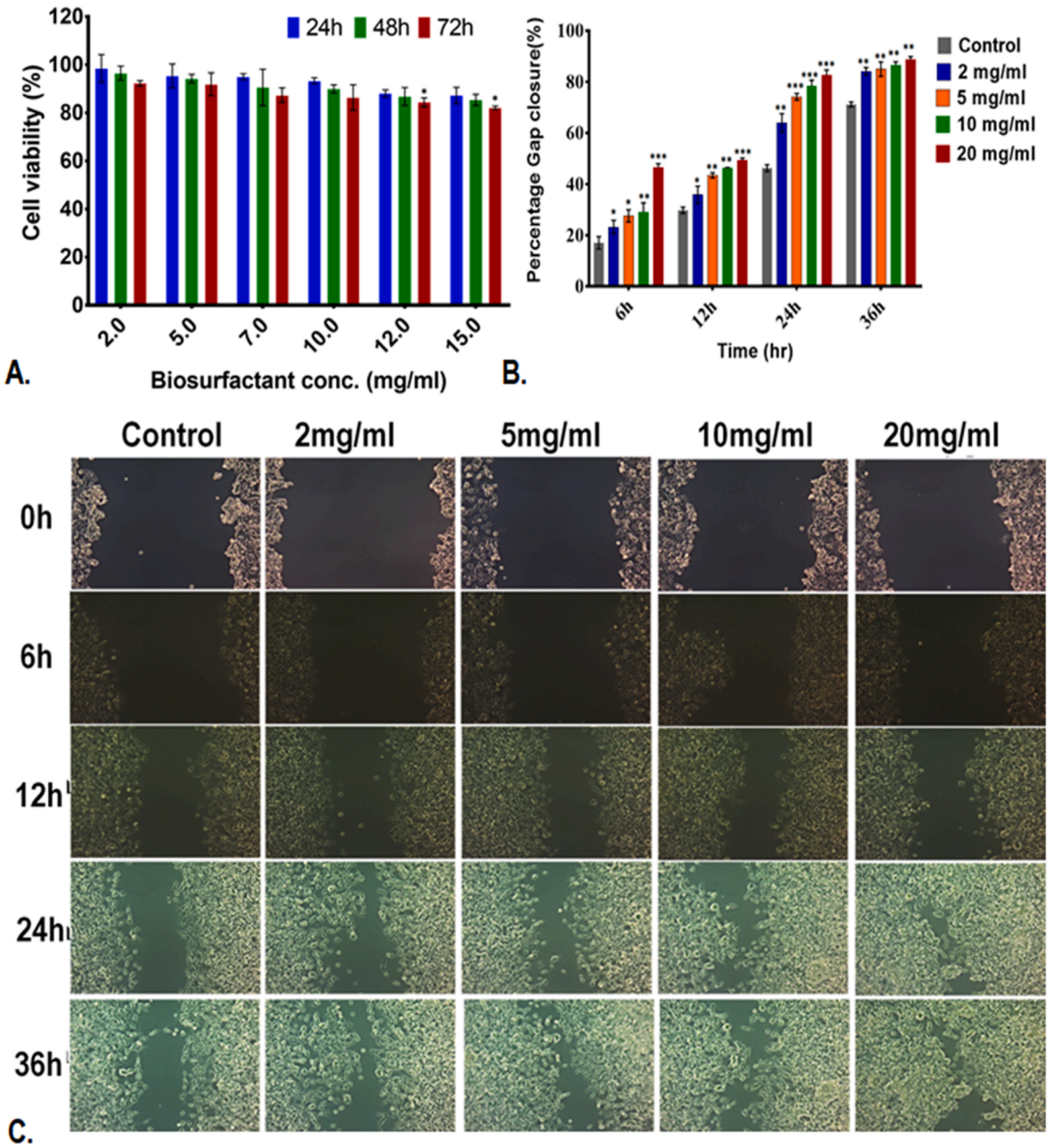

Fig. 6. Biocompatibility analysis of the *B. casei* strain LS14 biosurfactant (A) Cell survivability analysis by MTT assay (B) Bar plot showing the percentage of gap closure after 6 h, 12 h,24 h, and 36 h following the scratch in cells treated with different concentrations of biosurfactant. The values show Mean ± SD. All the experimental analysis was done in triplicate and thrice independently. The values represent the mean ± SD of three independent experiments. *P > 0.5, * *P > 0.01, and * **P > 0.001 denote the compared significant change at each exposed concentration as obtained from post hoc analysis after one-way ANOVA. (C) Bright field image of the cell migration in presence of different concentration of biosurfactants at different time points.

unexploited.

