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Osmoregulation by choline-based deep eutectic solvent induces electroactivity in *Bacillus subtilis* biofilms

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ABSTRACT

Gram-positive *Bacillus subtilis* is a model organism for the biotechnology industry and has recently been characterized as weakly electroactive in both planktonic cultures and biofilms. Increasing the extracellular electron transfer (EET) rate in *B. subtilis* biofilms will help to develop an efficient microbial electrochemical technology (MET) and improve the bioproduction of high-value metabolites under electrofermentative conditions. In our previous work, we have shown that the addition of compatible solute precursors such as choline chloride (ChCl) to the growth medium formulation increases current output and biofilm formation in *B. subtilis*. In this work, we utilized a low-carbon tryptone yeast extract medium with added salts to further expose *B. subtilis* to salt stress and observe the osmoregulatory and/or nutritional effects of a D-sorbitol/choline chloride (ChCl) (1:1 mol mol^{-1}) deep eutectic solvents (DESs) on the electroactivity of the formed biofilm. The results show that ChCl and D-sorbitol alleviate the osmotic stress induced by the addition of NaH_2PO_4 and KH_2PO_4 salts and boost biofilm production. This is probably due to the osmoprotective effect of ChCl, a precursor of the osmoprotectant glycine betaine, and the induction of electroactive exopolymeric substances within the *B. subtilis* biofilm. Since high ionic strength media are commonly used in microbial biotechnology, the combination of ChCl-containing DESs and salt stress could enhance biofilm-based electrofermentation processes that bring significant benefits for biotechnological applications.

1. Introduction

Microorganisms known as electricigens are capable of transferring electrons to solid electrodes or minerals via extracellular electron transfer (EET). EET can occur via two mechanisms: (i) a direct pathway, which uses protein-based structures, attached or embedded in the cell membrane, such as bacterial nanowires, pili, filaments, and multiheme cytochromes, or (ii) an indirect pathway, involving redox mediators that are either soluble or membrane-bound [1–4]. Electricigens are employed in microbial electrochemical technology (MET) for energy recovery and bioelectrosynthesis [5,6]. MET aims to manipulate the metabolism of electricigens through the application of electrochemical potential or current at electrodes, which serves as electron acceptors or donors, thereby enhancing the production of platform chemicals [7], active pharmaceutical ingredients [8], and biopolymers [9], among others.

B. subtilis is a Gram-positive bacterium and a model organism in the bioprocess industry due to its ability to produce extracellular enzymes, proteins, and secondary metabolites. The ability to grow in harsh environments and its potential to secrete large amounts of proteins, make *B. subtilis* an excellent cell factory for drug discovery, food processing, agricultural and pharmaceutical production [10,11]. Recent experiments in potentiostat-controlled electrochemical cells have shown that *B. subtilis* exhibits weak electricigen properties, producing a low current output when exposed to an oxidizing potential [12,13]. In fact, *B. subtilis* is known to produce outer membrane cytochromes and

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membrane-bound flavins, both of which are redox-active agents involved in EET [14,15].

The growth of *B. subtilis* at a certain electrochemical potential can increase the yield of high-value metabolites, such as alkaline proteases, biopolymers, biosurfactants, and antimicrobial peptides [16–18]. However, proximity between the microorganisms and the electrodes is required to increase the EET rate and achieve efficient MET processes [19]. Therefore, immobilized microorganisms should be used [20]. Cell immobilization can be achieved with a coating, in which viable microorganisms are embedded in a biocompatible and conductive polymeric matrix [21]. While this method allows controlling the coating thickness and the concentration of microorganisms [22], it requires careful optimization and is susceptible to biodegradation of the polymer [23], resulting in lower activity and mechanical detachment of the coating [24]. On the other hand, immobilization can be achieved by the formation of biofilm on the electrodes. Biofilm formation is a spontaneous process that can be enhanced by modifying the media composition, nutrient concentration, and temperature [25]. However, biofilms are heterogeneous and dynamic systems, making it difficult to achieve repeatable coating thickness and performance over time. In biofilms, cells are encased in self-secreted extracellular polymeric substance (EPS) [24], which plays a pivotal role in the EET mechanism [22]. The EPS shields cells from their environment and serves as a carbon and energy source for the biofilm. In addition, the components of the EPS matrix, such as polysaccharides, proteins, nucleic acids, and lipids, can interact with redox-active substances and be involved in stabilizing redox protein complexes, which may then act as electron conduits through the biofilm [25]. Under flow conditions and in continuous processes, biofilms are subject to localized dispersal in the presence of specific physicochemical stress [26]. Previous studies have determined the effects of pollutants at sub-inhibitory concentrations, such as heavy metals and organic compounds, on biofilm formation and dispersal [27,28].

Stimulation of biofilm production is expected to increase the EET rate and consequently the biosynthesis rate of high-value metabolites in electrofermentation (EF) processes [19,20]. The EET rate has been improved by various strategies including physical and chemical pretreatment of the inoculum [29], supplementation of the growth medium with specific nutrients, media optimization, and biofilm engineering [29–31]. The EET rate can also be improved also by increasing the conductivity of the growth medium, for example through the addition of salts. However, salts impair the membrane potential of microorganisms, resulting in lower metabolic activity [32]. Therefore, novel media formulations that protect biofilms from osmotic stress should be investigated for the purpose of EET improvement.

In our previous work [12], we had enhanced the EET in *B. subtilis* using deep eutectic solvents (DESs). DESs are mixtures of two or more pure compounds (e.g., Lewis or Brønsted acids and bases, anionic and/or cationic species) in a well-defined stoichiometric proportion, with a melting point far below that of an ideal liquid mixture [33]. DESs are investigated as substitutes for toxic and volatile organic compounds (VOCs) in bioprocesses [34], such as protein crystallization, photosynthesis reactions [35] and bioelectrochemistry [12,36]. The high biodegradability, non-toxicity, low cost, low volatility and eco-friendliness, make DESs attractive candidates to replace conventional fossil-based volatile organic compounds.

Using a DES with the formula choline chloride (ChCl): glycerol (Gly) (1:2 mol mol^{-1}), we previously observed an increase in the electroactivity of *B. subtilis* biofilms when grown in the presence of low DES concentrations [12]. This occurred simultaneously with biofilm enhancement, and we hypothesized that this was most likely due to the osmoprotectant effect of ChCl. This hypothesis was based on the role of ChCl as a precursor of glycine betaine, a compatible osmolyte that contributes to osmoregulation and improves electroactivity by regulating ionic flow and inducing transmembrane potential [37–39]. However, the osmoprotective effect of ChCl-based DESs and/or glycine betaine should be assessed when the bacteria are grown under ionic stress conditions.

