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Research Article

Surface Charge Characteristics of *Bacillus Subtilis* NRS-762 Cells

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Received date: January 06, 2022; **Accepted date:** January 20, 2022; **Published date:** January 25, 2022

Citation: Wenfa Ng (2022) Surface Charge Characteristics of Bacillus Subtilis NRS-762 Cells. *J, Biotechnology and Bioprocessing* 3(2); DOI: 10.31579/2766-2314/074

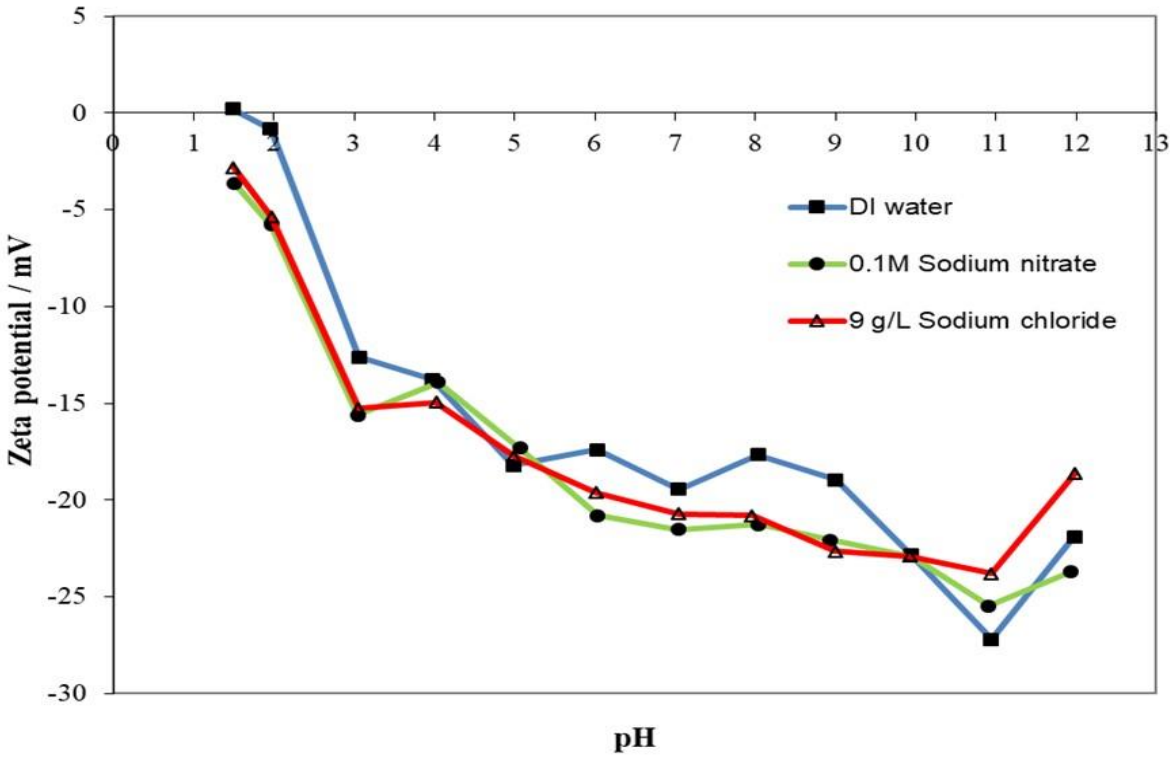
Abstract

Bacterial cell surface carries an electrical charge due to the myriad functional groups present, as well as assortment of ions and molecules non-specifically adsorbed to the cell surface. Thus, solution in contact with the bacterial cell surface play a critical role in influencing the overall surface charge characteristics through conferring non-specifically adsorbed ions and molecules. Various wash buffers are commonly used in removing non-specifically adsorbed ions and molecules for revealing the real surface charge of the bacterium. Using electrophoretic mobility measurement of zeta potential, this study attempted to understand the surface charge characteristics of *Bacillus subtilis* NRS-762 (ATCC 8473) with the help of three wash buffers: deionized (DI) water, 0.1M sodium nitrate, and 9 g/L sodium chloride. Experiment results revealed that *B. subtilis* NRS-762 was negatively charged over the entire pH range from 1.5 to 12. Specifically, with deionized water as wash buffer, the point-of-zero-charge (pH_{zpc}) was at pH 1.5, which indicated that large amount of negatively charged functional groups were present on the cell surface. Comparison between the zeta potential-pH profiles of *B. subtilis* NRS-762 cultivated at 30 ^oC and 37 ^oC revealed that the profile for growth at 37 ^oC was more negatively charged over the entire pH range compared to that for growth at 30 ^oC. This highlighted that physiological adaptation might had occurred on the cell surface for coping with growth at a higher temperature. Zeta potential-pH profiles obtained revealed that DI water could not remove significant quantities of the non-specifically adsorbed ions and molecules. On the other hand, the zeta potential-pH profiles of cells washed with 0.1M sodium nitrate and 9 g/L sodium chloride overlapped each other substantially and were more negatively charged over the pH range from 2 to 11, compared to that of cells washed with DI water. This revealed substantial removal of non-specifically adsorbed ions and molecules with the use of 0.1M sodium nitrate (0.1M ionic strength) and 9 g/L sodium chloride (0.15M ionic strength), which helped reveal the actual surface charge of *B. subtilis* NRS-762 cells. Collectively, actual surface charge of *B. subtilis* NRS-762 was masked by non-specifically adsorbed ions and molecules, which could be removed by 0.1M sodium nitrate and 9 g/L sodium chloride wash buffer. Thus, in the case of *B. subtilis* NRS-762, 0.1M ionic strength wash buffer was the threshold at which there was complete removal of non-specifically adsorbed ions and molecules from the cell surface.

Keywords: zeta potential; electrophoretic mobility; surface charge; bacillus subtilis; wash buffer; resuspension buffer; deionized water; sodium nitrate; sodium chloride; growth temperature

