Electroactivity of weak electricigen *Bacillus subtilis* biofilms in solution containing deep eutectic solvent components

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Abstract

Bacillus subtilis is a Gram-positive, spore-forming bacterium with a versatile and adaptable metabolism, which makes it a viable cell factory for microbial production. Electroactivity has recently been identified as a cellular characteristic linked with the metabolic activity of *B. subtilis*. The enhancement of *B. subtilis* electroactivity can positively enhance bioproduction of high-added value metabolites under electrofermentative conditions. Here, we explored the use of deep eutectic solvents (DESs) and DES components as biocompatible nutrient additives for enhancing electroactivity of *B. subtilis*. The strongest electroactivity was obtained in an aqueous choline chloride: glycerol (1:2 mol mol^{-1}) eutectic mixture. At low concentration (50-500 mM), this mixture induced a pseudo-diauxic increase in planktonic growth and increased biofilm formation, likely due to a nutritional and osmoprotectant effect. Similarities in electroactivity enhancements of choline chloride-based eutectic mixtures and quinone redox metabolism in *B. subtilis* were detected using high performance liquid chromatography and differential pulse voltammogram. Results show that choline chloride-based aqueous eutectic mixtures can enhance biomass and productivity in biofilm-based electrofermentation. However, the specific mechanism needs to be fully elucidated.

Keywords: Deep eutectic solvents, electroactivity, *Bacillus subtilis*, glycerol, choline chloride

1. Introduction

Bacteria capable of extracellular electron transfer (EET) to solid conductive surface maintained at set electrochemical potential are generally termed 'electricigens' [1] and this phenotype has been investigated for bioenergy production and biosensor application [2–4]. While EET phenomena can be observed in planktonic cells, they are particularly relevant in bacterial biofilms. Biofilms are microstructured bacterial communities that form on solid surface, in which microbial cells grow to high concentration, encased in self-produced biopolymers matrix [5]. Biofilm microstructure and thickness varies according to nutrient concentration and environmental conditions [6]. The close proximity between the cells and the solid conductive surface enables rapid EET [7]. Strong electricigens, like the anaerobic Gram-negative *Geobacter sp*. grown on graphite electrodes, produce high current density at strong oxidizing potentials (~1-10 A m^{-2} at 0.2 V vs. Ag/AgCl) via outer membrane cytochromes or conductive protein nanowires. Most microorganisms show instead low current output under the same conditions (~0.1 A m^{-2}) or require exogenous redox mediators to facilitate EET, thus can be classified as weak electricigens [8].

Biofilms comprising weak electricigens have been observed in numerous environments [9] and are relevant to electrofermentation processes, which find applications in fine chemical production and resource recovery [10]. Thus, it is interesting to study how biofilm electroactivity interact with the synthesis of key metabolites and how biofilm-based bioprocesses can be enhanced through the application of set electrical current or potential [11]. Enhanced electroactivity can be achieved through genetic modifications, such as induction of redox mediators and metabolic re-wiring of EET chain [2,12]. However, genetic modifications and addition of exogenous redox mediators are time-consuming and may result in product alteration [13]. Alternatively, media optimization has been proposed for EET enhancements in weak electricigens [14,15].

Bacillus species are Gram-positive, spore-forming weak electricigens and are relevant to the production of metabolites like alkaline proteases, biopolymers, biosurfactants, and antimicrobial peptides [16]. Electroactivity is related to the growth and metabolic activity of *B. subtilis*, with reports further linking electroactivity to its survival under extreme temperatures and pH [17]. Coupling EET to biosynthetic systems in *Bacillus* sp. can lead to improved yield and faster production rate in bioprocesses [18]. Herein,

we investigate, for the first time, the use of aqueous deep eutectic solvent (DESs) added to bacterial growth medium at sub-toxic concentrations (50-500 mM) to boost *B. subtilis* electroactivity.

DESs are an emerging class of neoteric solvents of two or more species (Brønsted or Lewis acids and bases) that, when mixed in the right molar ratio, form eutectic mixtures with a depressed melting temperature far below those of the parent compounds [19]. Typical DES components [e.g., choline chloride (ChCl), urea (U), L-lactic acid (LA), glycerol (Gly), amino acids, polyalcohols] are biodegradable and show low toxicity. Due to their shallow ecological footprint, ease of preparation, and tunable physicochemical properties, DESs are progressively replacing toxic and volatile organic compounds (VOCs) in organic synthesis and catalysis [20], photosynthesis [21], electrochemistry [22], and solar technology [23]. DESs may play a role in whole bacterial cell interactions and in biosynthetic processes, being involved in redox activities of enzyme and bacterial systems [24]. In the crystallization of lysozyme, DESs at low concentration were found to reduce solvent evaporation during the crystallization process, thereby increasing the dissolution time of the protein crystals [25]. The toxicity of DESs as minor component in growth medium has also been investigated [26] to assess their effect on the environment.

In this work, the effect of selected DESs on the electroactivity of *B. subtilis* is investigated. At sub-toxic concentration, a solution of the eutectic mixture ChCl/Gly was found to increase planktonic cell growth, biofilm formation, and electroactivity of *B. subtilis*. These results might lead to higher production of key metabolites in DES-enhanced *B. subtilis* electrofermentation. Chemical analyses indicated production of quinone mediators under the influence of Gly and ChCl, and voltammetry results are consistent with the possibility of an induced quinone-like redox metabolism. Understanding the mechanisms of electroactivity enhancements by ChCl-based DESs is necessary for electrofermentation and microbial cell factories application of *B. subtilis* and other weak electricigens.