Since the formation of biofilms can be influenced by environmental salinity, exopolymeric substances secreted within the biofilm could be accelerated under salt-stress conditions as a protective mechanism for bacterial cells. Glycine-betaine synthesis, which is among the initial responses of *B. subtilis* to osmotic stress at high salinity, could regulate the ionic membrane flow of *B. subtilis* and increase its cytochrome oxidase activity, leading to increased electroactivity [38,40–42]. In furtherance to our previous work, where we had observed that low concentrations (<10 % wt/wt) of ChCl DES increased electroactivity [12], in this work, we attempted to intentionally induce increased salt stress on the bacteria and observe the electroactivity responses in terms of assisted osmoregulation. We challenged the bacteria with additional salt stress and determined the counteractive osmoregulatory effects induced by the addition of a ChCl-containing DES, namely D-sorbitol/ChCl (1:1 mol mol^{-1}). We hypothesized that this might attenuate the osmotic effect of additional salt stress, while increasing biofilm formation and electroactivity of *B. subtilis*. The results show that both DES and their individual components promote biofilm formation. Furthermore, in the presence of the salts sodium dihydrogen phosphate (NaH_2PO_4) and potassium dihydrogen phosphate (KH_2PO_4) in subinhibitory concentrations, the DES components mitigate osmotic stress. These findings could contribute to the development of more effective MET processes for the production of high value metabolites in *B. subtilis* produced under osmotic stress.

2. Materials and methods

2.1. Materials

A chemically defined TY medium (tryptone 20 g L^{-1}, yeast extract 6.7 g L^{-1}) with pH adjusted to 6.5 was used as prepared and modified with a biocompatible DES (D-sorbitol/ChCl (1:1 mol mol^{-1}), its individual components (ChCl and D-sorbitol), two inorganic salts monosodium phosphate and monopotassium phosphate (NaH_2PO_4, KH_2PO_4) and a compatible solute (betaine anhydrous $(CH_3)_3NCH_2CO_2$)). Since natural uptake of choline by *B. subtilis* from the environment is possible, choline is oxidized to betaine, which is considered here for its its osmoprotective property. All media were prepared with deionized water before sterilization at 121 °C and 104 kPa for 20 min.

All chemicals were of analytical grade and were purchased from Sigma Aldrich and Thermo Fisher Scientific, Kazakhstan, and used according to the manufacturers instructions.

The bacterial strain *B. subtilis* ATCC 6051 (kindly provided by Prof Cinzia Calvio, University of Pavia, Italy) was subcultured and maintained on TY at 37 °C throughout the experiments.

2.2. Bacterial growth curves

The aerobic growth curves of *B. subtilis* at different concentrations of ChCl, D-sorbitol, their eutectic mixture (DES) and their combination in the presence of different concentrations of NaH_2PO_4 and KH_2PO_4 salts in TY medium were determined in 48-flat-bottom well plates using a Gen5TM microplate reader and Imager Software (BioTek Instruments).

Optical density was measured at 570 nm (OD_{570}) and experiments were conducted in four replicates (i.e., four wells), and values reported as mean ± standard deviation (SD). The concentration of ChCl, D-sorbitol, DES and their combination ranged from 5 mM to 6210 mM. The combination of DES components was prepared by adding the equivalent mass concentration of DES components to the growth medium. In each experiment, the added components were first dissolved in the prepared TY medium, and then sterilized using a 0.2 μm filter. Each well of the 48-well plate was filled with 1 mL of TY medium.

The incubation temperature and time were 37 °C and 48 h, respectively. For the inocula, 10 mL of a fresh overnight culture was grown in sterile Falcons tubes with a volume of 50 mL for 16 h at 37 °C under

constant agitation (180 rpm), and the resulting bacterial suspension was used as the inoculum. The wells were inoculated with an $OD_{570} = 0.1$, which had previously been determined to be approximately 10^6 colony forming units (CFU) per mL.

2.3. Biofilm assay

The crystal violet assay was used for the semi-quantitative detection of biofilms formed in the wells of the microtiter plate at the end of the growth curve determination described in the section on bacterial growth curves.

Following the 48-h incubation period, the medium and unattached cells were gently decanted from the microtiter plate without disrupting the biofilm matrix, afterward, the plate was submerged in a small tub of water to remove any remaining non-adherent cells and medium components. Subsequently, the microtiter plate was allowed to air dry for 20 min at room temperature until completely dry and devoid of dust or other extraneous materials.

Subsequently, 1 mL of a 0.1 % crystal violet solution (Crystal Violet, ACROS organics) was added to each well, and the microtiter plate was incubated at 25 °C for 15 min. Subsequently, 1000 μL of 33 % acetic acid in water was added to each well of the microtiter plate and incubated at 25 °C for 15 min to solubilize the biofilm-bound crystal violet dye. Subsequently, the solubilized crystal violet was transferred to a new microtiter plate, and the OD_{570} was measured using the microplate reader. Four replicates (i.e., four wells) were analyzed for each experimental condition and the values expressed as mean ± SD.

2.4. Microscopy

At the end of the bioelectrochemical experiment, the biofilms formed on the SPEs were observed using an Axio Zoom V16 microscope (Carl Zeiss). The electrodes were removed from the electrochemical cells and immersed in a 0.1 % wt. crystal violet solution. for 1 h. Subsequently, the SPEs were immersed in deionized water to remove the excess crystal violet and air-dried. The stained biofilm was then observed with excitation (590 nm) and emission (635 nm) filters and 25x magnification. The biofilm coverage on the SPE surface was later quantified using a MATLAB code developed by our group.

2.5. Enrichment and determination of riboflavin in the supernatant

Riboflavin concentration in the supernatant from the bioelectrochemical experiments was determined. Cell-free supernatants were collected, sterile-filtered, and then injected into the HPLC for quantification in comparison to the riboflavin standard. Riboflavin concentration was also assayed by collecting samples from identified peaks at 3.7 min and assayed using UV–visible spectrophotometry [43]. Four independent biological replicates (i.e., four electrochemical cells) were analyzed for each experimental condition.

2.6. Bioelectrochemical analysis

Screen-printed Carbon Electrodes (SPE) with a diameter of 4 mm and 0.126 cm^2 surface carbon working electrode (WE), carbon counter electrodes (CE) and Ag pseudo-reference electrode (Metrohm-Dropsens SPE Ref. C110) were used in all electrochemical experiments. All the potentials in the following are reported as E (volts) vs. Ag pseudo-reference electrode. Polymethacrylate electrochemical cells of 10 mL volume with 8 mL working volume were used in all the electrochemical experiments.

Chronoamperometry (CA) and differential pulse voltammetry (DPV) were performed sequentially to study the electrochemical activity of *B. subtilis* using a VSP multichannel potentiostat (Bio-Logic, France). The SPEs were surface sterilized in 70 % v/v ethanol, washed twice in sterile deionized water, and air-dried in a sterile Petri dish in a fume hood before use. The electrochemical cells were kept at 37 °C in a dry steel bead bath throughout the experiments. For the CA, the WE was set at 0.4 V for 48 h. For 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. The cumulative charge output (mC) for each experiment was also calculated through the integration of the current output with time using the EC Lab® software (Bio-logic, France).