Here, we investigated the cytotoxicity of biosurfactant against HEK293 cells using MTT assay, which revealed that the biosurfactant showed negligible cytotoxic effect on the HEK293 cell line. A shown in Fig. 6A, the cell viability of the HEK293 cells were found to decreased with increase in concentration and exposure time of surfactant. The HEK293 cell line was found to be viable in an MTT assay, with a viability range of 90.21–82.12% after being treated with 2–15 mg/ml of silica purified bio- surfactants. The LC50 for the surfactant was calculated as 55.6 ± 2.3 mg/ml depicting the surfactants to be non-cytotoxic for HEK293 cells [67].

3.9.2. *Effect of biosurfactant on the migration of HEK293 cells*

The effect of biosurfactants isolated from *B. casei* was investigated for their influence on cell migration activity of HEK293 cells. The surfactant was found to enhance the cell migration activity in a dose and time-dependent manner (Fig. 6B, C). Biosurfactants have been reported [68–70] to aid the wound healing process both in clinical and in vitro studies, however, the mechanism of action of biosurfactants is still not completely deciphered. Potential of the biosurfactant to accelerate wound healing may be linked to several functions, including antimicrobial activity, tissue repair, wound cleansing, stabilization of the antimicrobials, and protein aggregation. This would be expected to speed up autolytic exfoliation in the wound by degrading damaged collagen and protecting the healthy collagen. Surfactants aid the autolytic exfoliation process by activating matrix metalloproteinase (MMP), which help in the breakdown of collagen debris [69]. Surfactants have also been proven to seal or regenerate the cell/tissue membranes, preventing