Subject areas: biogeochemistry, biotechnology, microbiology, biochemistry, bioengineering

Graphical Abstract



Short Description

Nonspecific adsorption of ions and molecules on cell surface would mask the real surface charge. Thus, various wash buffers are commonly used in removing non-specifically adsorbed ions and molecules from the cell surface. Using the phenomenon of ionic strength mediated charge screening that could remove non-specifically adsorbed ions and molecules, this study attempted to understand the surface charge characteristics of *Bacillus subtilis* NRS-762 cells (ATCC 8473) after treatment with 3 different wash buffers: deionized water, 0.1M sodium nitrate, and 9 g/L sodium chloride, with deionized water as resuspension buffer in microelectrophoresis analysis of surface charge. Experiment results revealed that the zeta potential-pH profiles of cells washed with 0.1M sodium nitrate (0.1M ionic strength) and 9 g/L sodium chloride (0.15M ionic strength) overlapped each other substantially and were more negatively charged over the pH range from 2 to 11 compared to cells washed with deionized water. This suggested that non-specifically adsorbed ions and molecules were removed from *B. subtilis* NRS-762 cell surface and helped unmask the real surface charge of the cells. Thus, 0.1M ionic strength wash buffer could help remove non-specifically adsorbed ions and molecules and reveal the real surface charge characteristics of *B. subtilis* NRS-762 cells.

Significance of the work

Bacterial cells carry a "memory" of the solution it contacted with in the form of a layer of ions and molecules non-specifically adsorbed to the cell surface. Such a layer of ions and molecules would thus mask the real surface charge characteristics of the cell. In micro electrophoretic measurement of surface charge of bacterial cells, various wash buffers had been used in removing the non-specifically adsorbed ions and molecules, but their relative efficacy remain poorly understood. In this study, the surface charge characteristics of *Bacillus subtilis* NRS-762 (ATCC 8473) was determined using electrophoretic mobility measurement, where three wash buffers (i.e., deionized water, 0.1M sodium nitrate, and 9 g/L sodium chloride) helped remove non-specifically adsorbed ions and molecules from the cell surface. Experiment results revealed that *B. subtilis* NRS-762 cell surface was negatively charged over the entire pH range from 1.5 to 12. Furthermore, zeta potential-pH profiles of cells washed with 0.1M sodium nitrate and 9 g/L sodium chloride overlapped each other substantially and were more negatively charged than that obtained with deionized water as wash buffer. This indicated that both 0.1M sodium nitrate (ionic strength 0.1M) and 9 g/L sodium chloride (0.15M ionic strength) could remove the non-specifically adsorbed ions and molecules through charge screening and helped unmask the real surface charge of *B. subtilis* NRS-762 when suspended in deionized water. Thus, 0.1M ionic strength was the threshold where there was complete removal of the non-specifically adsorbed ions and molecules from *B. subtilis* NRS-762 cells which reveal the real surface charge characteristics.

