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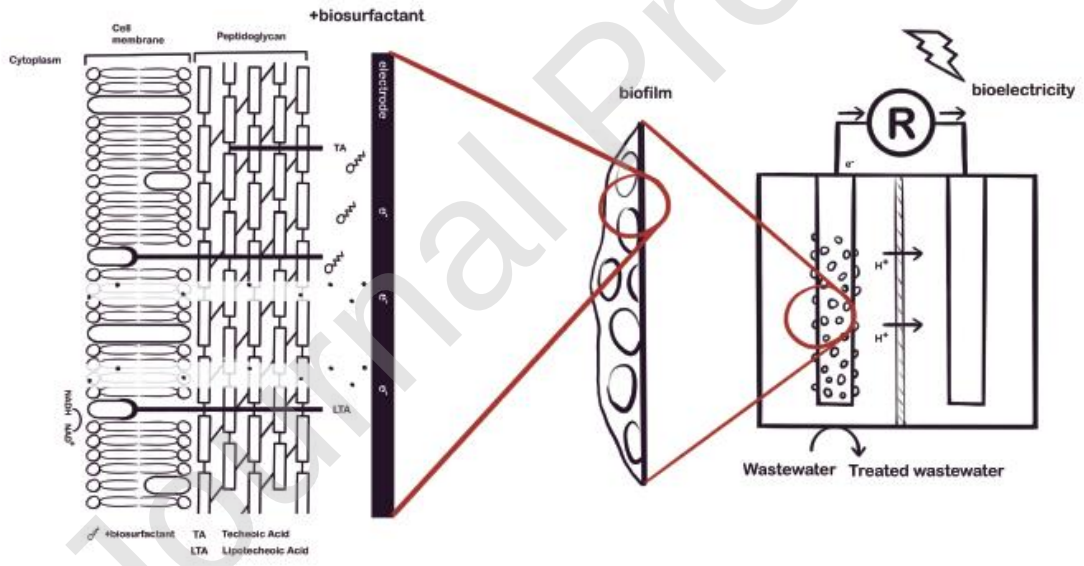
Graphical abstract



Highlights

- The time point of adding biosurfactant to bacterial cells determines its effect
- Biosurfactant in low concentrations induces conductivity and biofilm formation
- Manipulation of bacterial cells is an effective way to accelerate Microbial Fuel Cell performance

Abstract

A biosurfactant producing Gram positive bacterium isolated from anodic biofilm of textile wastewater fed MFC was identified as *Bacillus* sp. MFC (Accession number: MT322244). Scanning Electron Microscopy of the bacterium showed appendages, the bacterium forms biofilm on Congo red agar medium. The obtained results showed that the addition of 5 mg/l endogenous biosurfactant to the bacterial cells resulted in 19-fold increase in bacterial surface-bound exopolysaccharides (EPS) and 1.94-fold increase in biofilm. However, when the biosurfactant concentration increased to 20 and 40 mg/l, EPS and biofilm decreased and the cells lost their colony forming ability. The dielectric properties of the bacterial cells showed increase in conductivity and relative permittivity with increasing biosurfactant concentrations. The shape of the voltammogram currents peak, their location and Electrochemical impedance spectroscopy (EIS) suggest the involvement of biofilm as direct electron transfer pathway. The average voltage obtained was 0.65V as compared to 0.45V for the control MFC. Decolourization was tested for Congo red in a double chamber Microbial Fuel Cell (MFC), the results showed 2-fold increase in decolourization when biosurfactant is added post biofilm formation. The results confirm that *Bacillus* sp. MFC possess electrogenic properties and that adding low concentrations of endogenous biosurfactant to 24 h biofilm accelerates electron transfer by inducing perforations in the cell wall and increasing EPS as an electron transfer transient medium. Therefore, MFC performance can be enhanced.

Keywords: **Gram positive bacteria**; **Biofilm**; **Permeability**; ***Bacillus* sp.**; **Microbial Fuel Cell**; **Biosurfactant**.

Introduction

Microbial Fuel cells (MFC) depend on microorganisms, as biocatalysts, that drive oxidation/ reduction reactions at electrode surfaces. The electron transfer from microorganisms to electrodes is considered one of the key aspects that controls MFC operation and sustainability [1]. MFC anodic electron transfer is one of the rate-limiting steps in the process, therefore, biofilm formation on anode plays a key role in electron transfer. Electrogenic microbes are present in different ecosystems and have been found to belong to diverse families of Gram negative and Gram positive bacteria [2,3]. Gram negative bacteria have been extensively studied with their electron transfer pathway categorized as direct (via membrane associated cytochromes, or conductive through pili) or mediated (by redox mediators) **[4].** However, little has been reported on electron transfer in Gram positive bacteria. Gram positive bacteria have a thick cell wall (10 to 80 nm) that maybe encased in a glycoprotein S layer. Due to the structural differences between Gram negative and Gram positive bacteria, the latter was assumed not to follow direct electron transfer to insoluble electron acceptors; hence they require addition of mediators [5]. However, evidence emerged that Gram positive bacteria follow a direct electron transfer pathway as well [6]. Generally, different approaches have been adopted to overcome bacterial electron transfer limitations such as including anode surface

modification [7], bacterial cell perforations [8], addition of synthetic or natural mediators [9, 10] or biosurfactants in the anode chamber [11]. The latter is the focus of our study. There are different groups of biosurfactants, such as glycolipids, phospholipids and lipopeptides. Gram positive bacteria are known to produce lipopeptides, they can be of different chain length, generally cationic and have been reported to have diverse effects on membrane lipid bilayer [12]. The biosurfactant produced by *Bacillus* sp in our study is short chain lipopeptide. The charge of the short chain lipopeptide was reported to lay within the range from +1 to +4 [13]. The addition of biosurfactants to microbial cells was reported to induce different effects on bacterial cells. It increased cell permeabilization in *Bacillus* cells and membrane fluidity [14], induced diverse effects on membrane lipid bilayer [12]. Cationic hydrophobic containing peptides were reported to favour and aid its insertion and interaction with bacterial membranes causing neutralization of the membrane that is followed by permeabilization at a later stage [15]. Cationic peptides interact with negatively charged parts of the bacterial membrane, interact with the lipid head group in what is termed the "carpet model" causing "toroidal pores" [16]. A biosurfactant added to anodic MFC compartment was reported to increase biofilm formation by making the electrode surface more hydrophilic, acting as a bridge that facilitated bacterial cell attachment to the electrode and this in return led to an increase in MFC performance [17]. From this standpoint, adding biosurfactant to anodic bacteria can either cause permeabilization that would lead to mediated electron transfer or attachment as biofilm that would lead to direct electron transfer. Therefore, the aim of the present study is to characterize the biochemical, biophysical and bioelectrochemical changes of *Bacillus s*p. cells after adding lipopeptide biosurfacant and correlate this effect to MFC performance by assessing the electricity production and decolorization of congo red as a model dye.

Materials and methods

Isolation and biochemical characterization of biosurfactant producing Gram positive exoelectrogenic bacterium

Bacterial biofilm grown on an electrode of textile wastewater-fed double chamber Microbial Fuel Cell was collected and identified as a *Bacillus* sp. rich biofilm in a previous study [18]. In the current study, a single bacterium was chosen to study its biochemical, biophysical and bioelectrochemical characteristics. Luria Bertani (LB) agar plate was used for recovery of the bacterium from the anodic biofilm sample. The plates were incubated at 37° C for 48 to 72 h. Single colonies were removed from these plates and sub-cultured for isolation, purification and selection of the highest biosurfactant producer based on oil dispersion assay [19].