2.7. Statistics

The number of independent biological replicates N is indicated in each figure. Statistical significance for each treatment and for the different experimental conditions was determined using one-way ANOVA and Tukey's post hoc test. The normality of the data distribution was checked using the Prosme normality and lognormality test before the post hoc tests. The data were presented as mean ± SD. All analyses were performed at a 0.05 level of significance. Data were analyzed using Prism and Origin 8.5 software.

3. Results and discussion

3.1. Planktonic growth

The addition of D-sorbitol/ChCl (1:1 mol mol^{-1}) DES increased the maximum growth and prolonged the growth phase of planktonic cells when concentrations in the range of 20–200 mM were used. However, the highest effects on growth were observed at both 40 mM and 80 mM, as the addition of DES at these concentrations resulted in continuous growth over 24 h. However, in Fig. 1A, a comparison between the of 40 mM and 80 mM concentrations showed that 40 mM resulted in the highest individual $OD_{570\ nm}$ values. For this reason, 40 mM was used for further analysis.

At lower concentrations (< 20 mM), the effect was not visible, while at higher concentration (>200 mM), the DES had a toxic effect on planktonic growth (Fig. 1A). *B. subtilis* can rapidly metabolize sorbitol using sorbitol dehydrogenase (SDH), which converts sorbitol to fructose using NAD^+ as a coenzyme in cellular metabolism. Since DES contains both a carbon source (D-sorbitol) and a precursor of osmoprotective agent (ChCl) [40], experiments were performed with the individual DES components at the same concentrations to deconvolute their effect on planktonic growth (Fig. 1B–C).

From the growth curves, it can be deduced that DES at a concentration of 20–200 mM promotes growth especially during the transition from exponential to stationary phase by prolonging the onset of stationary phase. This effect was not observed when individual components of the DES were used (Fig. 1B, 1C), nor when equivalent mixtures of the DES components were used as a non-eutectic mixture (Fig. 1D), as there was an immediate decrease in growth and a more rapid transition to stationary phases. This indicates a specific advantage of DES for bacterial growth over simple equivalent liquid mixtures of the same individual components.

The addition of ChCl to the TY medium slightly increased maximum growth and delayed senescence (Fig. 1B). Tryptone is a carbohydrate-deficient nutrient source, and the addition of ChCl or ChCl-containing agents to the medium can compensate for the lack of carbohydrates in this medium while enhancing the carbohydrate metabolism. The latter in turn leads to further growth. Interestingly, there was no significant difference in the maximum OD_{570} at all the concentrations tested up to 200 mM. A prolonged stationary phase and a decrease in the maximum OD_{570} were observed at ChCl concentrations ≥ 200 mM. The ChCl results obtained here are slightly at variance with the results of ChCl-induced growth in our previous work [12]. There were slight differences in the growth pattern of ChCl induced *B. subtilis* cells at similar ChCl concentrations used in both papers. We attribute this difference in growth response to the different media used. Here we used a tryptone-yeast extract medium, whereas in the previous work we used

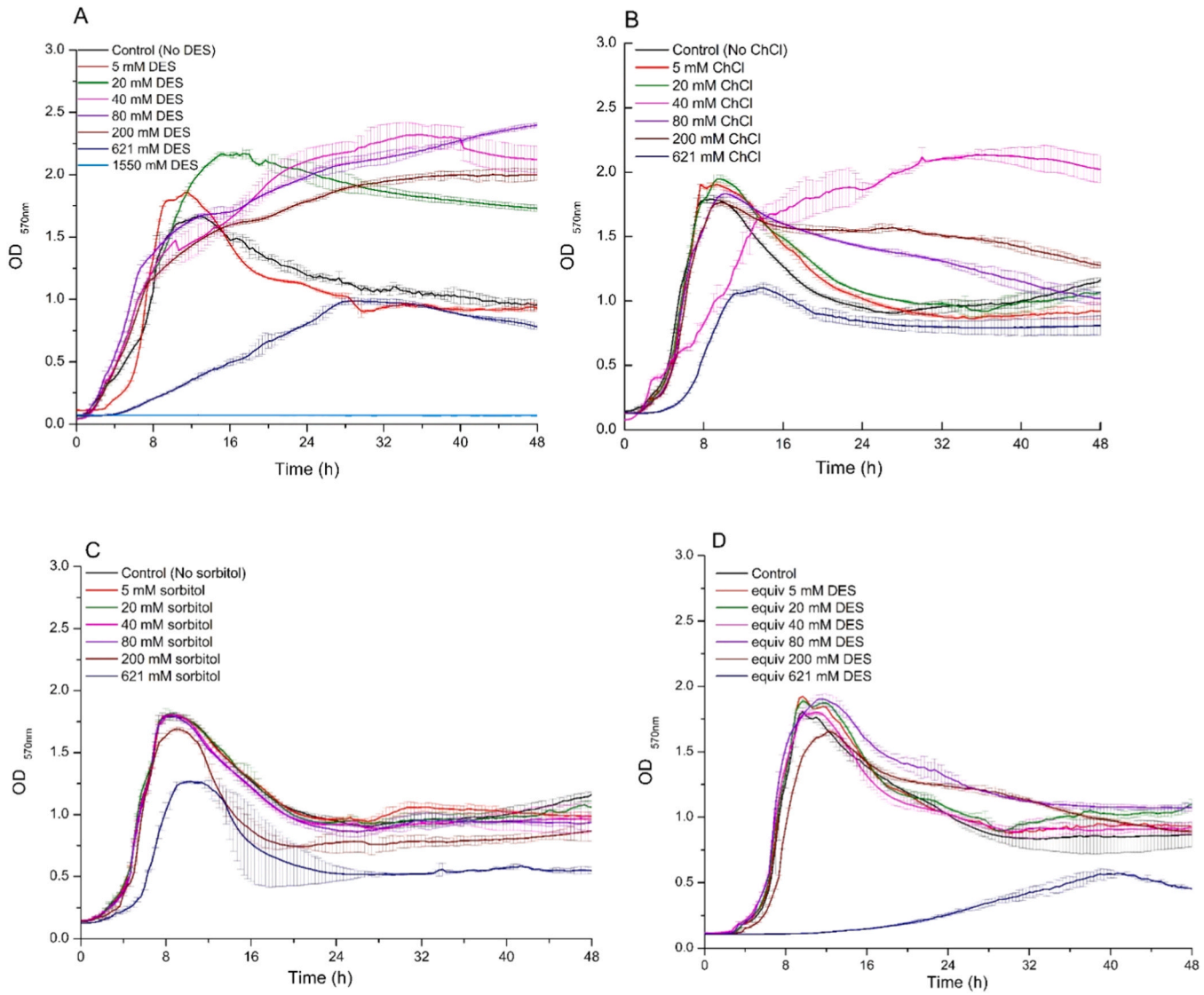

Fig. 1. Planktonic growth of *B. subtilis* over 48 h at different molar concentrations of A) DES (D-sorbitol/ChCl 1:1 mol mol^{-1}) B) ChCl; C) D-sorbitol; D) equivalent concentration of (D-sorbitol + ChCl). N = 4.

nutrient broth. Although both media are complex media, the specific mineral components may vary, resulting in slight differences in responses to ChCl metabolism of *B. subtilis* at similar ChCl concentrations.