Highlights

1) Nonspecific adsorption of ions and molecules on bacterial cell surface masks the real surface charge characteristics; thus, various wash buffers were used in removing the nonspecifically adsorbed ions and molecules through ionic strength mediated charge screening effect.

2) This study reports the surface charge characteristics of *Bacillus subtilis* NRS-762 (ATCC 8473) cells measured via the microelectrophoresis method with readout as zeta potential values. Three wash buffers: deionized water, 0.1M sodium nitrate, and 9 g/L sodium chloride were used.

3) Defined at the shear plane, zeta potential values could be erroneous representation of cell surface charge if nonspecifically adsorbed ions and molecules were not removed by wash buffers of suitable ionic strength.

4) Experiment results from cells washed with deionized water revealed that the *B. subtilis* NRS-762 cell surface was negatively charged over the pH range from 1.5 to 12.

5) Comparison of cells grown at 30 °C and 37 °C in LB Lennox medium with 2 g/L glucose revealed that the surface of cells cultivated at 37 °C was more negatively charged compared to those grown at 30 °C over the pH range from 2 to 11; thereby, suggesting that there might be physiological adaptations at the cell envelope for coping with higher growth temperature.

6) Zeta potential-pH profiles of cells washed with 0.1M sodium nitrate and 9 g/L sodium chloride overlapped each other substantially, and were more negatively charged than that obtained with deionized water wash buffer, which suggested that substantial amount of nonspecifically adsorbed ions and molecules were removed, that help unmasked the real surface charge characteristics of the cells.

Introduction

Surface of bacterial cell envelope is commonly covered with different functional groups that confer an electrical charge to the surface. For example, phosphate groups provide a predominantly negative charge to the bacterial cell surface [1, 2]. Similarly, amino groups confer a positive charge when the functional groups are protonated at low pH. Presence of an electrical charge naturally attract a counter-ion layer of opposite charge close to the cell surface, which partially neutralize the charge on the cell surface. Additionally, nonspecific adsorption of ions and molecules could also occur on the cell surface, which partially masks the cell surface charge [3].

Thus, understanding of the bacterial cell surface charge is in relation to the solution environment of the cell. Specifically, ions and molecules from the solution could non-specifically adsorb to the cell surface [3]. On the other hand, solution environment defines the conditions at which the bacterial cell surface would interact with the solution. For example, pH determines the protonation state of functional groups on the bacterial cell surface, while ionic strength affects the binding of molecules and ions onto the cell surface. Thus, is it possible to determine the true bacterial cell surface charge characteristics? The answer depends significantly on the type of solution in contact with the bacterial cell surface, and whether the non-specifically adsorbed ions and molecules could be removed without affecting the intrinsic charge of the cell surface [3].

Currently, no experimental method exists for direct measurement of bacterial cell surface charge. However, proxy methods for inferring a surface charge of the bacterial cell are available to help understand various phenomena that hinge on the bacterial surface charge, one of which is bacterial aggregation and flocculation during wastewater treatment [4-6]. Specifically, zeta potential measured by the microelectrophoresis technique determine an electrical charge at the shear plane of the cell, which is a short distance away from the actual surface [1, 7]. Thus, zeta potential approximates the cell surface charge, but this approximation could be influenced by a variety of factors such as types and amount of ions and molecules that non-specifically adsorbed within the space between the cell surface and the shear plane [3].