2. Materials and methods

2.1. Materials

The DESs used in this study had the following compositions (Table 1), DES1: ChCl/U (1:2 mol mol^{-1}); DES2: ChCl/LA (1:2 mol mol^{-1}); DES3: ChCl/Gly (1:2 mol mol^{-1}). Nutrient broth, NB (beef extract 3 g L^{-1}, peptone 5 g L^{-1}) and a chemically defined medium (CDM) containing glucose 10 g L^{-1}, NH_4Cl 5 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $FeCl_3$ 0.15 g L^{-1}, $MgSO_4$ 0.5 g L^{-1}, $CaCl_2$ 0.7 g L^{-1}, NaCl 0.5 g L^{-1}, $MnSO_4$ 0.104 g L^{-1}, both adjusted at pH 6.5, were used for all experiments. All media were prepared with deionized water before sterilization at 121 °C and 104 kPa for 15 min. The redox mediator, 2-hydroxy-1,4-naphthoquinone (2-HNQ) was obtained from Sigma Aldrich, Kazakhstan. All commercial chemicals and reagents were of analytical grade and prepared according to the manufacturer's instructions.

The bacterial strain *Bacillus subtilis* ATCC 6051 was sub-cultured and maintained on NB throughout the experiments. Screen-Printed Carbon Electrodes (SPE Ref. C110) obtained from Metrohm DropSens, Spain, with graphite working electrode (WE) of 4 mm diameter and 0.126 cm^2 surface area, graphite counter electrode, and Ag pseudoreference electrode were used in all electrochemistry experiments. In the following, all potentials are reported vs. Ag pseudoreference electrode. Electrochemical cells of 10 mL capacity were used, with 8 mL working volume.

Table 1

2.2 Bacterial growth studies

The growth curves of *B. subtilis* at different concentrations of DES1, DES2 and DES3 and in different concentrations of each component that made up DES3 in both NB and CDM were determined in 48-well plates using a Gen5TM Microplate Reader and Imager Software (BioTek Instruments). The absorbance was measured at 600 nm (OD_{600}) and experiments were conducted in quadruplicates, with values reported as mean ± standard deviation (SD). Varying concentrations of DESs and DES components, ranging from ~15 mM to ~1 M were prepared, filter sterilized with sterile 0.2 µm filters, and added to the final volume of 1000 µL per well. Incubation temperature and time were 37 °C and 48 h, respectively. For inocula, fresh cultures were grown overnight at 37 °C under constant agitation (180 rpm) and adjusted to an optical density of 0.1 (OD_{600}), which was earlier determined to be approximately 10^6 colony forming units (CFU) per mL. The redox mediator 2-HNQ (50 µM) was added in selected experiments.

2.3 Bioelectrochemical analyses

Differential pulse voltammetry (DPV) and chronoamperometry (CA) were carried out in sequence immediately after the inoculum addition, and at the end of the experiments using a computer-controlled VSP multichannel potentiostat (Bio-Logic, France). An inoculum size of 0.5 OD_{600} (approximately 5 x 10^6 CFU mL^{-1}) was used to minimize the effect of planktonic bacterial growth on current output. Under these conditions, the concentration of planktonic cells does not change significantly and the current output is mostly due to the viable cells in the biofilm [27]. Prior to experiments, SPEs were surface sterilized in 70% v/v ethanol, washed thrice in sterile deionized water, and air dried. The DPV parameters were set as follows: E_i = -0.4 V and E_f = 0.4 V, pulse height 50 mV, and pulse time 200 ms. DPV analysis was conducted immediately after the inoculum and at 48 h. For the CA, the working electrode was set at 0.4 V for 48 h. The electrical charge output (mC) for each experiment was also calculated using EC Lab® software (Biologic, France). The electrochemical cells were maintained at a temperature of 37 °C throughout the period of incubation in steel beads dry baths. Following results of DES effects on *B. subtilis* electroactivity, further bioelectrochemical analyses were carried out to determine the effects of individual components of the eutectic mixtures on *B. subtilis* electroactivity. In these experiments, similar molar concentrations of the individual components as found within the mixtures were used, and DPV and CA analyses were carried out in same fashion as earlier done for the DES analyses.

2.4 Biofilm assay

The biofilms formed on the carbon SPEs after electroanalyses were quantified using the crystal violet assay. In short, electrodes were removed from the electrochemical cells after each experiment at 48 h and immersed in sterile deionized water for media and planktonic cell removal. Subsequently, they were air dried and placed in 0.5 % wt. crystal violet solution and incubated for 10 min. The stained biofilm on the electrode was then removed and solubilized by placing in marked wells of a sterile 48-well microtiter plate containing equal volumes (1000 µL) of 33 % wt. glacial acetic acid. The absorbance was then measured at 570 nm (OD_{570}) using SmartSpec™ 3000 Spectrophotometer (Bio-Rad Laboratory automated Multiscan EX reader Lab systems, Helsinki, Finland). Four independent biological replicates were analyzed for each experimental condition.