Identification of most potent biosurfactant producing isolate

The isolate showing the highest oil dispersion was characterized according to Bergey's manual of determinative bacteriology. Detection of biofilm was performed using Congo red agar (CRA)

method. Brain heart infusion broth (BHI) was prepared according to manufacturer's instructions (Oxoid, UK). Colonies turning black were regarded as biofilm forming whereas those remaining white to pinkish were considered biofilm negative. Riboflavin was assayed in culture supernatant [10].

16S rRNA phylogenetic identification

DNA of a 24 h culture was extracted using DNAEasy extraction kit according to the manufacturer's instructions. 16S rRNA universal primers used were 27-8 GAGTTTGATCCTGGCTCAG and 1492 GGTTACCTTGTTACGA (Sigma Co.). The amplification was performed as follows: 35 amplification cycles at 94℃ for 45 sec, 55℃ for 60 sec and 72℃ for 60 sec. The visualized band amplified PCR products were submitted to Solgent Co Ltd (South Korea) for purification and sequencing. The resulting sequence was trimmed and assembled in Geneious software (Biomatters). The sequence was compared to the NCBI nucleotide database (www.ncbi.nih.gov\blst). Netwick file was generated using the free online software www.phylogeny.fr and phylogentic tree was constructed using the neighbour-joining method using Molecular Evolutionary Genetics Analysis software (MEGA X). The sequence was submitted to Genbank public database with accession number MT322244. G-C content (%) was calculated for the sequence using software www.sciencebodies.com.

Scanning Electron Microscopy (SEM)

Scanning electron micrographs of the isolated bacteria were captured using a JOEL JMS 5600 scanning electron microscope, cells were centrifuged and fixed using different alcohol concentrations and placed to dry on glass cover slip for 24 h. A suitable piece was cut using a clean sterile cutter then glued onto brass stub using a double-sided adhesive tape and was coated with a thin layer of gold under reduced pressure. The images were captured at magnifications of 7500 X using an electron beam high voltage of 30 kV.

Biosurfactant production and characterization

The media used for biosurfactant production was basal mineral salt solution (MSS; pH 7.0) containing (g/l): $MgSO_4 \cdot 7H_2O$, 0.3; KH_2PO_4, 5.0; $K_2HPO_4 \cdot 3H_2O$, 5.0; and NaCl, 5.0. The fermentation medium (BB; pH 7.0) used contained (g/l): beef extract, 3.0; peptone, 10.0; NaCl, 5.0; and brown sugar, 10.0 [11]. Bacterial cells were harvested by centrifugation at 10,000×*g* for 10 min at 4°C (Eppendorf, 5804R, Germany). The cell-free supernatant was acidified to pH 2 using 6M HCl, refrigerated overnight, extracted by solvent, evaporated and used as the crude biosurfactant. Blood agar lysis, oil dispersion and emulsion index. Oil dispersion assay was tested by placing 20 ml clean water in a clean petri dish, 200 µl of paraffin oil was added to the surface on the water. About 100 µl of the fermentation broth was placed at the centre of the paraffin oil surface, the created clear zone represented biosurfactant activity. The test was performed in replicates. Emulsifying activity was

determined by adding 5 ml of paraffin oil to 5 ml of the cell-free supernatant in a glass tube, then mixing it with a vortex for 2 min and incubating it at ambient temperature for 24 h. The emulsification index (E24 %) was calculated as the height of the emulsion layer (mm) divided by the total height of the liquid column (mm) multiplied by 100 according to the following equation:

$$E24\% = \frac{HE}{HT} \text{ x } 100$$

where HE and HT are the height of the emulsion layer and the total height of liquid column, respectively. For partial characterization of the crude biosurfactant, First, Thin layer chromatography was performed. In order to confirm the composition of the biosurfactant, the biosurfactant was dissolved in methanol and spotted on TLC sheets using capillary tube. TLC solvent was composed of chloroform: methanol: acetic acid (65:15:2 v/v/v). The compounds separated by TLC were visualized by spraying with ninhydrin 1 % (w/v, in water). The plates were heated at 110°C for 5 min until the appearance of the respective colours. Fourier Transform Infrared Spectroscopy (FT-IR) was used to detect the main functional groups of the biosurfactant. Scanning was performed from 400 to 4000 nm using FTIR, BRUKER VERTEX 70 device at NCRRT.

Biochemical changes in *Bacillus* sp. cells exposed to different biosurfactant concentrations

Bacillus sp. was incubated with different biosurfactant concentrations (0, 5, 10, 20 and 40 mg/l), the number of colony forming units (CFU/ml) was obtained from the appropriate dilution and log count was calculated and plotted. Biofilm formation was detected using crystal violet, the changes in the density of the biofilm were followed using ELIZA reader at NCRRT, readings were taken at 595 nm in 96 well round bottom plate. Exopolysaccharides *(*EPS) was quantified using the phenol-sulfuric acid method described by Chaplin and Kennedy [20], the absorbance was measured at 490 nm, the standard curve was prepared using different glucose concentrations. Surface-bound protein was measured as described by Castellanos et al [21].

Biophysical changes

Dielectric properties

The biophysical changes were assayed using dielectric measurements as described in [22]. The bacterium was grown in LB supplemented with different biosurfactant concentrations (0, 5, 10, 20 and 40 mg/l) for 2 h at 30ºC under static conditions. The cells were harvested by centrifugation at 6000 rpm for 15 min, washed twice with phosphate buffer saline (pH 7) and suspended in 50 ml distilled water for dielectric measurements.

The dielectric measurements were carried out using LCR meter HIOKI 3531, manufactured in Japan, in the frequency range 40 kHz to 1 MHz. All measurements were performed at 37±1 ◦C.

The measuring cell used was is a parallel plate conductivity cell with platinum electrodes of 4 cm^2 area and separating distance of 2 cm. The measured parameters were admittance (Y), phase angle (y),

reactance (X) and susceptance (B) which allows capacitance (C), conductance (G) and impedance (Z) analysis using the appropriate relations. All the calculations were carried out by means of the LCR meter software.

Dynamic Light Scattering and Zeta potential

The bacterial cell size distribution profile and cell surface charge was detected for *Bacillus* sp. cells grown in LB media, *Bacillus* sp cells in media supplemented with 5 mg/l biosurfactant and biosurfactant. Measurements were performed using DLS using Zeta potential/particle sizer (NICOMP380 ZLS, PSS. NICOMP particle sizing systems, Santa Barbara, California, USA) at NCRRT. The applied wavelength of the incident light was 632.8 nm from red He–Ne laser diode. Measurements were performed at 23°C.