The effect of D-sorbitol was negligible at concentrations < 200 mM. At higher concentrations, the maximum OD_{570} value decreased without a significant shift/change in the exponential phase of the growth curve (Fig. 1C). The addition of D-sorbitol and ChCl in the same proportions as in DES (Fig. 1D**)** did not significantly increase the growth rate and maximum growth compared to DES. These results are consistent with a synergistic effect of the components in the form of DES. A similar effect was shown by ChCl-urea DES on bacterial viability, membrane integrity, retention of metabolic activity, and the efficiency of Δ1,2-dehydrogeneration of cortisone acetate when whole cells of *Arthrobacter simplex* were used [44]. Fig. 1 includes only selected concentration for clarity. See Fig. S1 for all the concentrations tested.

B. subtilis is generally considered a halotolerant microorganism and selected strains can tolerate up to 30 % wt of salts [15,45,46]. Furthermore, the addition of a low salt concentration can serve as a source of ions that are beneficial for various cellular processes [47]. At NaH_2PO_4 or KH_2PO_4 concentrations lower than 5 % wt/wt, the maximum growth increased and mortality decreased. However, at higher concentrations, the lag phase was prolonged, and at 10 % wt/wt, planktonic growth was completely inhibited (Fig. 2A–B). The effects of sub-toxic concentration of DES were tested at two selected concentrations of NaH_2PO_4 and KH_2PO_4, one below the maximum tolerance (2 % wt/wt) and one above the maximum tolerance (5 % wt/wt) to verify whether the addition of DES can increase the growth of *B. subtilis*. The results show that the addition of 40 mM DES triggers a second growth phase at 11–12 h and delays the onset of death phase at 2 % wt/wt concentration for both salts, while it has no significant effect at a salt concentration of 5 % wt/wt (Fig. 2C–D). This is consistent with the osmoprotective effect of ChCl contained in the DES.

3.2. Biofilm production

Biofilm formation increased with increasing DES concentration in the tested range (5–100 mM) (Fig. 3A). Interestingly, the addition of ChCl had no effect biofilm formation (Fig. 3B), while D-sorbitol had a positive effect on biofilm formation only at concentrations below 60 mM, with the concentration of 10 mM showing the highest optical density value (Fig. 3C**)**. Higher concentrations of D-sorbitol (≥100 mM) likely lead to increased osmolarity and a decrease in water activity (a_w) of the nutrient medium [48,49].

These results are consistent with the effect of DES on planktonic

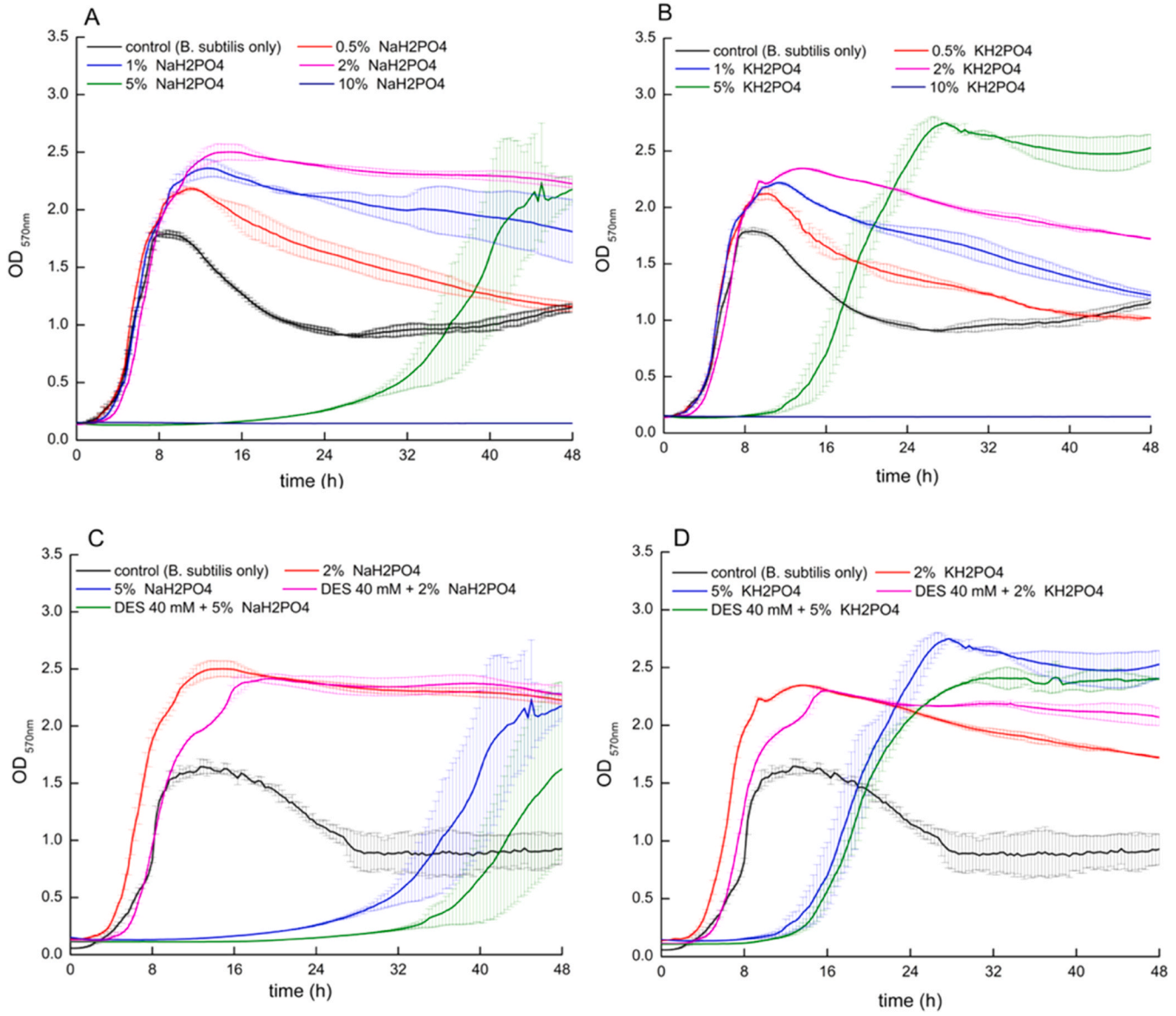

Fig. 2. Planktonic growth of *B. subtilis* in the presence of A) increasing concentrations of NaH_2PO_4 (wt/wt); B) increasing concentrations of KH_2PO_4 (wt/wt); C) 2 and 5 % wt/wt NaH_2PO_4 with 40 mM DES; D) 2 and 5 % wt/wt KH_2PO_4 with 40 mM DES. N = 4.