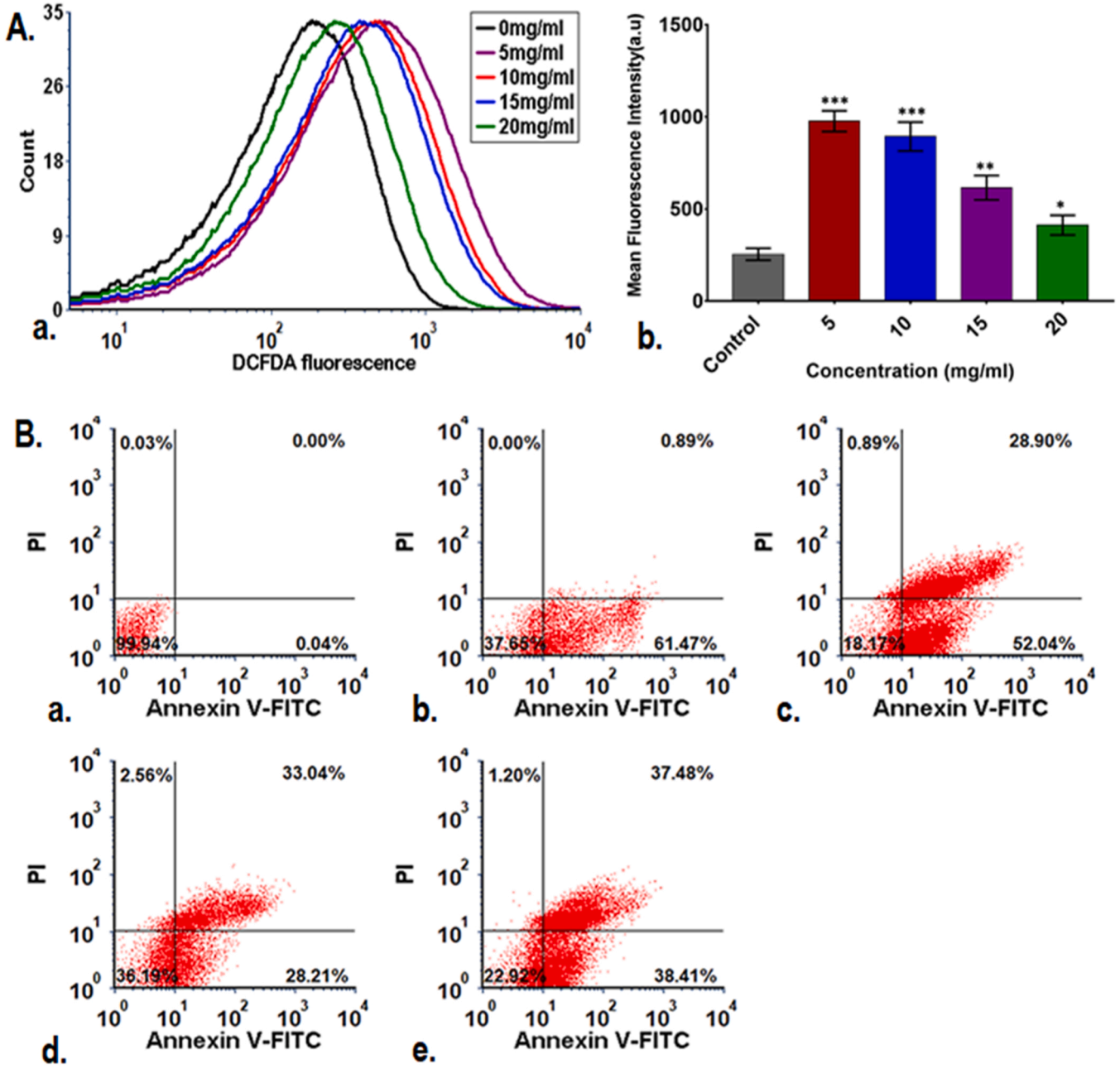

Fig. 7. Biocompatibility analysis of the *B. casei* strain LS14 biosurfactant (A) ROS analysis by flow cytometry in HEK293 cells treated with different concentration of biosurfactant; (a) Histogram presentation (b) mean fluorescent intensity. The treated and untreated cells were stained with DCFDA. (B) Apoptosis analysis in HEK293 cells treated with different concentration of biosurfactant by AnnexinV-FITC /PI staining. The values show Mean ± SD. All the experimental analysis was done in triplicate and thrice independently. The values represent the mean ± SD of three independent experiments. *P > 0.5, * *P > 0.01, and * **P > 0.001 denote the compared significant change at each exposed concentration as obtained from post hoc analysis after one-way ANOVA.

additional cellular damage [71,72]. Surfactants can improve the antimicrobial stability in wound dressings by reducing the surface tension between two phases, and hence can be used to boost antimicrobial activity [73].

3.9.3. *Mechanistic biocompatibility of B. casei LS14 biosurfactant*

Biosurfactants have been associated with a wide range of chemical groups, including fatty acids, lipopeptide, phospholipids, neutral lipids, and glycolipids [74]. Biosurfactants have been found to permeate the cell membrane by creating a channel or by a simple ion diffusion method, as well as a membrane-solubilization mechanism [75–77]. The mechanism of the *B. casei* LS14 biosurfactant induced cytotoxicity in HEK293 cells was further elucidated. Previous literatures have mentioned the reason of cytotoxicity of xenobiotic compounds like food chemicals, nanoparticles and other chemicals as an effect of oxidative stress imbalance leading to cell death through an influential effect on the cellular machinery. The cytotoxic effect of *B. casei* LS14 biosurfactant was hypothesized to be regulated by the ROS imbalance. As shown in Fig. 7A(a) and A(b), The mean fluorescent intensity of the DCFDA fluorescence indicating the ROS induction was found to be decreased with increase in concentration of the biosurfactant treatment in HEK293 cells. The outcome can be reasoned to the ROS scavenging capacity of the biosurfactants through H-donation due to the presence carboxyl and methoxy group. Moreover, it can also be hypothesized that the internalized molecule of biosurfactants inside the cells influence the structural and functional integrity of ROS regulating proteins like Superoxide