Bioelectrochemical study

The cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) for carbon cloth electrode (2 x 3 cm) were measured under the following conditions: 24 h biofilm grown in biosurfactant-inducing medium with biosurfactant as the anolyte, 24 h biofilm grown in LB medium with biosurfactant as the anolyte, and 24 h biofilm grown in LB media then PBS as the anolyte. The electrodes were tested at scan rate 50 mV/s. The 3-electrode set up consisted of 24h biofilm carbon cloth 2 x 3 cm as the working electrode, carbon electrode as a counter electrode and an Ag/AgCl (saturated KCl) as a reference electrode. Electrodes were connected to BioLogic science instruments Potentiostat/Galvanostat (Germany) at NCRRT. The electrochemical cell was sealed and gassed with nitrogen to ensure an anaerobic environment during the experiment. In order to determine catalytic activity and electroactivity of bacterial biofilm, cyclic voltammetry was measured in the potential region between -0.7 - +0.1 V vs Ag/AgCl , Scan rate 50 mV/s. EIS test was conducted over a frequency range of 100 kHz to 100 mHz,

Application for dye decolourization in double chamber Microbial Fuel Cell

A double chambered MFC system was used in the following study. The anodes and cathodes (2x3 cm) were made of carbon cloth. The anode and the cathode compartments were separated with a cation-exchange membrane CMI-7000 (Membranes International USA). The anaerobic anode compartment with 100 ml working volume was purged with nitrogen gas for 10 min through 0.22 µm pore size diameter filter prior to inoculation, the medium was MSM prepared according to Gomaa et al. [10] which contained the following (g/l): NH_4Cl 0.46, KCl 0.22, $MgSO_4.7H_2O$ 0.12, NaH_2PO_4 2.5, Na_2HPO_4 4.11, $(NH_4)_2SO_4$ 0.22, a vitamin mixture and trace mineral solution was added (1%), 500 mg/l casein hydrolysate and 2.2 g/l sodium pyruvate were also added. Air-saturated cathode contained 100 ml working volume of 100 mM potassium ferricyanide in 50 mM sodium phosphate buffer (pH 7). Two MFC systems were set up and incubated at 37ºC in an incubator, A) a 24 h inoculum (20% v/v) of induced endogenous biosurfactant from the isolated culture was added to the anodic chamber and B) 20% 24 h LB inoculum containing 5 mg/l of the extracted biosurfactant. Congo red dye was added at initial concentration of 50 mg/l. Decolorization

was followed at 497 nm at 0, 1, 3, 6, 10 and 15 days. Decolourization was calculated using the following equation at:

$$\text{Decol}(\%) = \frac{I-F}{F} \times 100$$

Where I indicates the initial colour and F the absorbance at the end of MFC performance.

Statistical analysis

All experimental data indicated on the graphs and tables represent the mean value of triplicate experiments; the error bars in the graphs represent the standard deviation of the mean (SD). Statistical analysis of the data was conducted by one-way analysis of variance (ANOVA) using Microsoft Excel statistics package.

Results

Isolation and characterization of anodic biosurfactant producing bacteria

Detailed work flow of the present work is illustrated in a schematic diagram (S1). The isolate was chosen based on oil dispersion test, its ability to form biofilm and iron reduction (S2). Table 1 shows phenotypic and some of the biochemical characteristics for isolate 9. Scanning Electron Microscopy (SEM) image shows that the cells possess appendages/tendrils that connect the bacteria together (Fig. 1). Fig (2) represents the generated phylogenetic tree, the isolate under study showed close similarity with different *Bacillus* strains. The isolate's calculated G-C content was 53.8%. The isolate was named *Bacillus* sp. MFC and deposited in the GenBank under accession number MT322244.

The *Bacillus* sp. under study was tested for its biosurfactant production. The results showed E24 was 50% as compared to 75% for Tween80 under the same test conditions. The speed of oil dispersion was very close to that of Tween80 (Table 2). The main functional groups of the biosurfactant was detected using FTIR (Fig. 3). The stretching peak at 3308.28 cm^{-1} indicated the presence -NH, peaks at 2959 and 2928 cm^{-1} indicate the presence of aliphatic CH of CH_3 and CH_2, peak at 1736 cm^{-1} indicate ester of carbonyl group –CO. Stretching peak at 1535 indicates –C=O-NH fatty acid linkage. The hydrophilic fraction of the biosurfactant is composed of 3 fragments as detected using TLC visualized with ninhydrin (Data not shown). The biosurfactant has a net positive charge of +2.11 mV using Zeta potential and size of 12154 nm as detected using dynamic light scattering (DLS) (Table 3).

Biochemical changes

The addition of different biosurfactant concentrations to the *Bacillus* sp. under study has led to different responses (Fig. 4). The log colony forming ability of the bacteria remained the same for control samples and those incubated with 5 mg/l biosurfactant, this was followed by a slight decrease at 10 mg/l, no colonies were detected when biosurfactant was added at 20 and 40 mg/l. Biofilm formation was detected using crystal violet in 96 well plates. The results showed a 2 fold increase in absorbance from 0.094 to 0.183 when 5 mg/l biosurfactant was added, this was followed by slight gradual decrease to 0.151, 0.129 and 0.098 when 10, 20 and 40 mg/l biosurfactant was added, respectively.

Exopolysaccharide assay showed an increase in surface bound EPS production in *Bacillus* sp. MFC cells exposed to 5 mg/l lipopeptide produced from 0.9 to 17.11 mg/ml which represents 19 fold increase. Above this concentrations, the EPS produced decreased to 14.06, 12 and 8.14 mg/ml when exposed to 10, 20 and 40 mg/l biosurfactant. Surface bound proteins decreased from 511.8 at 0 biosurfactant to 219.4 and 168.2 mg/ml upon the addition of the 5 and 10 mg/l biosurfactant, respectively. Above which, the surface bound proteins measured showed an increase to reach 263.3 and 475.3 mg/ml, at 20 and 40 mg/l biosurfactant, respectively.

Biophysical changes

The biophysical changes induced after adding different biodurfactant concentrations to *Bacillus* sp. cells for 24 h were detected using dielectric properties. The effect of the biosurfactant on the cell membrane resulted in an increase in relative permittivity and area under loss peak by 3 and 2.61 fold, respectively after adding 10 mg/l, the value slightly decreased at 20 & 40 mg/l for both tested parameters (Fig.5a&b). The measured conductivity of the bacterial cells under the same conditions exhibited a slight gradual increase from 0.075 to 0.11 (S/m) (Fig. 5c). The effective capacitance of native cells were 1 F was increased 9 fold to reach 9.2 F after adding 10 mg/l for 24 h, this was followed by gradual drop in effective capacitance that reached values of 7.6 and 4.9 F after adding 20 & 40 mg/l to the cells (Fig. 5d). The addition of biosurfactant to the bacterial cells has led to change in its net surface charge from -29.3 to 14.55 mV while DLS for bacteria was 5000 nm and dropped to 2000 nm after incubation with 5 mg/l biosurfactant, respectively (Table 3).

Bioelectrochemical test and MFC operation

Cyclic voltammetry was performed for *Bacillus* sp. biofilm under different conditions to evaluate redox characteristic and electrochemical activity of the developing biofilm. Fig (6a) represents the biofilm grown under biosurfactant inducing conditions and biosurfactant used as the anolyte, biofilm grown in LB media and biosurfactant used as anolyte and biofilm grown in LB and PBS was used as the anolyte. The voltammogram recorded shows oxidation peak at 0.83 V (vs SCE) in forward scan and small reduction peak at -0.65 V (vs. SCE) in the reverse scan for biofilm grown in LB media and biosurfactant supplemented anolyte (red line), while biofilm grown in biosurfactant inducing media and biosurfactant supplemented anolyte (black line) showed oxidation peak at 0.075V and reduction peaks at – 0.065V and – 0.13V indicating electrochemical activity of the biofilm on the electrode

surface in presence biosurfactant as anolyte. On the other hand, when PBS was used as the anolyte (blue line), no reduction peaks were detected. Electrochemical impedance spectroscopy (EIS) is a powerful tool for in depth analysis of resistances in MFCs. The result in Fig. (6b) represents the Nyquist plot for all tested samples including semicircle portion at high frequency which corresponds to charge limited process and linear region at low frequency which corresponds to mass transfer process at electrode-electrolyte interface. The EIS of biofilm grown on carbon electrode is fitted with equivalent circuit (inset b in fig. 6b).