growth and demonstrate a synergy between D-sorbitol and ChCl in promoting biofilm formation. However, while ChCl promotes planktonic growth, it has no significant effect on biofilms. This could be because planktonic cells are more susceptible to osmotic variations that can arise even in the medium without salt added, in comparison with protected cells within a biofilm. The osmoprotection induced by ChCl mostly influences single cells exposed to osmotic stress. In fact, both the cell size caused by water accumulation and membrane flow of ions are controlled by the activity of osmolytes [50]. Since cell size is related to cell volume, similarities could be observed when measuring optical density. On the other hand, D-sorbitol increases biofilm formation while it has little or no effect on planktonic growth. D-sorbitol can be used as an additional carbon source in biofilm production. After absorption, D-sorbitol is rapidly phosphorylated into sorbitol-6-phosphate by a phosphopyruvate-dependent phosphotransferase (PTS) sorbitol system. This converted sorbitol can then be incorporated into the sugar composition of the EPS within the biofilm, ultimately promoting biofilm production [51].

Thus, the combination of an additional carbon source (D-sorbitol) and an osmoprotectant precursor (ChCl) appears to be ideal for bioprocess applications, as it enhances both planktonic and biofilm growth [52,53]. In addition to its role as a secondary carbon source, D-sorbitol reduces the a_w of the medium due to the association of ions with water molecules [49], which could lead to the secretion of exopolysaccharides in the EPS as the main cellular response to unfavourable and stressful environmental conditions, thus contributing to the adhesion of *B. subtilis* cells to each other and to the substrate surface [49,52,53]. In addition, higher EPS production could improve the EET rate due to EPS semiconductive properties and its electrochemically active components [37].

EPS plays an important role in regulating proline-compatible solute and ions uptake. In fact, halotolerance positively correlates with cell cytoplasm activity and EPS microbial content, and EPS overproduction is the main response of the microorganism to water deficiency. The negatively charged groups in the EPS structure can bind cations and prevent the entry of positive ions through the membrane. Moreover, EPSs have a water-holding capacity due to the presence of hydroxyl groups in their structure, which holds the water around the cell and prevents its dehydration [21,54].

While biofilm formation decreases in the presence of NaH_2PO_4 and

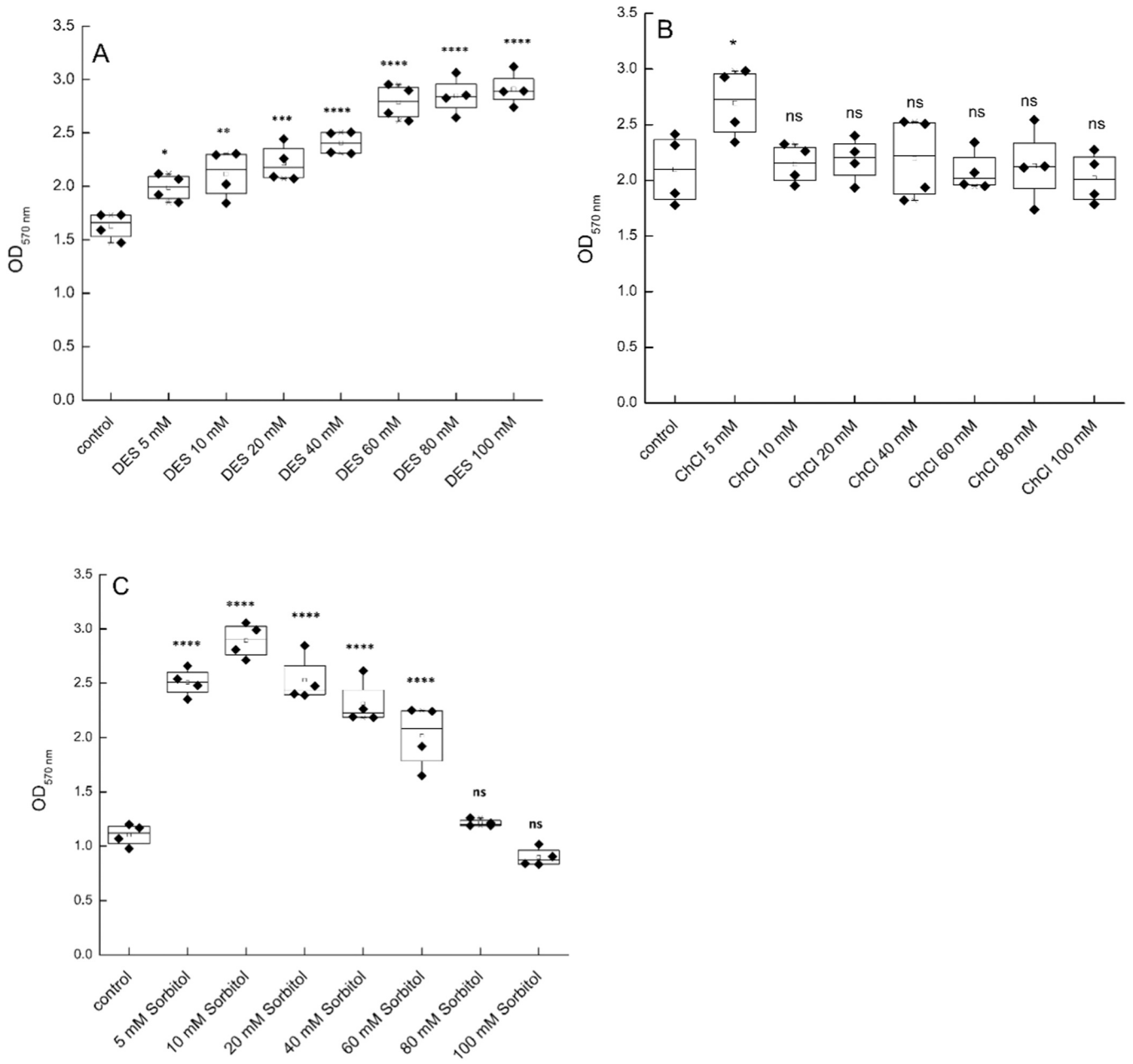

Fig. 3. Biofilm formation in the presence of increasing concentrations of A) DES; B) ChCl; C) D-sorbitol. N = 4. *, **, ***, **** indicate statistically significant difference between treated cells and control cells at $p<0.05$, $0.01<p<0.05$, $0.001<p<0.01$, and $0.0001<p<0.001$, respectively, following to Tukey's test from ANOVA.