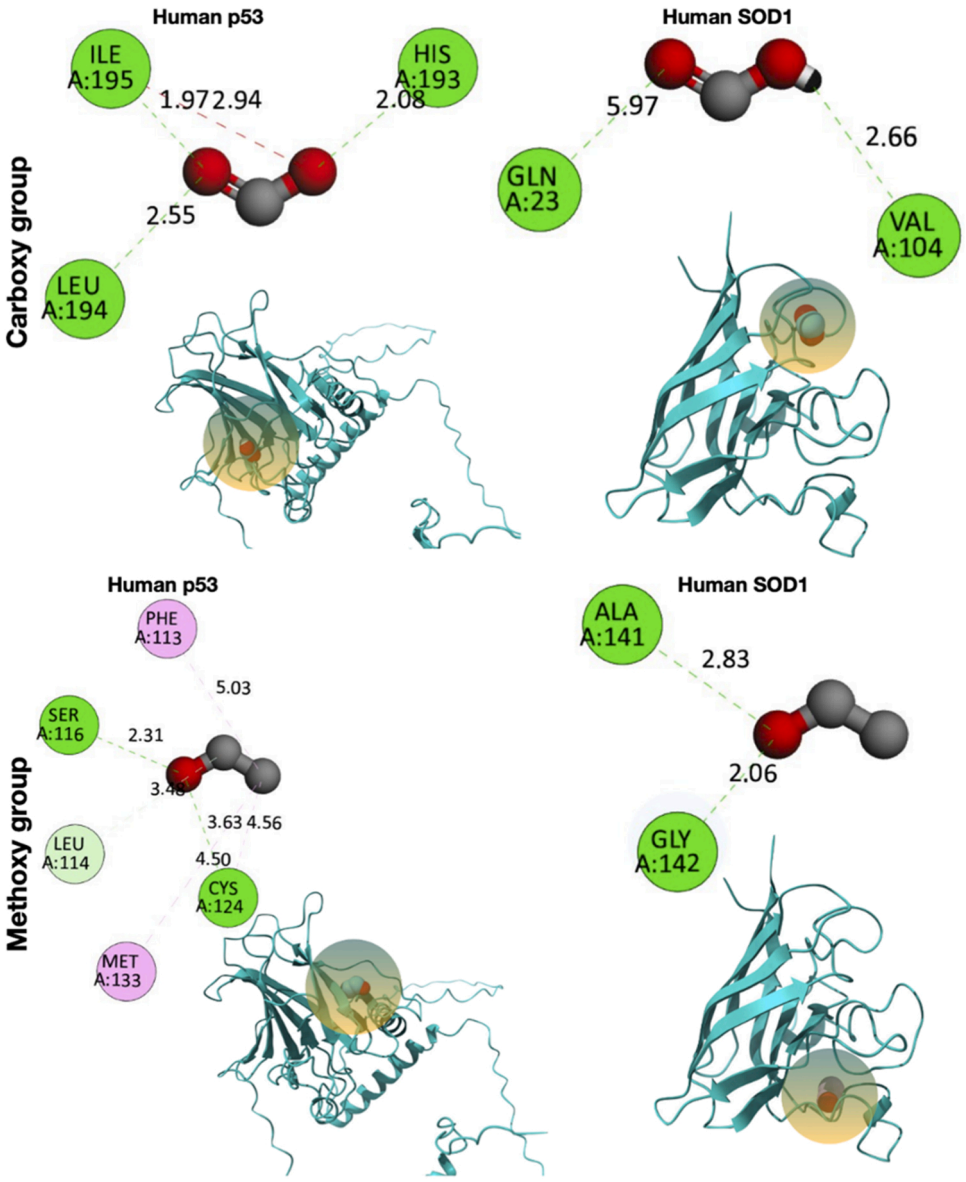

Fig. 8. *In silico* molecular docking analysis of interaction of carboxyl and Methoxy moieties of biosurfactant with Sod1 and P53 proteins of human cells.