The MFC operation depicts that adding biosurfactant to a biofilm formed anode has resulted in increasing both electricity production and decolorization of Congo red dye. Fig. 7a shows that there is an increase in average voltage when adding biosurfactant to LB grown culture media 0.45 to 0.65V. The decolourization increased in the first few days by 1.5 to 2.15 fold, the decolourization reached 100% for both MFC set ups (Fig. 7b). The images representing the decolorization using MFC can be found in Supplementary material (S3).

Discussion

Bacillus sp. MFC under study produced biosurfactant that lies within the range of potent production. Potent biosurfactant producing *Bacillus* isolates were reported to have E24 within the range from 45 to 60% [17 &19].

The main functional groups of the biosurfactant was detected using FTIR. The obtained functional groups confirm the resemblance to lipopeptides described in previous literature [17&19]. The charge of the short chain lipopeptide was reported to lay within the range from +1 to +4 [13]. *Bacillus* sp. under study showed a positive hemolytic test this indicates the production of hemolysins. It is noteworthy to mention that the hemolytic activity of lipopeptide highly depends on amino acid composition of hydrophilic portion and fatty acid chain length of the molecule [23].

The addition of different biosurfactant concentrations to the *Bacillus* sp. under study has led to different responses. The results obtained from biofilm assay using crystal violet indicates that the biofilm formation has slightly decreased with increasing biosurfactant concentratins. Crystal violet is a basic protein stain that binds with negatively charged molecules of the bacterial cells such as peptidoglycans, it also binds to the polysaccharide extracellular matrix [24]. Surface bound EPS was monitored as well, the results obtained exhibited a close pattern to the biofilm formation results which confirms that EPS is key step in biofilm formation. The exopolysaccharide production in bacteria plays different roles. and its production is an important step in biofilm matrix formation [25], The addition of biosurfactant to the bacterial cells led to change in its net surface charge. Bacterial EPS produced has been reported to neutralize bacterial net charge where it is reduced from -29.7 to 5.4 mV, this results in increasing surface hydrophobicity [26], and this would facilitate the attachment to the MFC electrode material also facilitating biofilm formation. The attachment of bacteria to the electrode decreases the start-up of MFCs [27]. EPS plays a pivotal role in biofilm

engineering, it was reported to act as a scaffold, it enhances cell adhesion and compensates for lack of pili or nanowires in exoelectrogenic bacteria [28].

Straus and Hanckock [29] reported in their review that the mechanism of interaction of cationic biosurfactant with Gram positive bacteria generally includes the following pattern: the hydrophilic moiety binds to the lipid bilayer of the bacterium followed by interaction with the peptide part of the membrane and thinning of the outer membrane, then aggregation and pore formation, and finally depolarization and/or peptide internalization and diffusion to intracellular targets. At this point, the cells lose their viability depending on the extent of the damage. This conclusion is confirmed by the dielectric properties results obtained in the current study where the membrane conductance is in a low range that doesn't exceed 1 S/m, which according to Patel and Markx [30], is considered a proof of viability, whereas non-viable cells are of higher orders. The measurement of dielectric properties in general is considered an effective and accurate online monitoring tool for cell viability, it provides important information about cell physiology [31]. In the dielectric study, an alternating electric field is applied to cell suspension. It influences the movement of ions in the solution, which run from the positively to the negatively charged electrode. The two major processes occurring in the structure of the cell membrane are the ion transport across the lipid bilayer and the spatial arrangement of the lipid and protein components. The relative permittivity can be correlated to the structural arrangement of the lipid bilayer and to the conformation and localization of proteins in the membrane, and consequently with the spatial distribution of charge and dipolar groups at the hydrophilic interface [32]. The area under the dielectric loss peak is a function of a number of dipoles irrespective to their relative positions. The relative permittivity ε' and dielectric loss ε" calculated as previously described [22, 30]. Generally, biosurfactants induce permeability in Gram positive bacteria [14], it attacks the hydrophobic (lipid) part of the membrane, and releases the hydrophilic (protein) part. This effect facilitates the movement of the charged parts of the membrane to be oriented in the direction of the applied electric field resulting in increase in relative permittivty (which reflects bacterial surface charge). As the concentration of the biosurfactant increases, it may disrupt parts of the cell membrane proteins or influence their charged groups and decrease the surface charge and the area under loss peaks as seen from our results, suggesting partial loss of the membrane polarizable groups. On the other hand, since increased permeability of a cell membrane is accompanied by increased membrane conductivity [33]. In the present work, conductivity was measured in bacteria exposed to different biosurfactant concentrations and the results show a slight increase in conductivity that was correlated to the added biosurfactant concentrations. This is attributed to the increase in ion flow from inside the cells to the surrounding media that was caused by biosurfactant induced permeability. This result coincides with Mier et al [34] who reported that lipopeptides induce cell permeabilization and membrane depolarization. Since the ions moving around the cells can be considered as its conductivity, the results can reflect the net transport of ionic species across the membrane through pores, ion channels, or defects in membrane structure under the influence of an applied field. It also involves contributions of the movement of external and internal charge. The results confirms a direct relationship between biosurfactant concentration and cell physiology.

Cyclic voltammetry results confirm that the addition of biosurfactant as the anolyte has resulted in the appearance of redox peaks. The detected peaks were reported to be attributed to redox system II which is known for direct electron transfer (DET) and biofilm attached to electrode surface [27]. This suggests that the biofilm formation is the main pathway for electron transfer for Bacillus sp, this is in agreement with Wringhton et al. [6]. Fricke et al [35] discussed the use of fresh media in cyclic voltammetry experiments to demonstrate that all redox signals are attributed to the biofilm based redox compounds. It is noteworthy to mention that surface bound proteins show redox peaks vary in literature [27]. Christwardana et al [36] suggested the involvement of quorum sensing compounds in the culture, which in turn help in biofilm formation. In a study published by Koch and Harnish [3], it was reported that 91% of direct electron transfer took place via biofilm, while 38% of reported bacteria used a mediated electron transfer. However, it was also reported that excreted redox mediators were involved in accelerating direct electron transfer.

The MFC operation shows that addition of biosurfactant to a biofilm-formed anode resulted in the increase in both electricity production and decolourization of Congo red dye. Zhang et al [11] reported that the role of biosurfactants in MFCs is to promote biofilm formation, especially because the hydrophilic part of the biosurfactant can attach to the hydrophilic bacteria and help in attaching the cells to the electrode surface. On the other hand, the increase in EPS produced in response to biosurfactant addition may play a role in promoting bacterial electron transfer, since it is considered a transient medium that facilitates hopping of electrons from bacteria to electrode surface [37]. While a hydrophilic compound cannot cross the cell membrane and can only interact with outer cell proteins, a lipophilic compound can cross the membrane barrier and enter inside the cells [38]. The role of biosurfactant addition in dye decolourization can be attributed to increasing dye dispersal and improving contact between the dye and the bacterial cell [39, 40], this may explain why the cells acquired Congo red colour at the start of the MFC operation and then decolourization followed by time until complete decolorization took place (S3). It was reported that surfactants change cell membrane ultrastructure and promote transmembrane channels; both are effective contributors to increasing microbial cell permeability, reducing membrane resistance and increasing substrate degradation [11]. The presence of EPS secreted due to the addition of 5 mg/l biosurfactant could have contributed to this. EPS secreted by *Enterobacter cloacae* strain TU has been reported to possess high emulsifying activity. The EPS could increase the hydrophobicity of the bacterial cell surface and also neutralize the surface charge of the cells [26]. This is in agreement with our zeta potential results.