KH_2PO_4 at a concentration higher than 1 % wt/wt (Fig. 4A), the addition of betaine alone had no effect on biofilm formation in the concentration range of 5 – 200 mM (Fig. S2). This is because betaine primarily acts as an osmoregulatory compatible solute, exhibiting its main property during instances of osmotic stress. In the current study, both the control medium and the medium with betaine addition presented similar, insignificant osmotic challenges.

This could be the reason for the observed consistency in biofilm yield during the experiments. Also, betaine mainly serves to increase cell size and ionic flow to compensate for osmotic stress by regulating turgor pressure, a feature that is more pronounced in planktonic cell growth than in biofilm growth.

It has been proven that biofilm and planktonic cells react differently to osmotic stress [55]. The effect of increasing concentrations of betaine on biofilm formation without salt challenge (Fig. S2) is similar to the effect of ChCl treatment, as shown in Fig. 3B. This suggests that osmoregulation serves to maintain the biomass density in the biofilm at a regulated level, regardless of the extracellular concentration of the compatible solute, as long as the osmotic stress remains similar. Subsequently, when salt stress was introduced under a certain concentration of betaine (40 mM), betaine stabilized the biofilm biomass with increased concentrations of 1–2 % (wt/wt) of NaH_2PO_4 and KH_2PO_4 salts. In addition, the introduction of 40 mM betaine to a medium containing 2 % KH_2PO_4 (wt/wt) resulted in an increase in biofilm formation (Fig. 4B). However, this increase was not observed with 2 % NaH_2PO_4 (wt/wt). This indicates an ion-dependent (Na vs. K ions) influence on biofilm production with betaine treatment. In contrast to ChCl, which is a precursor of betaine, betaine is a very basic, compatible solute used by most bacteria. It primarily influences the transmembrane ionic flow and potential. This influence is based on ion-specific regulatory mechanisms

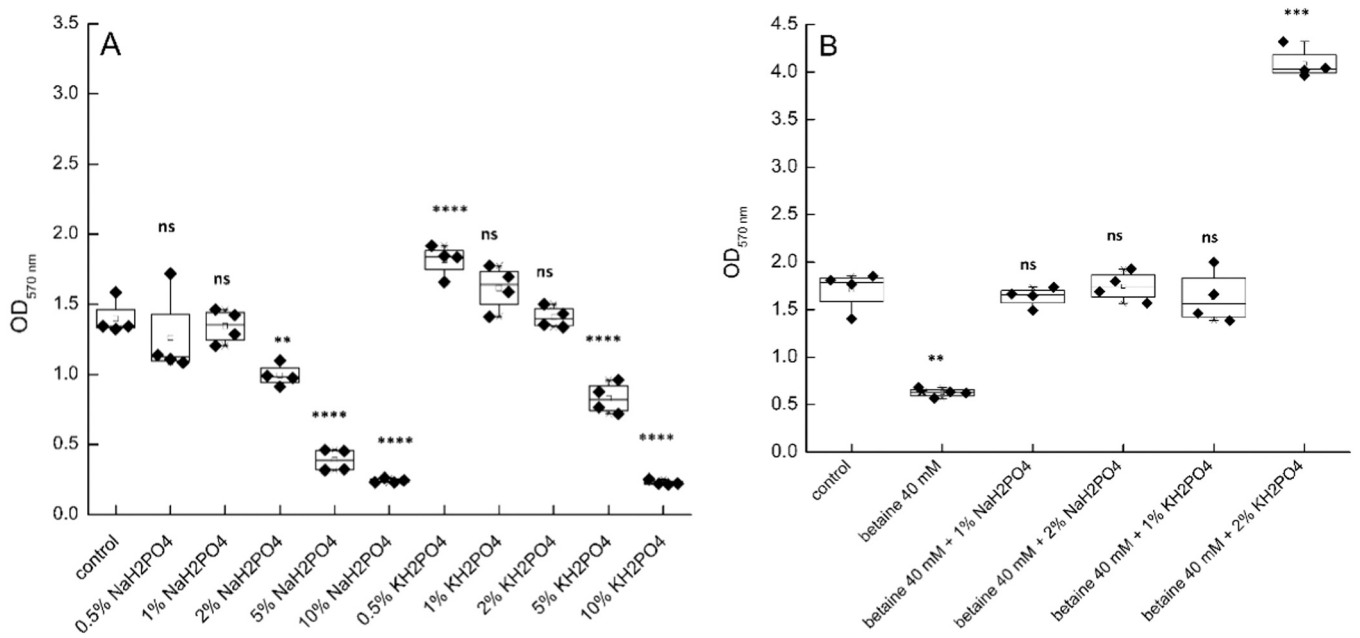

Fig. 4. A) Biofilm formation in the presence of NaH_2PO_4 and KH_2PO_4; B) the effect of betaine and salts on biofilm formation. N = 4. *, **, ***, **** indicate statistically significant difference between treated cells and control cells at $p<0.05$, $0.01<p<0.05$, $0.001<p<0.01$, and $0.0001<p<0.001$, respectively, following to Tukey's test from ANOVA.

that can alter intracellular pH. Fast acting osmoregulators such as betaine immediately maintain cell size and volume during osmotic variabilities. However, in the case of ChCl, a betaine precursor, the osmoregulatory process is a longer process, and therefore the cell may use complementary strategies, such as increasing EPS secretion to adjust the concentration of salts present in the environment [56]. In proper perspective, this means that the increased EPS production combined with increased transmembrane potentials when ChCl is used may result in increased electroactivity, which is higher than when betaine is used. When the cell is exposed to osmotic stress, there is an immediate assimilation of compatible solutes from the immediate extracellular environment. However, further experiments with other compatible solutes as osmoprotective agents are required to confirm this mitigating effect.

The effect of DES and its components at 40 mM concentration on *B. subtilis* biofilm formation in the presence and absence of the salts NaH_2PO_4 and KH_2PO_4 is different **(**Fig. 5**).** DES and ChCl moderately increase biofilm formation in the presence of the salts at 1 and 2 % (wt/wt), while the effect of sorbitol is much stronger, although it does not change between 1 % and 2 % (wt/wt) salt.