2.5 HPLC analyses

Following completion of electrochemical experiments, cell free supernatants were prepared by filtration of culture broths using sterile 0.22 µm cellulose membrane filters. Approximately 1.5

mL samples were subsequently injected into the HPLC system (Accela 600, Thermo Scientific, USA). A C18 analytical column (1.9 µm particle size with length of 150 mm and diameter 2.1 mm) maintained at 30 °C was used as the stationary phase. The solvents used for sample mobility were prepared using HPLC grade water/65% acetic acid (solvent A) and methanol 35% (solvent B) and utilised at a flow rate of 0.2 mL min^{-1} for sample mobility.

3. Results

3.1 Growth experiments

B. subtilis cells grew rapidly in NB and their concentration, measured by OD_{600}, peaked at 12–14 h (Figure 1). Cell growth in CDM was slower and the OD_{600} did not reach a plateau within 48 h (Figure 1). Thus, NB was chosen for further experiments. The cell concentration was higher than in unmodified NB for DES3 at concentrations lower than ~1 M (Figure 2). However, cell concentration in CDM was maximum for DES1 (Figure 3). DES2 resulted in low growth, likely because of the low pH of Lactic acid, which affects the cell membrane, causing loss of cell viability [26]. Xu et al. reported that organic acid-based DESs inhibit bacterial growth [24]. DES1 reduced the growth of *B. subtilis* in NB, but increased growth in CDM. Increased growth in CDM could be a result of the additional urea as a nitrogen source supplied by DES1 in nitrogen-limited CDM [28]. Further experiments were carried out with DES3 using NB, as it best supported growth of *B. subtilis*.

In previous studies, DESs showed low microbial toxicity, with little effect on microbial growth in Mueller-Hinton broth at concentration below 200 mM (LC_{50} approximately 400-500 mM) [29]. However, studies based on antibiograms may be insufficient to determine long-term DES toxicity. A 48 h study on the DES acetylcholine chloride (AcChCl):acetamide (1:2) found no toxicity below 300 mM, partial inhibition between 300 and 450 mM, and complete inhibition of growth above 600 mM [30]. Our results are consistent with previous reports, indicating that ChCl-based DESs are non-toxic below 200 mM, and can serve as supporting nutrients. Higher DES concentration curb both cell growth and extracellular respiration, suggesting that DES formulation should be improved to increase their biocompatibility.

Figure 1

Figure 2

Figure 3

At 55 mM concentration, DES3 increases planktonic cell growth without changing its pattern, whereas at concentrations higher than 110 mM, a pseudo-diauxic growth is observed (Figure 4C), which is consistent with the metabolization of ChCl when added alone (Figure 4A). Notably, Gly alone neither significantly affects the cell growth over 48 h nor changes the growth pattern (Figure 4B). Diauxic growth is a bi-phasic bacterial growth response to the presence of two different carbon sources (mainly carbohydrates) in the medium, which are used sequentially, as indicated by the switch point on the growth curve [31]. Since ChCl is a nutrient that undergoes amino acid-like metabolism, it may not be used as a substitute of carbon source. However, previous studies reported that bacteria can use ChCl as a sole carbon source [32]. Thus, we envisage that ChCl could either act as a substitute of nutrient or trigger transcription of regulators, thus affecting the carbohydrate metabolism, and in turn leading to further growth. In the presence of DES3, there was a further increase in growth at concentration higher than 110 mM (Figure 4C). Overall, these results show a synergistic effect of ChCl and Gly when present together even at high dilution. However, experiments with Gly and ChCl at different molar ratios. (e.g., 1:1 and 1:3 mol mol^{-1}) might be needed to identify the optimal medium. Control experiments with 50 µM 2-HNQ, a redox mediator concentration commonly adopted in bioelectrochemical experiments (Figure 4D) do not impact bacterial growth.

Figure 4

Gly as a medium amendment for *B. subtilis* has been investigated in microbial fuel cells [33], however, co-utilisation of ChCl and Gly as media amendments for bacteria remains elusive. Several studies[29][34] have shown that at high concentrations, DESs such as DES3 (Table 1) inhibit microbial growth in *Escherichia coli and Listeria monocytogenes*, which is consistent with the results reported here. At the small concentrations used in this study, the effect on planktonic growth is not due to the solvent properties of DESs. In fact, the hydrogen-bonding structure of a DES appears at concentrations higher than 50 % w/w, which correspond to 4-5 M DES, depending on the DES used [35]. In conclusion, DES3 concentration range 55-547 mM was selected for the bioelectrochemical experiments.

3.2 Biofilm analysis – Crystal violet

Since electroactivity correlates strongly with the concentration of biofilm [36,37,38,39], the latter was quantified with the crystal violet method after 48 h of growth. While DES3 at any concentration tested has either negative or slightly positive effect on biofilm concentration, ChCl at concentrations higher than 36 mM strongly increases biofilm biomass, while Gly has a smaller effect (Figure 5). Overall, the biofilm quantification confirms that ChCl promotes both planktonic growth and attachment of *B. subtilis* on graphite electrodes.