Conclusion

Our results demonstrate that adding biosurfactant to *Bacillus* sp. culture can be used to manipulate the cells on different levels. We can propose that adding biosurfactant to *Bacillus* sp. cells can trigger two pathways depending on the time they are added. It can act as 1) a quorum sensing molecule when it is endogenously induced and accelerate biofilm production and 2) it can also induce permeability of cell membrane and enhance EPS production when added post biofilm formation. In terms of MFC application, the obtained data indicate that spiking biosurfactant post biofilm formation is better than

endogenous induction over the experiment duration. The use of biosurfactant to manipulate cell performance is worth further investigation as a safe and effective strategy to improve electron transfer in MFCs.

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CRediT author statement

Ola M. Gomaa, Conceptualization, project administration, investigation, writing original draft, final editing and correspondence, **Nabila Selim** Conceptualization, investigation, writing original draft, review **Reham Fathy** investigation, **Heba Hamed Maghrawy** investigation, **Marwa Gamal** investigation, **Hussein Abd El Kareem** supervision, **Godfrey Kyazze**, supervision, editing, **Tajalli Keshavarz** project administration, supervision, editing.

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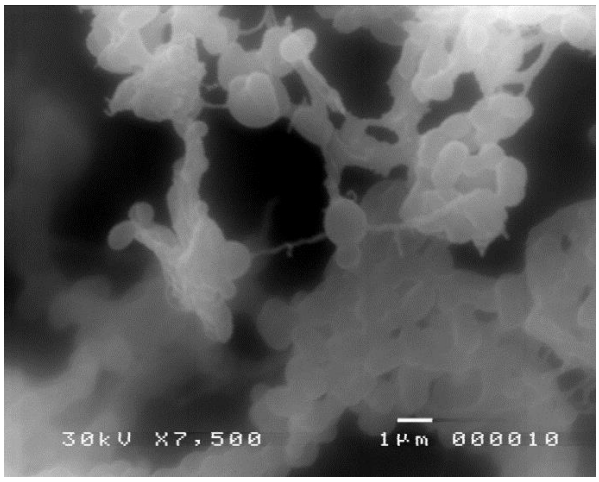


Fig. (1): Scanning Electron Micrograph showing the isolated *Bacillus* sp. under study.

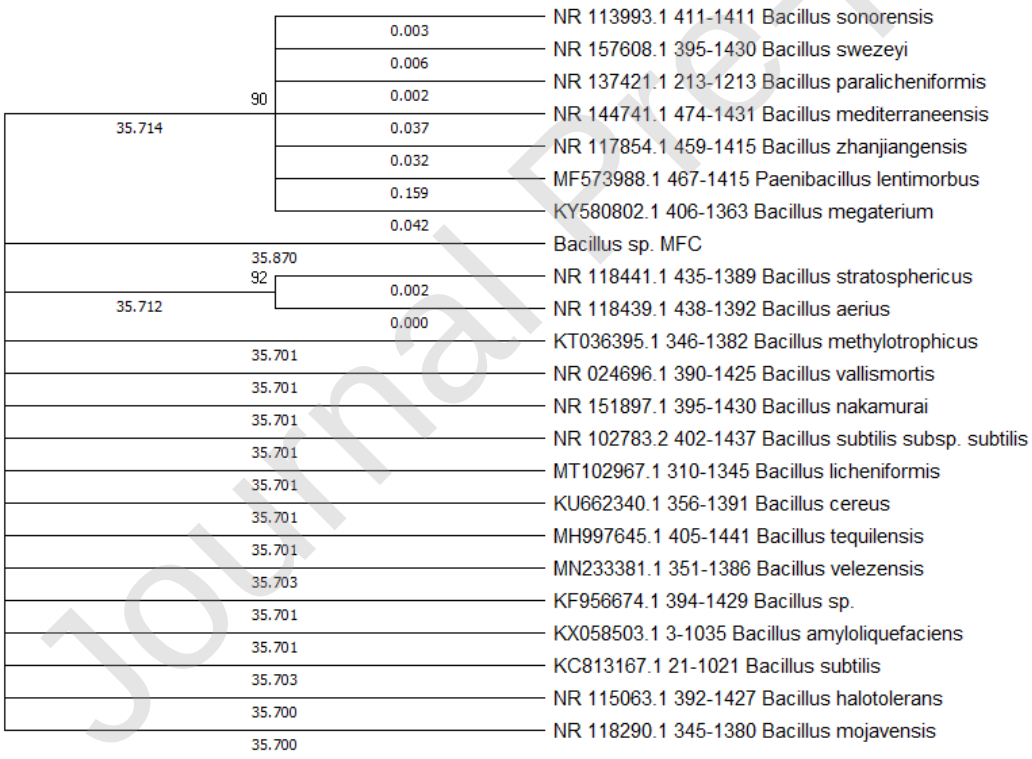


Fig. (2): Phylogenetic tree of the isolated *Bacillus* sp. (Accession number: MT322244) under study based on 16S rRNA sequence.