Prior to measurement of zeta potential, cell samples are typically washed by various wash buffers as part of sample preparation. Depending on the ionic strength of the wash buffers, non-specifically adsorbed ions and molecules could be removed; thereby, revealing more aspects of the bacterial cell surface charge [3]. On the other hand, high ionic strength wash buffers such as 0.1M sodium citrate could potentially damage the cell surface [3]. Thus, choice of wash buffers play an important role in allowing a better approximation to the actual cell surface charge to be determined. For example, 0.15M ionic strength wash buffer such as 9 g/L sodium chloride was found to be effective in removing almost all the non-specifically adsorbed ions and molecules and helped unmask the real surface charge of *Escherichia coli* DH5α [3].

Thus, this study sets out to understand the cell surface characteristics of *Bacillus subtilis* NRS-762 (ATCC 8473) through the zeta potential measurement method, where wash buffer of 0.1M ionic strength (0.1M sodium nitrate) and 0.15M ionic strength (9 g/L NaCl) were chosen to help remove non-specifically adsorbed ions and molecules that mask the actual surface charge. A Gram-positive bacterium, the cell surface of *B. subtilis* NRS-762 is defined by a thick peptidoglycan layer meshed with arrays of teichoic acid molecules. Thus, nonspecific adsorption of various ions and molecules likely mask the actual surface charge of the *B. subtilis* NRS-762 cell surface.

Materials and Methods

Materials

LB Lennox medium was purchased from Difco and used as is. Composition of LB Lennox medium was [g/L]: Tryptone, 10.0; Yeast extract, 5.0; NaCl, 5.0. Composition of LB Lennox medium with 2 g/L glucose was [g/L]: Tryptone, 10.0; Yeast extract, 5.0; NaCl, 5.0, D-Glucose, 2.0.

Growth of Bacillus subtilis NRS-762 in growth medium

Stock cultures of *B. subtilis* NRS-762 were prepared in 40% glycerol and kept at -70 ºC until use. One glycerol stock culture of *B. subtilis* NRS-762 was used in inoculating 100 mL of LB Lennox medium in a 250 mL glass conical flask as seed culture. Incubation conditions were 30 ºC and 230 rpm rotational shaking in a temperature-controlled incubator. After 8 hours of culture, 1 mL of seed culture was withdrawn and used as inoculum for 100 mL of LB Lennox medium with 2 g/L glucose in a 250 mL glass conical flask. Incubation conditions were 30 ºC and 230 rpm rotational shaking in a temperature-controlled incubator (Yih Der LM-570D, Taiwan). Two biological replicates were prepared.

Sample preparation for zeta potential analysis

After 15 hours of incubation, 2.5 mL of culture broth was withdrawn and diluted with 37.5 mL of non-sterile wash buffer in a 50 mL polypropylene centrifuge tube and mixed vigorously by hand and vortex mixer. This content was centrifuged at 3300 x g for 10 minutes at 25 ºC. Following centrifugation, wash buffer was carefully decanted off without disturbing the cell pellet. 40 mL of wash buffer was added to the centrifuge tube and the contents resuspended by vigorous mixing by hand and vortex mixer. The centrifugation and washing steps were repeated a total of three times prior to use of 40 mL of deionized water for resuspending the cell pellet through vigorous shaking by hand and vortex mixer. Nitric acid and sodium hydroxide was added to the samples for pH adjustment with pH measured by an Orion 9156 BNWP pH probe outfitted to a Mettler Toledo Delta 320 pH meter.

Zeta potential analysis

Samples were vigorously shaken by hand prior to zeta potential analysis by Malvern Zetasizer Nano ZS instrument. The microelectrophoresis cell was rinsed with deionized water 3 times prior to each analysis, and care was taken to avoid bubble formation during addition of sample into the measurement cell. Measurements were made at 25 ºC. The ionic strength of wash buffers was estimated by the Debye-Huckel theory.