3.3 Bioelectrochemical analyses

Bioelectrochemical experiments using 0.5 OD_{600} *B. subtilis* cells incubated at 37 °C, at oxidative potential (0.4V vs Ag/AgCl), showed that the addition of DES3 delays the onset of current and increases the maximum current (Figure 7). The highest charge output (Figure 8) is observed upon addition of ChCl at 36 and 73 mM, followed by Gly (73 and 146 mM) and DES3 (110 and 219 mM). The charge output after addition of ChCl is even higher than that measured with 50 μM of 2-HNQ, suggesting a specific role of ChCl in the EET process. HPLC analyses indicate the presence of menadione and riboflavin in the cell-free supernatant of *B. subtilis* grown for 48 h in electrochemical cells with 36 mM ChCl and 219 mM DES3, respectively (Figure S2-S3). However, the DPV analysis of the cell-free supernatant did not show any strong peak at potential higher than 0 V (Figure S1), indicating that the redox mediator might be located in the biofilm or adsorbed/embedded in the bacterial membrane. A much smaller charge output of 3.73±0.74, 3.11±1.42, 2.27±2.18 mC was observed in experiments supplemented at 55,110 and 547 mM DES3, indicating that bioavailable nitrogen source is needed to stimulate electroactivity under the challenging conditions imposed by the presence of Gly and ChCl. As previously mentioned, the addition of DES at high dilution (< 1 M) is likely not to cause a significant alteration on hydrogen-bonding structure of the solvents. In fact, a control experiment with pure ChCl and Gly mixed with the NB medium at the same concentration of the DES3 showed no significant difference in charge output, confirming that the solvent effect on electroactivity was negligible in the concentration range tested (Figure 8).

Figure 6

Figure 7

Figure 8

Differential pulse voltammetry (DPV) was carried out to investigate the redox active species in the bioelectrochemical system after 48 h of growth (Figure 9). Two main peaks were observed. While the peak at low potential (~0.1 V) may be attributed to cell biomass and did not change following Gly addition, the peak at higher potential (~0.2 V) is likely due to ChCl metabolization. In two experiments at 219 and 547 mM DES3, a low potential peak (~-0.1 V) was observed (Figure S4). This may be due to DES3 toxicity at the concentrations tested. In fact, the peaks at such concentrations could be attributed to partial cell lysis and release of intracellular enzymes and other redox-active compounds. It is also possible that the peak at low potential corresponds to a redox mediator produced in DES3. However, CA experiments at 0 V did not show any current or charge output, indicating that EET was not possible at such potential (Figure 10). Therefore, we conclude that the peak at (~-0.1 V) occasionally observed at 219 and frequently observed at 547 mM DES3 was due to partial cell lysis or cell damage, and was not indicative of EET at low potential. The decrease of charge output at 219 and especially at 547 mM DES3 is consistent with this interpretation. Overall, the DPV results (Figure 9) show that both ChCl and Gly increase *B. subtilis* electroactivity when added separately, while their synergistic effect is lower, particularly at high concentration (547 mM). The position and height of the peak at ~0.1 V suggest that the electroactivity increases upon Gly addition and is likely related to the cell electroactivity rather than to the presence of additional redox-active species. However, both biofilms biomass and the planktonic cell concentration are not significantly affected by Gly. Thus, Gly appears to enhance the specific electroactivity of *B. subtilis* cells. Instead, the observed increase in electroactivity upon ChCl addition is a combined effect of planktonic and biofilm growth and specific electroactivity, as shown by the peak at ~0.2 V, which is similar to the one observed upon 2-HNQ addition, suggesting the formation of a quinone or hydroquinone in the metabolization of ChCl and Gly. However, further experiments are needed to ascertain the nature of the putative redox mediator(s).

Figure 9

Figure 10

4. Discussion

With respect to DES growth, Gly could serve as a potential secondary carbon source for utilization by bacterial cells in a booster or diauxic fashion, especially at the onset of nutrient depletions in the NB medium [40]. However, this may not be the main reason for the unique growth pattern, as Gly alone did not show the pseudo-diauxic effect on growth of *B. subtilis*. The growth pattern in DES 3 (110-219 mM) specifically was a gradual exponential rise in biomass up till the 7-12 h where there was a pseudo-diauxic switch point to another short lag phase, subsequently resulting in an extended log phase and overall increased growth (Figure 4C). Gly accumulation by bacterial cells is concentration dependent, and at values higher than 0.05 mM, it can trigger bacteriostatic processes due to intracellular saturation of the compound [41].

The high cell growth over extended time suggests a synergistic effect of the two DES3 components (Gly and ChCl). As the nutrients in the medium are consumed over time, the osmolarity decreases, leading to increased turgor pressure on bacterial cells, subsequently reducing cell replication, and potentially increasing cell death [42]. Bacteria have devised a number of mechanisms with which they can balance osmolarity and evade osmotic shock [42]. Bacteria can balance osmolarity through the synthesis of compatible solutes like proline and glycine betaine, which act as fine osmoregulatory [43,44]. ChCl has been identified as the precursor of glycine betaine, a compatible solute used by *B. subtilis* to regulate cytoplasmic osmolarity [45]. In this regard, it is likely that as nutrients in NB are gradually used up, osmolarity builds up outside the cell causing decreased growths of *B. subtilis*, necessitating an intracellular osmoregulatory mechanism for cell survival.

Gly functions both as a humectant and a carbon source. Its humectant property slows down the exit of water molecules from the cells. As a carbon source, Gly is transported into the cell through facilitated diffusion, though its utilization in the presence of other more desirable carbon sources like glucose is blocked by the phosphoenolpyruvate phosphotransferase system [46]. Primary nutrient depletion in the medium causes the cells to gradually accumulate and convert Gly (as a secondary carbon source) into metabolic products for ATP generation [47]. ATP generated via this process could be utilised by specific ATP binding cassette (ABC) transporters responsible for choline

absorption as they uptake choline into the cytoplasm, thereby balancing cellular osmolarity [45].