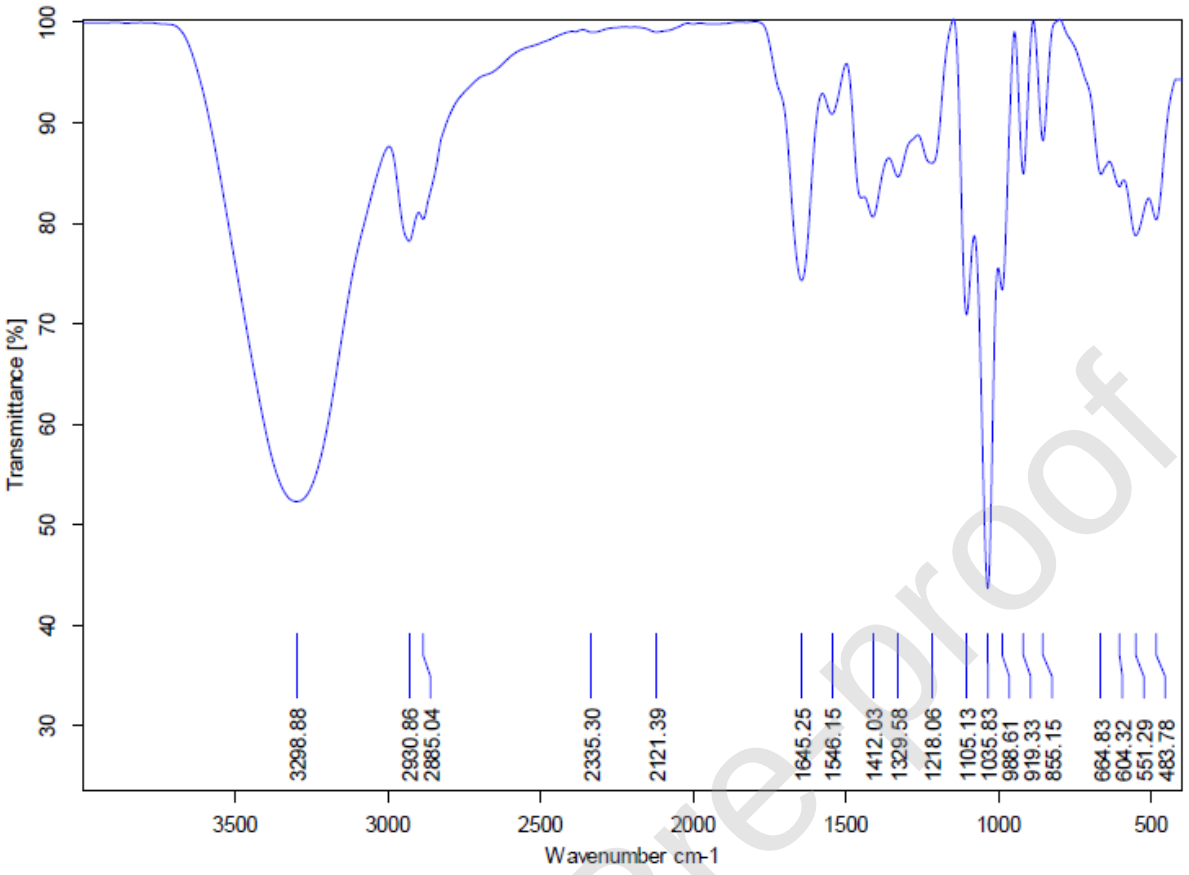
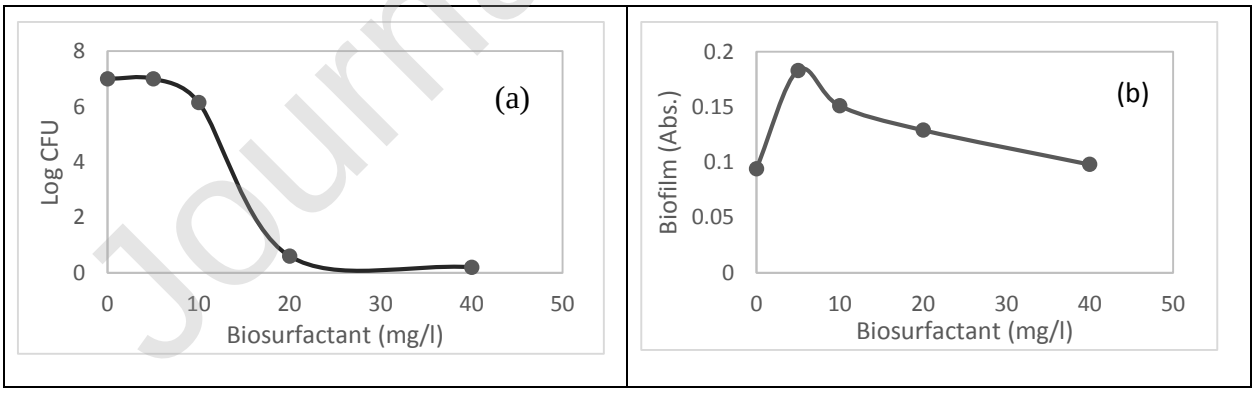


Fig. (3): FTIR spectrum of the *Bacillus* sp. produced biosurfactant.



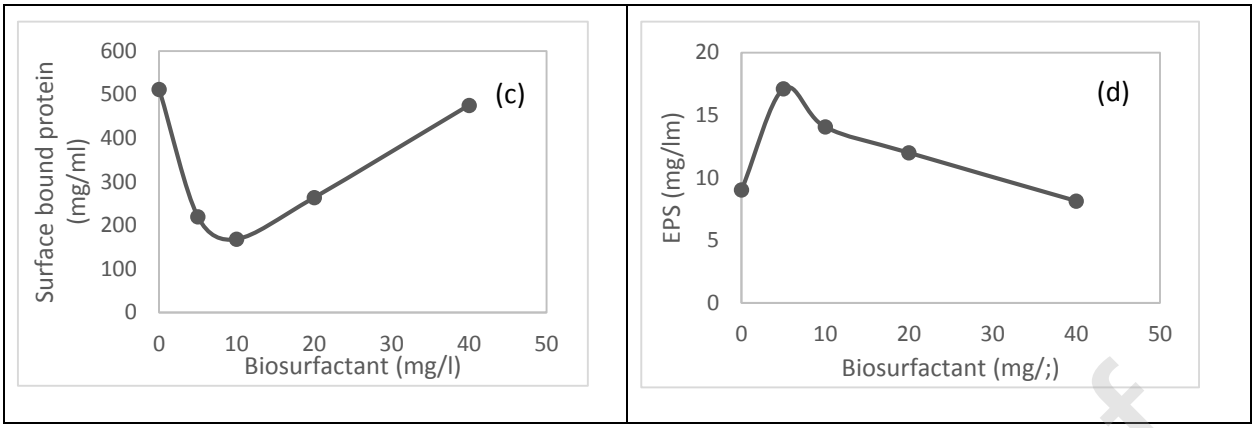


Fig. (4): The effect of different biosurfactant concentrations on colony forming ability (a), biofilm formation (b), surface bound protein (c) and EPS (d) of *Bacillus* sp. after 24 h incubation.