Results and Discussion

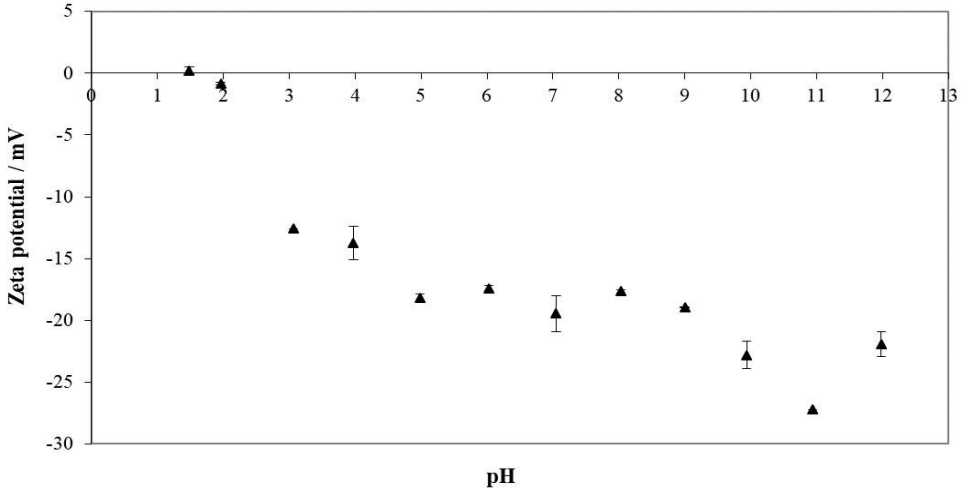


Figure 1: Variation of zeta potential with pH for *Bacillus subtilis* NRS-762 cells grown in LB Lennox with 2 g/L glucose at 30 ºC and 230 rpm rotational shaking. Deionized water was used as wash and resuspension buffer. Note that this is essentially a titration curve of *B. subtilis* NRS-762 cell surface against pH.

For *B. subtilis* NRS-762 cultivated in LB Lennox with 2 g/L glucose, the zeta potential-pH profile obtained with deionized water as wash buffer revealed that the cell surface was negatively charged within the pH range from 1.5 to 12 (Figure 1). Specifically, the point-of-zero-charge (pH_{zpc}) was pH 1.5, which defined a pH where the cell surface charge was zero. In general, with increasing pH, the zeta potential of the cell surface became more negative, which indicated that more negatively charged functional groups are exposed. In transiting from pH 4 to 5, there was a decrease in zeta potential, which indicated that carboxyl functional groups likely deprotonated with increasing concentration of OH^- ions in the solution. In general, the zeta potential-pH profile of *B. subtilis* NRS-762 resembles a titration curve and comprised three segments: an initial rapid decrease in zeta potential values between pH 2 and 5, followed by a region of relatively stable zeta potential values between pH 5 and 9, and finally, a region of rapid decrease in zeta potential between pH 9 and 11, which culminated in an uptick in zeta potential at pH 12. Other studies have shown that the composition of the cell surface of *B. subtilis* changes as a function of pH due to the presence of different functional groups [8-11]. Specifically, potentiometric titrations of *B. subtilis* cells have revealed that the cell surface of the bacterium is negatively charged over the pH range from 2 to 10 with a point-of-zero-charge (pH_{zpc}) < 2 [9], which agrees well with results from this study. Finally, as the *B. subtilis* NRS-762 cell population was highly heterogeneous due to the presence of a variety of cell lineages [12], some scatter in the zeta potential data was expected given that cells belonging to different differentiation pathways likely exhibit different cell surface characteristics.