B. subtilis cells use cytochromes and NAD^+/NADH pathways for EET [17], and flavin involvement as redox mediator has been suggested [48]. The potentials of DESs to modulate membrane or increase mediator-based electroactivity has not been explored yet. DPV results show the involvement of metabolites from ChCl degradation in EET, while Gly increases the specific electroactivity of *B. subtilis* biofilm. The latter is consistent with previous work showing enhanced EET in the presence of small Gly concentration [33]. However, the effect of ChCl on biofilm electroactivity has not been previously investigated. Glycine betaine is a product of choline metabolization, and can increase the concentration and activity of cytochrome oxidase, especially under stress conditions [49], leading to higher electroactivity. Glycine betaine is also an osmoprotectant and regulates membrane ionic flow in *B. subtilis* [44]. This could contribute to the observed electroactivity. Nicotinamide Adenine Dinucleotide (NAD) [17] has been proven as a signalling molecule for EET in *B. subtilis*, while acetylcholine [50,51] has been hypothesised to have similar role in other species. These signalling molecules can be extracellularly produced under choline metabolization. However, further work is needed to validate this process. The charge output enhancement observed is likely due to changes in the EET as the *B. subtilis* biofilms adapt to osmotic fluctuations in the environment.

5. Conclusion

The DES mixture choline chloride/glycerol (1:2 mol mol^{-1}) increased planktonic growth of *B. subtilis* in NB without changing its pattern at low concentration (55 mM), whereas, a pseudo-diauxic growth is observed at higher concentration (>110 mM). This was consistent with the metabolization of choline chloride when added alone at 18 and 36 mM, respectively. Glycerol alone neither significantly affected the cell growth over 48 h, nor change the pattern of the growth curve. A switching point in the growth curves was observed when DES and choline chloride were used as secondary carbon sources. This supports the hypothesis that choline chloride could either act as a substitute nutrient or trigger transcription of regulators affecting cell growth. The DES mixture choline chloride/glycerol (1:2 mol mol^{-1}) at varied concentrations had negligible effect biofilm concentration, however independent addition of choline chloride at concentrations >36

mM strongly increased biofilm biomass. Similarly, glycerol had a small effect on biofilm biomass in NB but increased the biofilm biomass at concentration >364 mM in CDM. Overall, choline chloride promotes both growth and attachment of *B. subtilis* under the tested electrochemical conditions. Charge output results show that electroactivity of *B. subtilis* is enhanced in presence of the DES mixture choline chloride/glycerol (1:2 mol mol^{-1}) in the concentration range 55-547mM. The highest charge output was observed upon addition of choline chloride at 36 and 73 mM. The charge output after addition of choline chloride was even higher than that of 2-HNQ, suggesting a specific role of ChCl in the EET process. Overall, DES like choline chloride/glycerol (1:2 mol mol^{-1}) can be added at sub-toxic concentrations to growth medium to boost the electroactivity of the weak electricigen *B. subtilis*. While the observed effect is related to DES components rather than its solvent properties, it is likely that non-toxic DES formulations can be used at higher concentrations to facilitate EET between biofilm and electrodes.

Authors contribution

Neda Eghtesadi: Investigation, Data curation. **Kayode Olaifa**: Data curation and Formal analyses. **Obinna Aiunwa**: Conceptualization, Methodology, Writing - Original Draft, Writing - Review & Editing. **Enrico Marsili**: Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Filippo Perna**: Methodology, Writing - Review & Editing.
Vito Capriati: Methodology, Writing - Review & Editing **Massimo Trotta**: Methodology, Writing - Review & Editing.

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Conflict of interests

There are no conflicts of interest to declare.

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Table 1: Composition and properties of the DES tested in this study.

#	C1	C2	Molar ratio C1:C2	Density
1	Choline chloride	Urea	1:2	1.19
2	Choline chloride	Lactic acid	1:2	1.17
3	Choline chloride	Glycerol	1:2	1.18

Figure 1: Growth curves of *B. subtilis* in NB and CDM. The complex nitrogen source in NB was more bioavailable than NH_4Cl in CDM, thus resulting in rapid microbial growth.

Figure 2: Growth curves of *B. subtilis* in NB at two different concentrations of the three DESs: ChCl/U (1:2) (red trace), ChCl/LA (1:2) (blue trace), ChCl/Gly (1:2) (green trace). The control experiment in unmodified NB is also shown (black trace). DES3 shows the highest effect on growth over 48 h, followed by DES1. DES2 inhibits growth at both concentrations tested.

Figure 3: Growth curves of *B. subtilis* in CDM modified with the three DES tested. DES1 shows the highest effect on growth.

Figure 4: Growth pattern of *B. subtilis* at different mass concentrations of ChCl (A), Gly (B), and their mixture DES3 (Gly/ChCl 2:1 mol mol^{-1}) (C) for 48 h. Gly has small effect on growth but increases growth when mixed with ChCl. Control experiments with 50 μM 2-HNQ do not affect cell growth (D).

Figure 5: Biofilm concentration at different DES3 concentrations and concentrations of its components in NB and CDM. ChCl increases biofilm concentration 50% in NB and nearly 500% in CDM.

Figure 6: Charge outputs for *B. subtilis* after 48 h growth in NB modified with DES3 and its separate components.

Figure 7: chronoamperometric traces for different concentrations of DES3 and HNQ mediator

Figure 8: Charge output of *B. subtilis* grown in NB supplemented with DES3 and its components (Gly and ChCl) mixed to NB without heating at the same concentration. No significant difference of charge output was observed.