dismutase (Sod1, Sod2) leading to the ROS imbalance. The results were in line with the antiradical analysis by DPPH assay. The imbalance in ROS has been mentioned to be responsible for apoptosis in cells. Hence, apoptosis analysis was performed using AnnexinV-FITC assay. Fig. 7C showed the increasing intensity of AnnexinV-FITC with increase in concentration of biosurfactant (5–20 mg/ml) exposure. The data depicted the enhanced phosphotidyl serine at the surface of biosurfactant treated HEK293 cells. Interestingly, the cells were not found to be fully dead or apoptotic even at highest exposed concentration (20 mg/ml) indicating towards the biocompatible nature of the biosurfactants. The results indicated to a molecular interaction of biosurfactants carboxyl and methoxy moieties with oxidative stress and apoptosis metabolic protein Sod1 and P53. The hypothesis was checked by *in silico* interaction analysis. As shown in Fig. 8, the carboxyl moieties were found to interact with Sod1 protein through Gln and Val via hydrogen bond while the methoxy moieties were interacting by Ala and Gly. Similarly, the interaction of P53 protein was predicted to interact with carboxyl and methoxy moieties of surfactant via Ile, His, Leu and Phy, Ser, Cis, Met. It can be argued that the combined effect of interaction of both the moieties from biosurfactant were influencing the structural and functional integrities of proteins leading to ROS imbalance and apoptosis.

With reference to the experimental and computational results, it can be depicted that the antibacterial and biocompatibility of the *B. casei* LS14 biosurfactant are the mechanistic action of ROS scavenging capacity of the biosurfactants due to the presence of several chemical moieties **(**Fig. 9**).** Most of these biosurfactants, were reported to act as an anti-proliferative agents and can act as biologically active chemicals with medical relevance [78–80]. The detailed study advocated the use of novel *B. casei* LS14 biosurfactant in biomedical application in a dose-dependent balance.

4. Conclusion

Increased yields of the targeted product necessitate the factor optimization, which generally entails the application of a competent reasonable statistical approach [81,82]. Taguchi Design of Experiment is a robust and persuasive statistical procedure [83–85]. In the current study Biosurfactant production by *Brevibacterium casei* strain LS14 isolated previously by [19] was evaluated using Taguchi Design Of