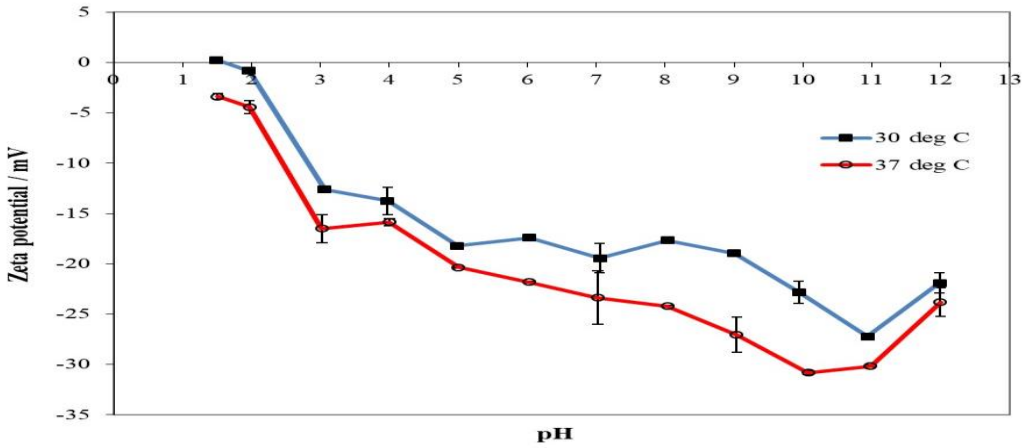


Figure 2: Zeta potential-pH profiles of *B. subtilis* NRS-762 grown at 30 ºC and 37 ºC in LB Lennox medium with 2 g/L glucose, and with deionized water as wash and resuspension buffer.

Comparison of zeta potential-pH profiles of *B. subtilis* NRS-762 grown at 30 ºC and 37 ºC in LB Lennox medium with 2 g/L glucose revealed that the cell surface of *B. subtilis* NRS-762 was more negatively charged after cultivation at 37 ºC compared to 30 ºC (Figure 2). Specifically, at all measured pH, zeta potential of cells cultivated at 37 ºC was more negative compared to that of cells cultivated at 30 ºC. This observation could arise due to fundamental changes to the cell surface of *B. subtilis* NRS-762 after cultivation at 37 ºC, for example, through the generation of more negatively charged functional groups on the cell surface. On the other hand, cells grown at 37 ºC could also secrete more acidic metabolites that non-specifically adsorbed to the *B. subtilis* NRS-762 cell surface that confer a more negative cell surface charge. Overall, cells cultivated at 37 ºC likely had a different set of surface functional groups compared to those cultivated at 30 ºC.

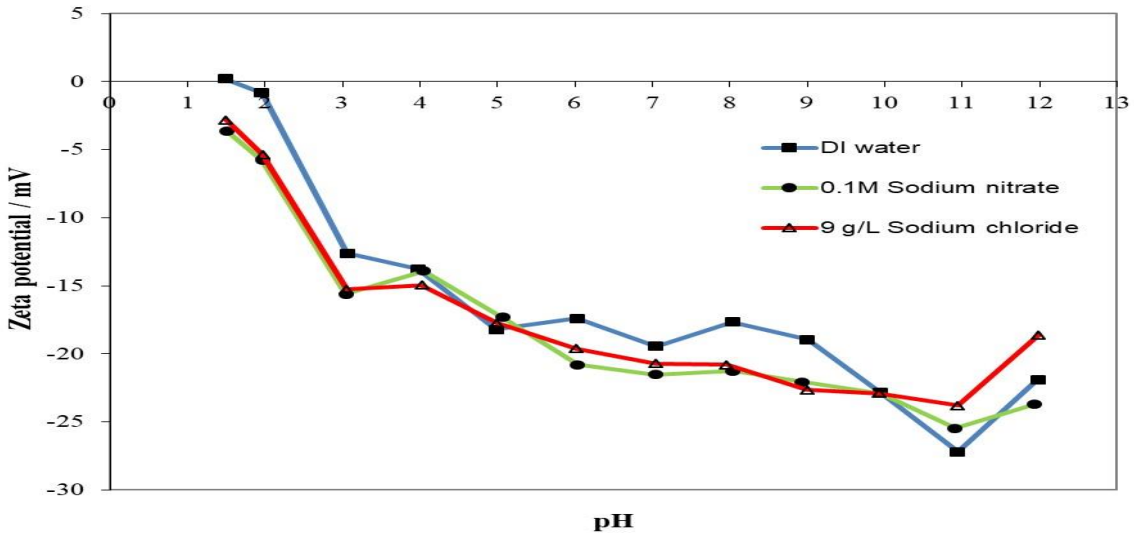


Figure 3: Zeta potential-pH profiles of *B. subtilis* NRS-762 cultivated in LB Lennox with 2 g/L glucose medium at 30 ºC and 230 rpm rotational shaking, and washed with different wash buffers (i.e., deionized water, 0.1M sodium nitrate, and 9 g/L sodium chloride). Deionized water was used as resuspension buffer. Error bars were not plotted to reduce potential confusion of the data from closely spaced curves.