Figure 9: Selected DPV curves of *B. subtilis* after 48 h of growth at 0.4 V. Curves have been translated for readability.

Figure 10: Charge output of *B. subtilis* grown in DES3 and NB on electrodes maintained at 0.4 and 0 V for 48 h. Negligible charge output was detected at 0 V.

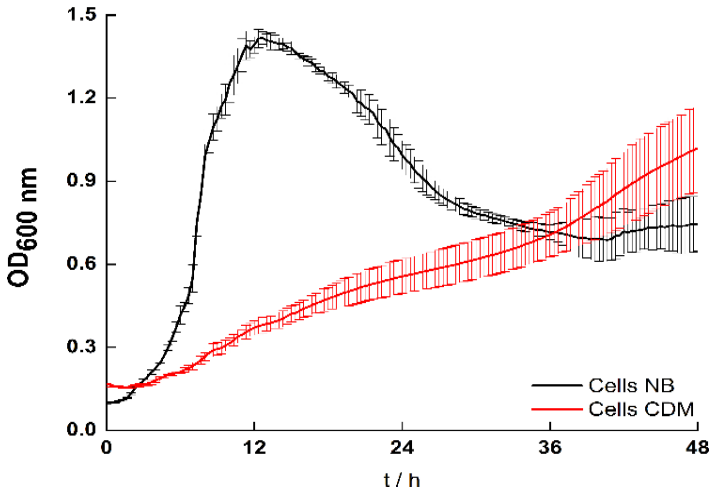


Figure 1

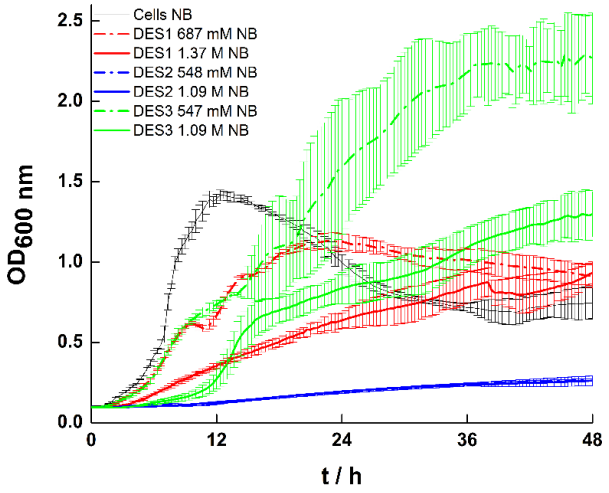


Figure 2

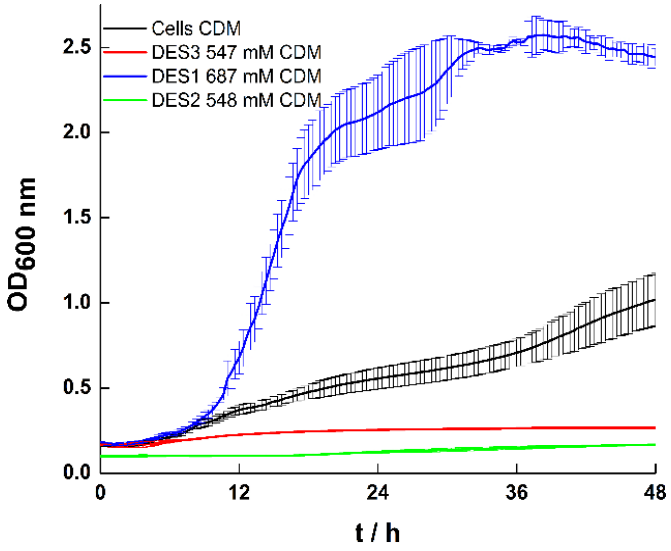
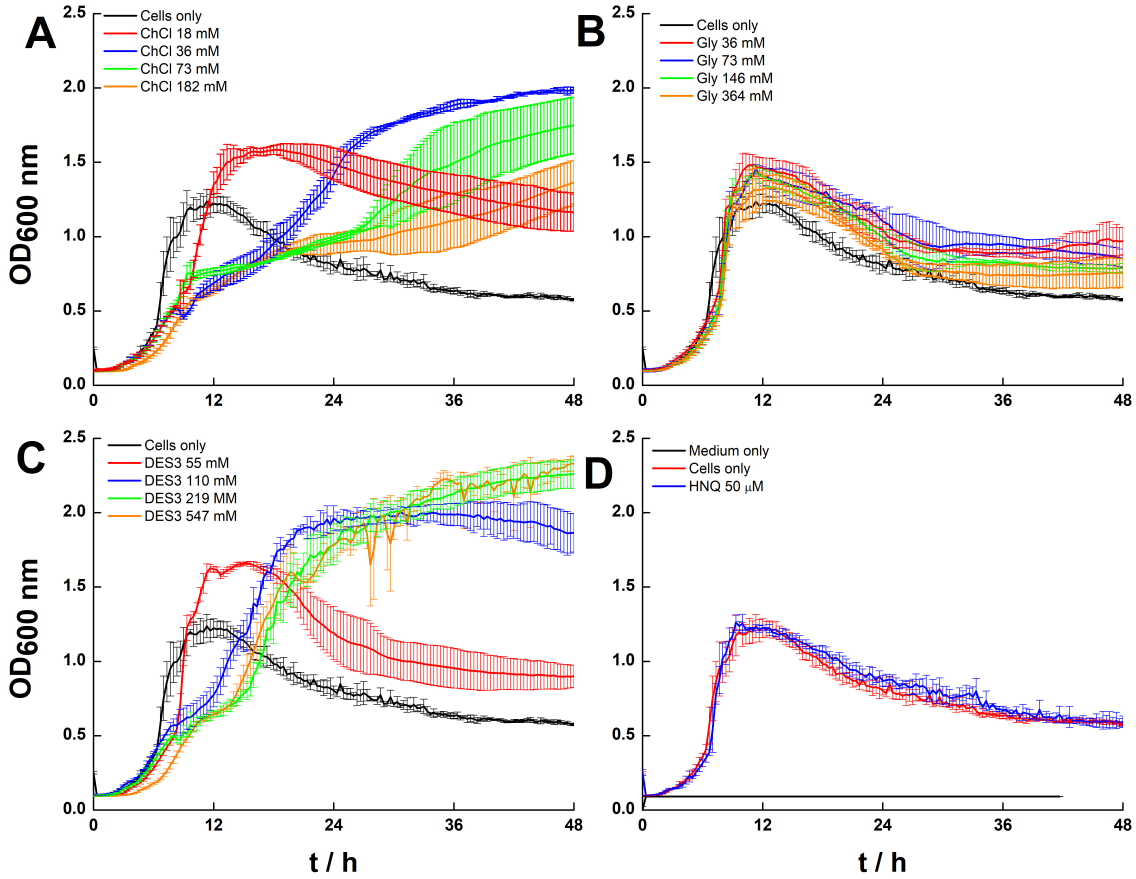