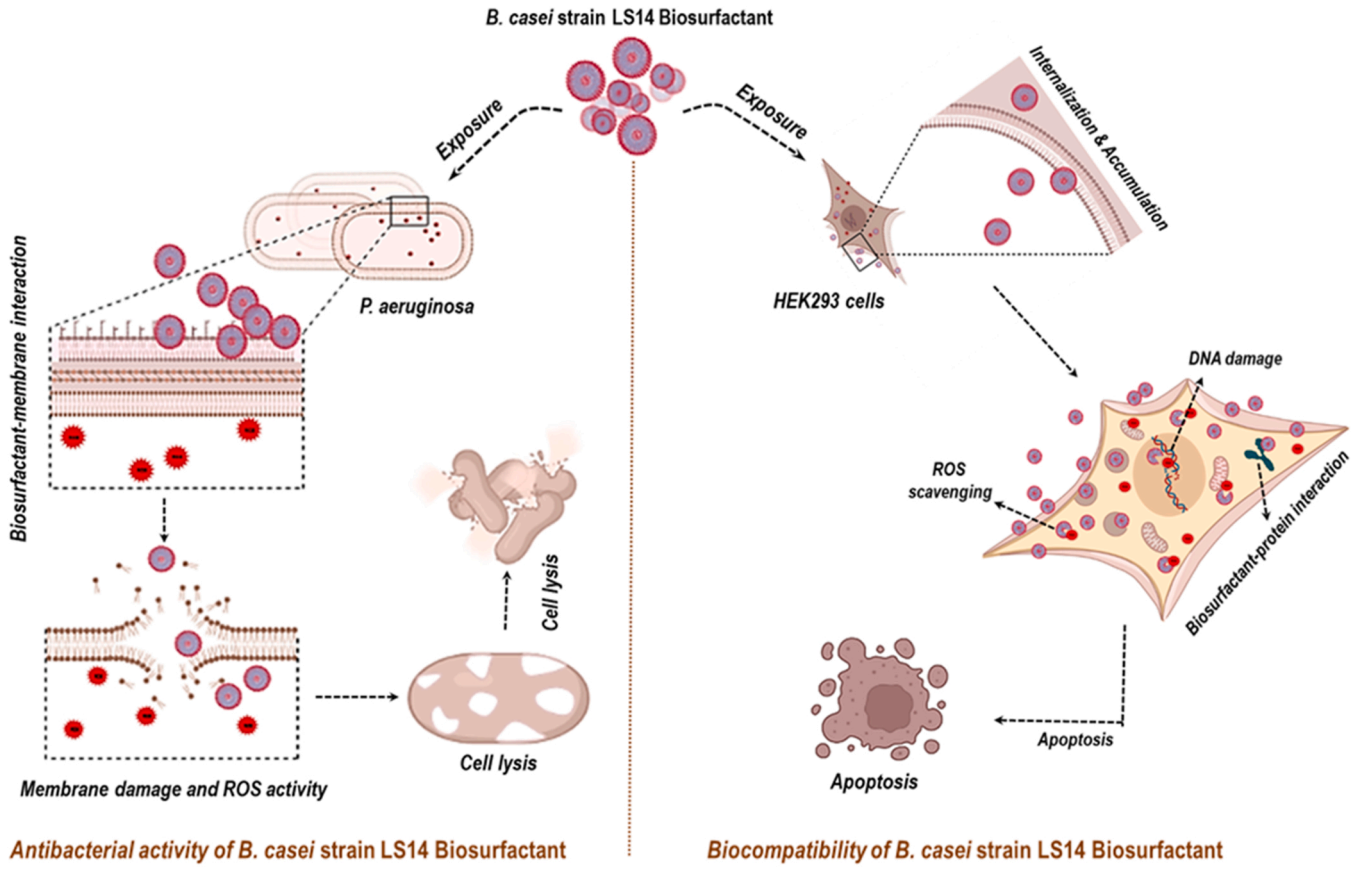

Fig. 9. Schematic diagram presenting the mechanistic antibacterial and biocompatibility of *B. casei* strain LS14 biosurfactant with *P. aeruginosa* and HEK293 cells respectively.

Experiment. The addition of optimized parameters i.e. pH-6, NaCl concentration (0.4% w/v), carbon source (waste glycerol), and nitrogen source (peptone) to the media showed approximately six-fold, and three-fold increase in oil displacement, and emulsification index respectively as compared to the un-optimized medium. It further decreased the surface tension of the biosurfactant by 1.2 times, when optimized factors were added to the culture media. One of the most important factors in production of biosurfactant is the carbon source present in the culture medium [86]. Waste Glycerol has been used a substrate in a variety of bioprocesses [87]. The use of low cost-carbon sources, such as waste glycerol, as a source of biosurfactant biosynthesis by microorganisms is an intriguing alternative. The purified biosurfactant extracted after optimization condition was characterized using NMR. The mechanistic evaluation for antimicrobial, anti-radical, anti-proliferative and cell migration activity depicted the mechanism as an influential regulation of oxidative stress(ROS) both in bacterial and human cells. The study concluded that biosurfactant from *Brevibacterium casei* has significant biological potential as compared to standard drugs and could be an interesting alternative for natural therapy.

CRediT authorship contribution statement

Khushbu Singh: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. **Himadri Tanaya Behera:** Methodology, Formal analysis, Investigation, Writing – original draft. **Priyadarshini Pratikshya Nayak:** Investigation, Methodology, Formal analysis, Writing – review & editing. **Adrija Sinha:** Methodology, Formal analysis, Software, Investigation, Validation, Writing – review & editing. **Aditya Nandi:** Methodology, Investigation, Formal analysis, Validation, Writing – review & editing. **Aishee Ghosh:** Methodology, Formal analysis, Investigation, Validation, Writing – review & editing. **Utsa Saha:** Methodology, Investigation, Validation, Writing – review & editing. **Mrutyunjay Suar:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Supervision, Funding acquisition. **Pritam Kumar Panda:** Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Suresh K. Verma:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Vishakha Raina:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114493.

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