Observation of zeta potential-pH profiles of *B. subtilis* NRS-762 cells cultivated in LB Lennox medium with 2 g/L glucose at 30 ºC and 230 rpm rotational shaking, and washed with deionized water, 0.1M sodium nitrate, or 9 g/L sodium chloride revealed that washing of cells with 0.1M sodium nitrate and 9 g/L sodium chloride rendered the cell surface to be more negatively charged over the entire pH range from 2 to 9 compared to that of cells washed with deionized water (Figure 3). This could be due to the removal of non-specifically adsorbed ions and molecules from the cell surface of *B. subtilis* NRS-762 by wash buffers. Specifically, the zeta potential-pH profile of cells washed with 0.1M sodium nitrate and 9 g/L sodium chloride overlapped each other substantially; thereby, indicating that the small difference in ionic strength between the two wash buffers (i.e., 0.1M ionic strength for 0.1M sodium nitrate, and 0.15M ionic strength for 9 g/L sodium chloride) did not result in significant differences in the types and amounts of non-specifically adsorbed ions and molecules removed. Overall, deionized water was not able to remove substantial amount of non-specifically adsorbed ions and molecules.

Taken together, 0.1M sodium nitrate and 9 g/L sodium chloride helped unmask the real surface charge of *B. subtilis* NRS-762 over the pH range from 2 to 12 through the charge screening effect that helped remove the non-specifically adsorbed ions and molecules on the cell surface. While possibilities exist that the ionic strength of the wash buffers might have damaged the cell surface, previous studies have shown that wash buffers

of 0.1M and 0.15M ionic strength are unlikely to result in cell surface damage [3]. In contrast, with respect to *Escherichia coli* DH5α, 0.15M ionic strength is the threshold upon which there was complete removal of non-specifically adsorbed ions and molecules from the cell surface of the Gram-negative bacterium [3]. This is slightly higher than the 0.1M ionic strength threshold for complete removal of non-specifically adsorbed ions and molecules from *B. subtilis* NRS-762 cell surface. Differences in cell wall structure and composition of functional groups on the cell surface could account for the observed difference in threshold ionic strength for unmasking the real surface charge of *E. coli* DH5α and *B. subtilis* NRS-762.

Conclusion

Zeta potential measurement revealed that the cell surface of *B. subtilis* NRS-762 was negatively charged within the pH range from 1.5 to 12. Thus, the Gram-positive bacterium holds application potential for use as a biosorbent in understanding the binding of heavy metals to the cell surface, which is relevant to many aspects of environmental geochemistry. Zeta potential-pH profiles of *B. subtilis* NRS-762 cultivated in LB Lennox medium with 2 g/L glucose at 30 and 37 ºC exhibited different characteristics. Specifically, cells cultivated at 37 ºC were more negatively charged over the entire pH range from 2 to 11 compared to that of cells cultured at 30 ºC. This suggested that the cell surface composition of *B. subtilis* NRS-762 was different upon cultivation at different temperatures and could be due to physiological adaptation to growth at a higher temperature. The point-of-zero-charge (pH_{zpc}) of *B. subtilis* NRS-762 cultivated at 30 ºC was pH 1.5, which indicated that large amount of negatively charged functional groups were present on the cell surface. With deionized water as wash buffer, no substantial removal of non-specifically adsorbed ions and molecules occurred. More importantly, more negative charges on the cell surface were revealed with 0.1M sodium nitrate and 9 g/L sodium chloride wash buffers, where higher ionic strength of 0.1M and 0.15M, respectively, helped removed non-specifically adsorbed ions and molecules. Finally, removal of the layer of non-specifically adsorbed ions and molecules from *B. subtilis* NRS-762 cell surface by 0.1M sodium nitrate and 9 g/L sodium chloride revealed the real surface charge of the cells with deionized water as resuspension buffer. Thus, 0.1M ionic strength is the threshold ionic strength below which there would be no complete removal of non-specifically adsorbed ions and molecules from *B. subtilis* NRS-762 cell surface.

Conflicts of interest

The author declares no conflicts of interest.

Funding

The author thanks the National University of Singapore for Financial support.

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DOI: 10.31579/2766-2314/074