Figure 3



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638 **Figure 4**

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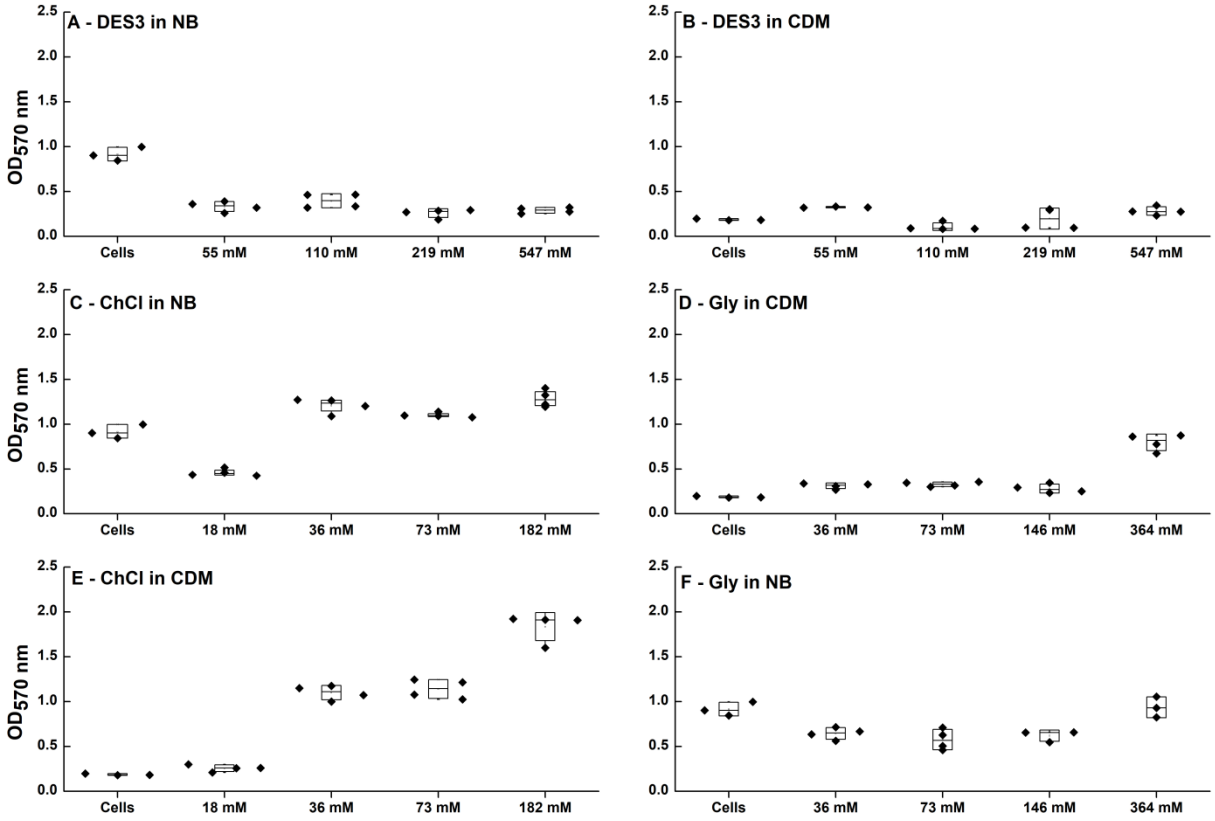