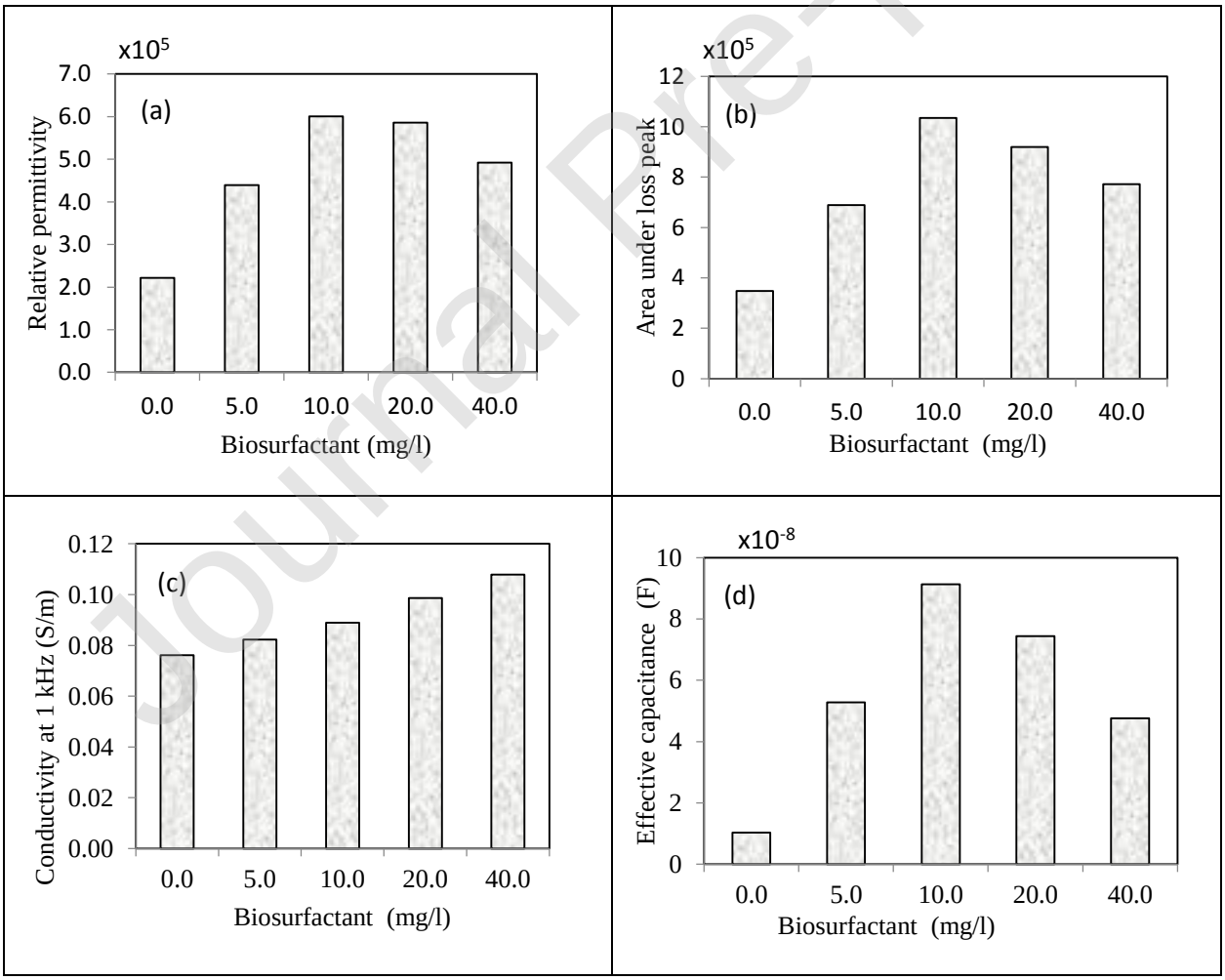


Fig. 5: Dielectric properties showing relative permittivity (a), area under loss peak (b), conductivity (c) and effective capacitance (d) of *Bacillus* sp. MFC behaviour under different biosurfactant concentrations.

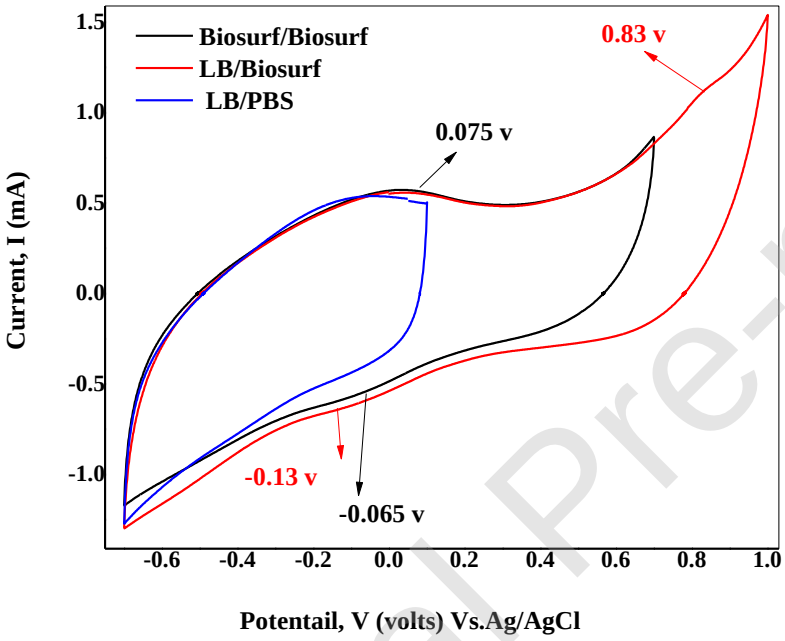


Fig. (6a): Cyclic Voltammetry for *Bacillus* sp. grown in LB medium in PBS as anolyte (blue line), LB in biosurfactant as the anolyte (red line) and cells grown in biosurfactant inducing medium in biosurfactant as anolyte (black line).

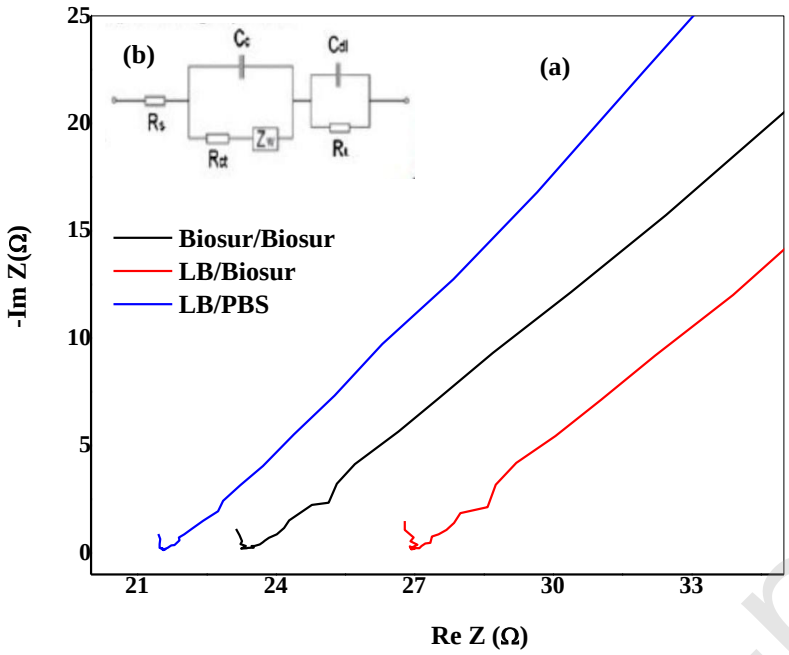


Fig. (6b): EIS showing Nyquist plot (a) and equivalent circuit (b) for *Bacillus* sp. grown in LB medium in PBS as anolyte (blue line), LB in biosurfactant as the anolyte (red line)and grown in biosurfactant inducing medium in biosurfactant as the anolyte (black line).