The addition of ChCl could facilitate the EET between biofilm and electrodes. This could be due to the extracellular production of NAD^+ as a signaling molecule in the context of choline metabolism in *B. subtilis*, which increases electroactivity. Moreover, ChCl acts as a precursor and inducer for glycine betaine synthesis, which contributes to osmoadaptation of *B. subtilis*. The uptake and synthesis of glycine betaine from choline in *B. subtilis* is mediated by the enzymes GbsB and GbsA [29]. The synthesis of glycine betaine under high salinity conditions could promote the release of exogenous electron shuttles such as flavins and C-type cytochromes, leading to higher electroactivity as observed in *Bacillus pumilus* biofilms [57]. Under osmotic stress conditions, salt ions movement, density, and membrane ionic flow are subject to change. The alterations are closely linked to the activity of the Na/K pumps, which could possibly also influence the electroactivity of the bacteria [58]. The initial reaction of *B. subtilis* to osmotic up-shock is potassium ion accumulation by several transporters (KtrAB and KtrCD, KimA). Although the accumulation of K^+ ions cannot be regarded as a sustained method of adaptation to high osmolarity, it can be recognized by the bacteria as a signal indicating the need for intracellular accumulation of more compatible solutes [37].

The results of the bioelectrochemical experiments at 0.4 V showed that the addition of DES and NaH_2PO_4 salts delayed the onset of the current and increased the maximum current (Fig. 6A). The highest charge output (Fig. 6B) is observed with the addition of DES 40 mM and ChCl 40 mM + NaH_2PO_4 2 % (wt/wt), respectively, which is consistent with the biofilm amount detected on the working electrode of the SPE.

Fluorescence microscopy analysis was used to visualize the biofilm formation on the WE surface area after 48 h of electrochemical experiment (Fig. S4-S5), and the calculated biofilm coverage is shown in Fig. S6. Since the fluorescence microscopy approach used here was two-dimensional, the coverage is not proportional to the biomass on the electrode and is reported here as a qualitative comparison. The biofilm coverage increases significantly with the concentration of DES and ChCl, especially in the presence of NaH_2PO_4. This is consistent with the increased biofilm production measured on SPE with crystal violet (Fig. 7) and the charge output in bioelectrochemical experiments **(**Fig. 6B), and confirms that EPS is produced to mitigate the osmotic stress.

DPV was performed to investigate the redox-active species produced in bioelectrochemical experiments (Fig. S7). After 48 h, the control biofilms (cells only) showed a main peak at 0.03 V, which moved to a higher potential (~0.25 V) after the addition of 40–100 mM ChCl **(**Fig. S7A**).** This potential shift suggests that the reactions at the biofilm/electrode interface become less reversible with the addition of ChCl. However, this was not confirmed by other experiments with the ChCl-containing DES **(**Fig. S7C**).** The addition of 1 % and 2 % KH_2PO_4 and NaH_2PO_4 (Fig. S7D and Fig. S7E**,** respectively) resulted in an increase in peak height, consistent with the increased biofilm production in the presence of the inorganic salts tested (Fig. 5). Further work is required to identify the chemical species corresponding to the DPV peaks and to understand the dynamics of these peaks under different experimental conditions and the involvement of metabolites from ChCl degradation in EET [59].

Electrical stimulation at 0.4 V was able to improve ionic flux, transmembrane potentials and electroactive EPS production at high salinity. The riboflavin concentration in selected supernatants was

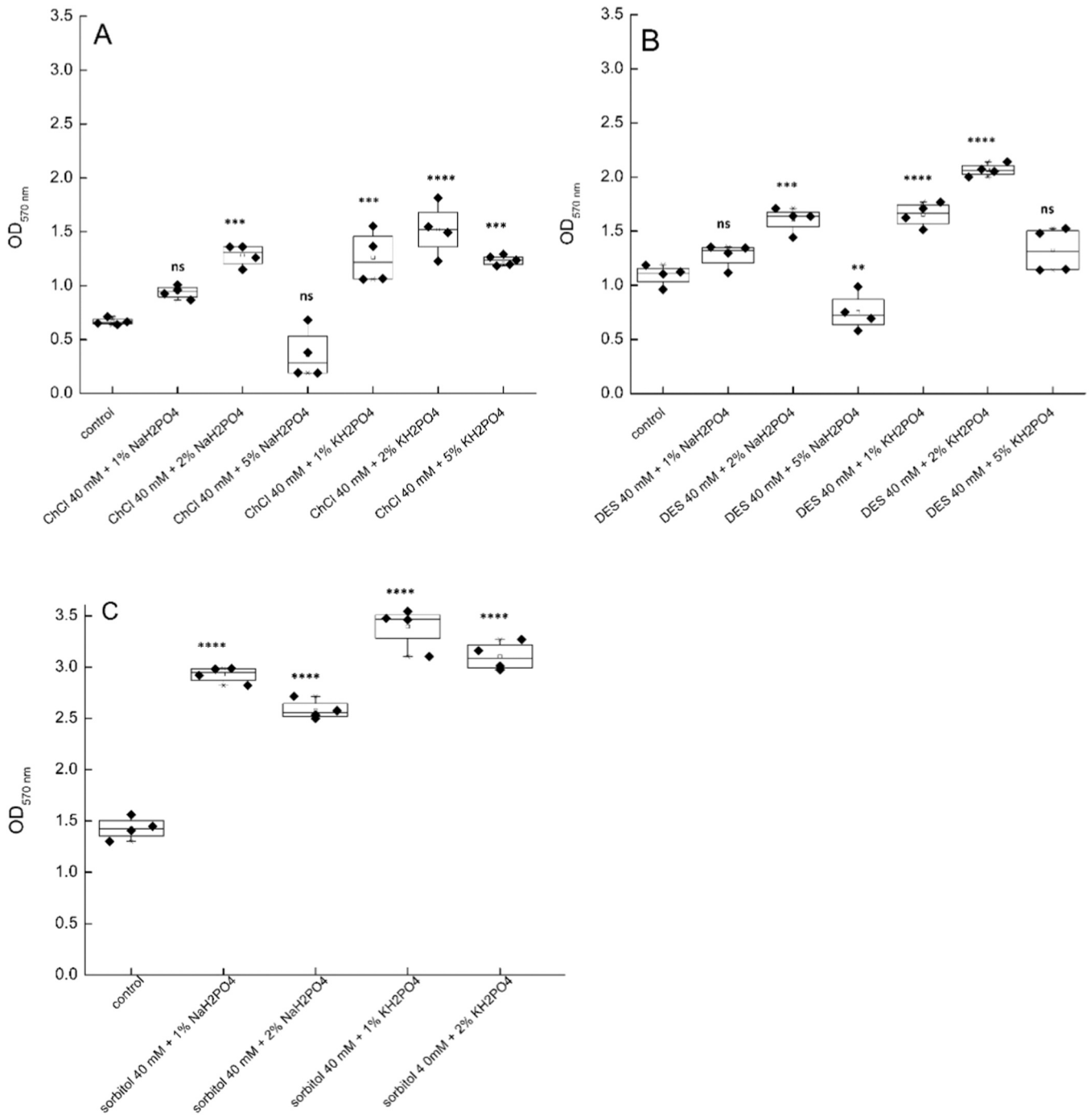

Fig. 5. Effect on biofilm formation of A) ChCl and salts; B) DES and salts; C) sorbitol and salts. N = 4. *, **, ***, **** indicate statistically significant difference between treated cells and control cells at $p<0.05$, $0.01<p<0.05$, $0.001<p<0.01$, and $0.0001<p<0.001$, respectively, following to Tukey's test from ANOVA.