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643 **Figure 5**

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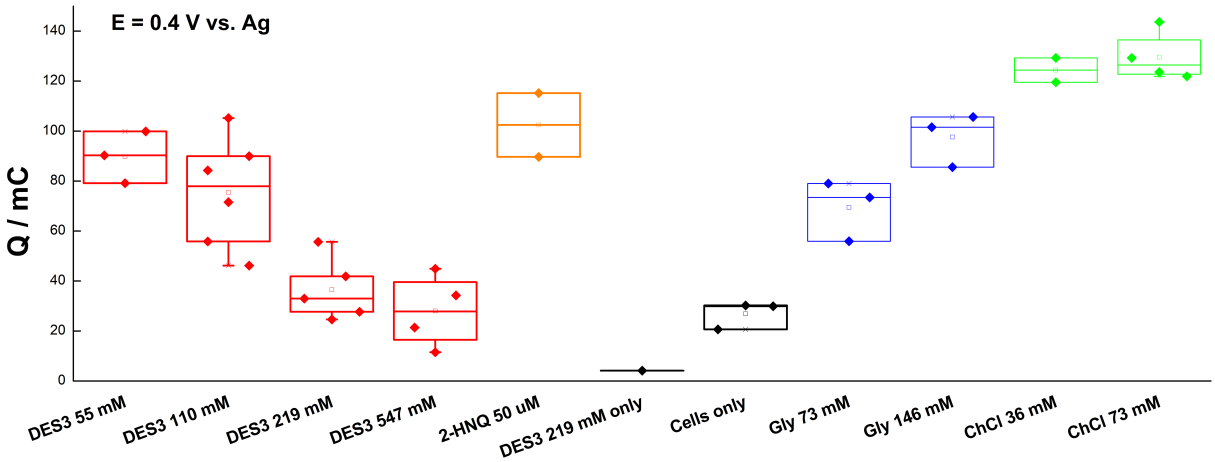

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647 **Figure 6**

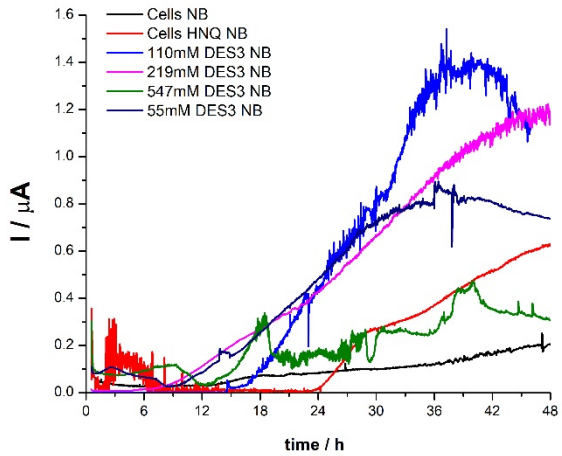

Figure 7

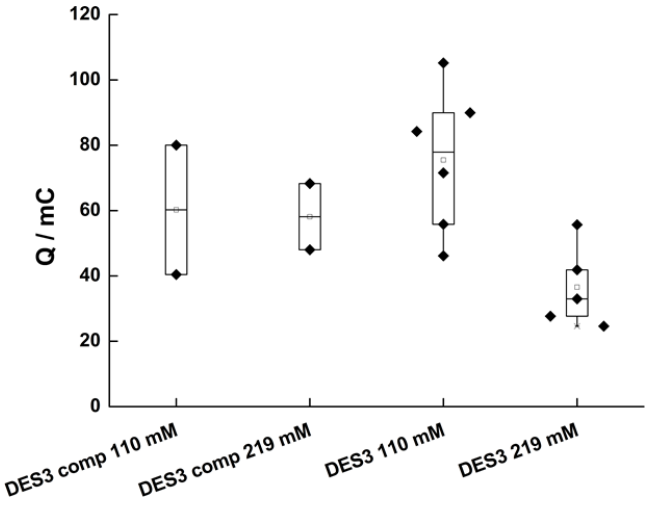

Figure 8

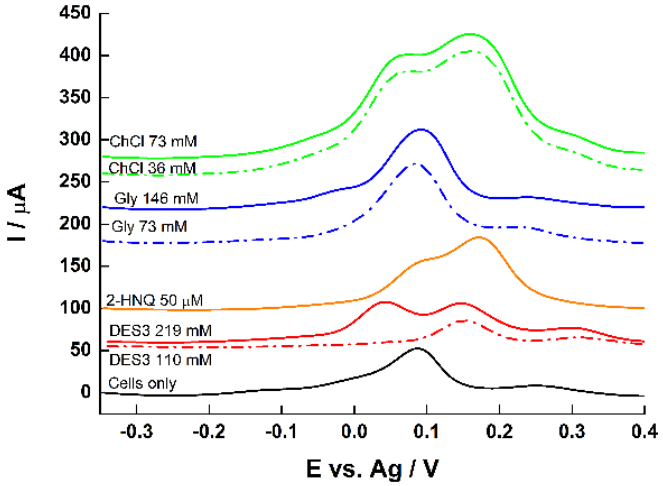

Figure 9

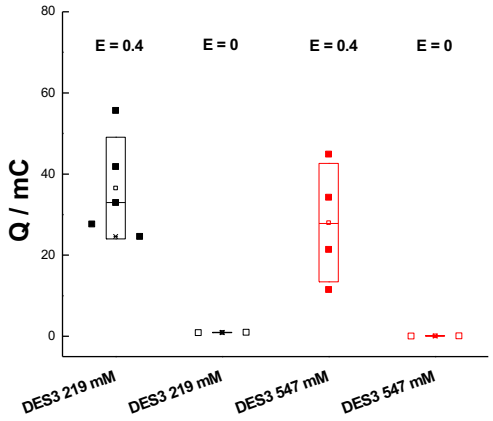

Figure 10