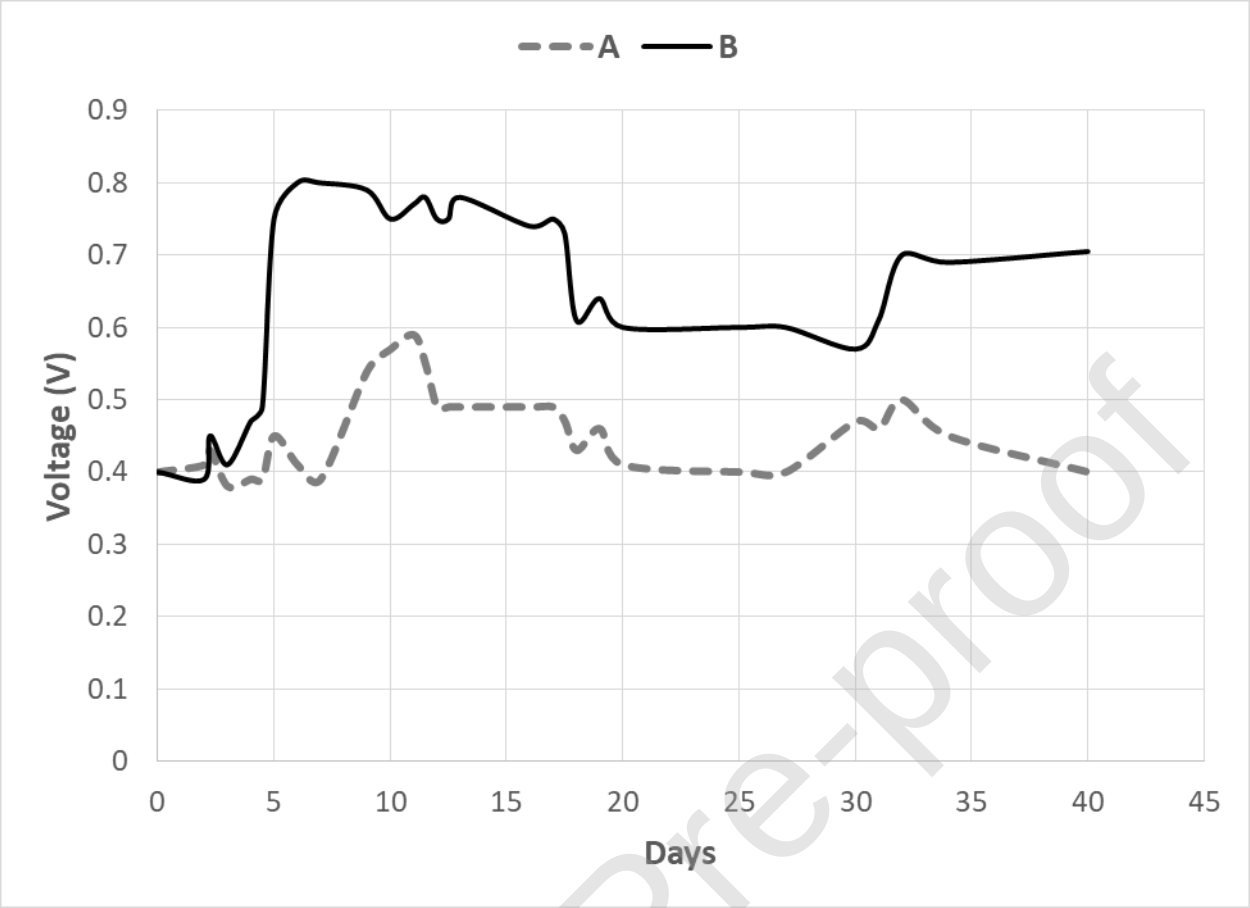


Fig. (7a): MFC performance of double chamber containing Congo red in anodic chamber and *Bacillus* sp. grown in A) LB medium with biosurfactant as the anolyte and B) biosurfactant inducing medium with biosurfactant as the anolyte

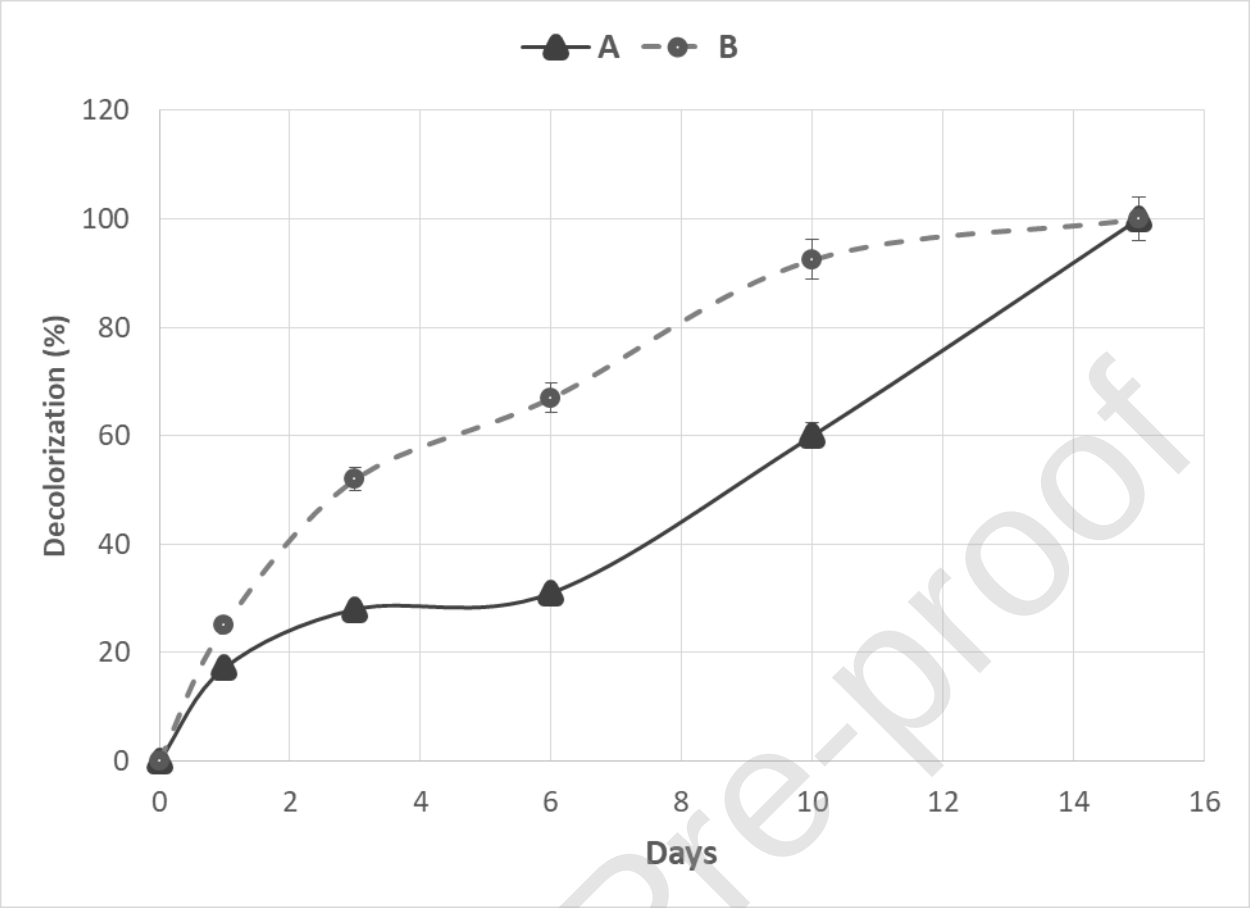


Fig. (7b): Congo red decolorization in MFC. Samples withdrawn after 0, 1,3,6,10 and 15 days for *Bacillus* sp. grown in A) 24 h biofilm grown in LB medium with biosurfactant as the anolyte, B) 24 h biofilm grown in biosurfactant inducing medium with biosurfactant as the anolyte

Table (1): Phenotypic and some biochemical characterization of isolate 9

Test	Phenotypic and Biochemical Characterization
Gram stain	+ve
Morphology	Bacilli
Catalase	+ve
Oxidase	-ve
Growth on Blood agar	Clear zone indicating blood heamolysis
Mobility	Sliding motility
Growth on 10% NaCl	-ve
Growth on pH 5.7	-ve
Fermentation with gas production:	
Mannitol	+ve
Glucose	+ve
Fructose	+ve
Sucrose	-ve
Biofilm formation (Congo red agar)	+ve
Phenazine	+ve
Riboflavin	+ve
Biosurfactant (oil spreading method)	+ve

Ferrous reduction	+ve

Table 2: Emulsification index and collapse drop for biosurfactant produced from *Bacillus* sp. as compared to positive control (Tween 80) and negative control (water).

Sample	Emulsification index 24%	Collapse drop*
Tween 80	75	++++
Biosurfactant present	50	+++
H_2O	0	-

*+ refers to the speed of the collapse drop visually.

Table 3: Zeta potential (mV) and Dynamic Light Scattering (nm) for Biosurfactant and *Bacillus* sp. mixed with different volumes of the biosurfactant.

Sample	Zeta Potential (mV)	Dynamic Light Scattering (nm)
Biosurfactant	2.11	12154
Bacillus sp.	-29.30	5000
Bacillus sp+ Biosurfactant (5 mg/l)	14.55	2000