assayed by UV/VIS spectrophotometer **(**Table S2 and Fig. S3**)**. *B. subtilis* can synthesize riboflavin which serves as a precursor for cofactors and export it extracellularly. Extracellularly secreted riboflavin can serve as an active redox mediator [60]. Therefore, it was appropriate to assay for its presence. Riboflavin increases slightly in the presence of DES 40 mM and ChCl 60 mM. However, there is no clear correlation between the riboflavin concentration and the electroactivity measured here. This suggests that the observed EET under the influence of ChCl, DES and DES components, and concurrent salt stress is mediated by other electron transfer processes within the biofilm.

4. Conclusion

The combination of inorganic salts, especially NaH_2PO_4 and KH_2PO_4 and 40 mM DES (D-sorbitol/ChCl, 1:1 mol mol^{-1}) enhances biofilm production of *B. subtilis* grown under static anoxic conditions. The positive effect of DES on the biofilm appears to be related to the presence of sorbitol, which serves as a secondary carbon and ready carbon pool for EPS synthesis. However, in the presence of salts (1–2 % wt/wt), ChCl functions to mitigate osmotic stress, probably because ChCl is the precursor of the osmoprotective agent glycine betaine. Based on the charge output, the effect of ChCl on biofilm formation does not directly correspond to the effect of betaine when both were used in conjunction with

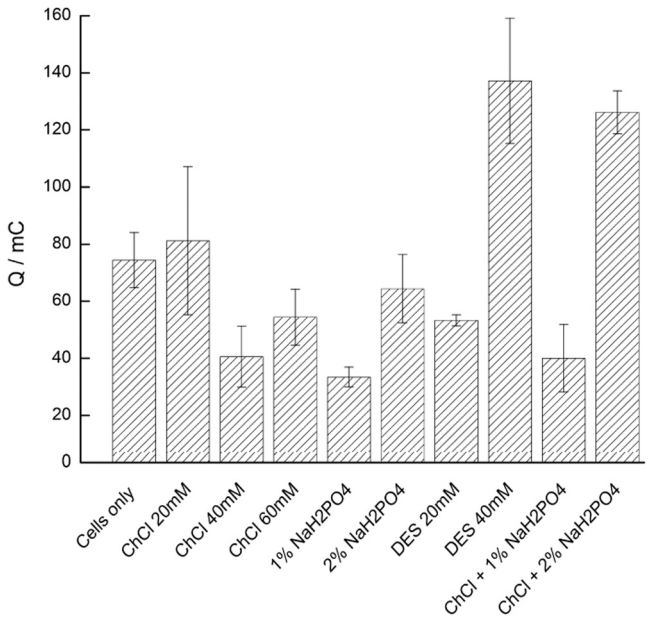

Fig. 6. Cumulative charge output for *B. subtilis* after 48 h growth at 400 mV in TY medium. N = 2.

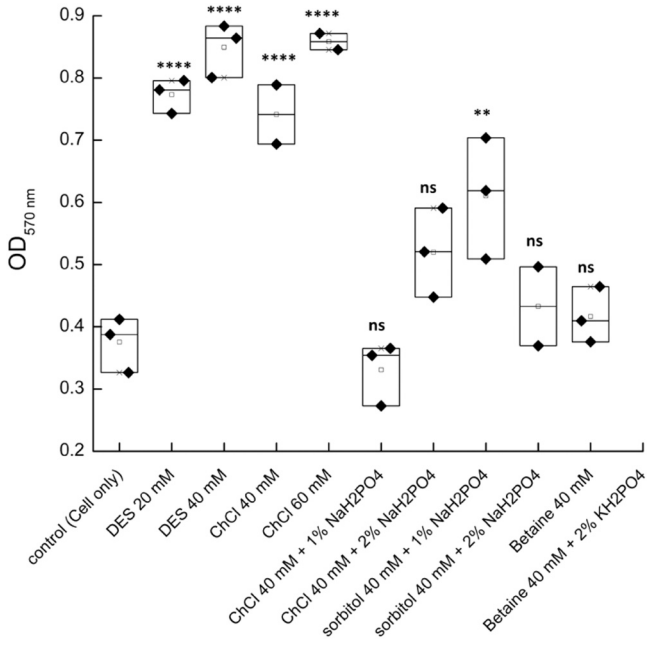

Fig. 7. Biofilm on SPE for selected conditions. N =3.

salts against osmotic stress. This is likely due to the fact that the synthesis of glycine betaine from choline in *B. subtilis*, mediated by the GbsB and GbsA dehydrogenases, and the presence of betaine in the medium can alter gene expression, leading to variations in biomass accumulation as an osmotic regulatory response. In the concentration range of 20–80 mM, DES has a strong effect on planktonic growth, which is only partially replicated when the two components, sorbitol and ChCl, are added separately or mixed in the aqueous solution. While this might indicate some effect of the hydrogen bonding of DES on planktonic growth, the tested DES inhibited growth at concentrations higher than 200 mM. Interestingly, DES does not mitigate the osmotic stress on planktonic growth. At concentration higher than 2 % wt. and 200 mM for salts and DES, respectively, both DES and salts hamper planktonic growth. Bioelectrochemical experiments indicate a small positive effect of ChCl on charge output in the presence of salts. Overall, the results indicate that the addition of ChCl or ChCl-containing DES at low concentrations enhances biofilm formation and mitigates osmotic stress. This observation could help in the development of more efficient bioelectrochemical processes, such as electrofermentation, especially in media containing concentrated electrolytes.

In conclusion, our study illuminates the intricate relationship between choline chloride (ChCl) and biofilm formation in *B. subtilis* under osmotic stress conditions. Our study provides novel insights into the interplay between osmotic stress regulators and biofilm formation. It highlights the need to further investigate of the molecular mechanisms controlling these processes and emphasizes the importance of considering the multifaceted nature of osmotic stress responses in bacterial systems.

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CRediT authorship contribution statement

Neda Eghtesadi: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Kayode Olaifa:** Methodology, Investigation. **Tri T Pham:** Writing – review & editing, Supervision, Project administration, Methodology, Data curation. **Vito Capriati:** Writing – review & editing, Methodology. **Obinna M Ajunwa:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Enrico Marsili:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Data Availability

Data will be made available on request.

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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.enzmictec.2024.110485.

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