Cell surface properties of the demulsifying strain *Alcaligenes* sp. S-XJ-1 governing its behavior in oil–water biphasic systems

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**Abstract**

Bacterial behavior in oil–water biphasic systems plays an essential role in hydrophobic contaminant degradation, oil recovery, and emulsion breaking. Less is known about the cell surface properties that govern their behaviors in oil–water biphasic systems. In this study, biphasic partitioning and aggregation of a demulsifying strain of *Alcaligenes* sp. S-XJ-1 were experimentally measured and evaluated based on the cell surface properties of surface charge, surface free energy, and cell surface hydrophobicity (CSH). The S-XJ-1 was cultivated with five different carbon sources, and the results showed a highly varied partitioning, aggregation behavior, and cell surface properties. The calculated interaction energies, based on the cell surface properties, were consistent with the results of their behavior. Among the cell surface properties, the electron-donor character ($\gamma^D$, range 8.8–57.0 mJ/m²), which correlated well with CSH ($\Delta G_{bwb}$), was an essential indicator of cell behavior. A low $\gamma^D$ value enhanced the cell–interface and cell–cell interaction energies, which promoted cell partitioning and aggregation eventually leading to demulsification. The results and analysis provide important information for researchers concerned with cell–cell and cell–interface interactions.

**Keywords**

Cell surface property; bacterial behavior; cell biphasic partitioning; cell aggregation; demulsifying strain

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**1. Introduction**

Microbial cell aggregation and interface adhesion are of great significance in many environmental and bioengineering applications, including biofilm formation in sewage treatment,[1] biofouling contamination of medical devices and food,[2–4] and adhesion to hydrocarbons for biodegradation.[5] Furthermore, the partitioning and aggregation of demulsifying cells in oil–water systems play important roles in the biological demulsification process. The behaviors of microbial cells in a two-phase liquid system are complex and generally governed by the extended Derjaguin–Landau–Verwey–Overbeek (XDLVO) theory by calculating cell–cell or cell–interface interaction energies.[6] According to the XDLVO...
theory, microbial cell behavior depends on the combination of Lifshitz–van der Waals (LW) interactions, acid–base (AB) interactions, and electrostatic (EL) interactions, which are mainly determined by the cell surface properties and the oil–water system.[6–8] However, our understanding of how the major components of cell surface properties affect demulsifying bacterial behavior in the emulsion, which ultimately leads to emulsion breaking, remains limited.

When whole bacterial cells are used as demulsifiers, physicochemical properties of the cell surface are used to understand the interactions between the demulsifying bacteria and emulsions.[9,10] Many studies have shown that cell surface hydrophobicity (CSH) of demulsifying bacteria has a positive correlation with their emulsion breaking performance.[11–13] Fernandes [14] reported that the correlation between CSH and the demulsifying capability was either absent, negative, or positive, depending on the microbial strain or on the previous growth conditions for a particular strain. However, little research has addressed the dynamic mechanisms of how cell surface properties affect demulsification, particularly how cell surface properties control their behaviors in an oil–water biphasic system.

The cell surface free energy of microbial cells, which can be calculated from the contact angles on the bacterial lawn deposited on membrane filters,[7] plays a crucial role in bacterial behavior in an oil–water biphasic system. The surface free energy includes contributions from LW and AB components. The LW component is apolar and generally attractive, while the AB component is polar and comprises electron-donor (γ−) and electron-acceptor (γ+) contributions.[15] The electron-donor parameter is a fairly good semi-quantitative indicator of the degree of bacterial CSH.[6] Moreover, the interfacial interaction energy between bacterial cells immersed in water (ΔG_{bwb}), calculated from cell surface free energy, is an ideal standard quantitative measure of the degree of CSH.[6] Therefore, the surface free energy is an important cell surface property that allows us to explore demulsifying cell behavior in an emulsion.

Different carbon sources have a noticeable impact on the demulsifying capability and cell surface properties of the demulsifying strain.[16,17] In our previous study, one highly efficient demulsifying bacteria, *Alcaligenes* sp. S-XJ-1, was screened from petroleum-polluted soil.[18] Its surface composition and demulsifying capability were significantly affected by different carbon sources including alkanes (paraffin or octadecane), fatty acid esters (rape oil or olein), and carbohydrates (glucose).[19] In this study, *Alcaligenes* sp. S-XJ-1 cultivated with five different carbon sources was used to investigate how cell surface properties govern bacterial behavior in oil–water biphasic systems. The surface charge, surface free energy, and CSH were used to characterize cell surface properties, and bacterial behavior and aggregation during biphasic partitioning were also investigated. The effect mechanism of the bacterial cell surface properties on their behavior in the oil–water system was elucidated using thermodynamic and XDLVO theories.

2. Materials and methods

2.1. Cultivation and preparation of bacterial cells

The tested strain of *Alcaligenes* sp. S-XJ-1 (CGMCC No. 2142), isolated from petroleum-contaminated soil, was kept at 4 °C in an agar slant culture.[18] After enrichment in a nutrient broth medium, the S-XJ-1 was cultivated in a modified mineral salts medium containing
4% (v/v) paraffin, octadecane, rape oil, olein, or glucose as carbon sources and harvested as in our previous study.[19] After a seven-day cultivation, the bacterial cells cultured with the various hydrophobic carbon sources were left to separate into the oil phase (at the top) and the aqueous phase (at the bottom). Almost all bacterial cells adhered to the suspended oil after 12 h. The bacteria were harvested from the oil phase by centrifugation at 13,000×g for 10 min. To remove the residual oil, the harvested bacteria were rinsed three times with n-hexane followed by centrifugal separation at 13,000×g. Bacterial cells cultivated with glucose were obtained by direct centrifugation of the fermented culture at 13,000×g and then rinsed with distilled water. The washed bacterial cells were dried in a freeze drier (Scientz-10 N; Ningbo Scientz Biotechnology, Zhejiang, China) at −50 °C for 24 h. The dried cells were used to characterize biomass, demulsifying capability, and cell surface properties.

2.2. Zeta potential Measurements

The Zeta potentials (ZPs) of demulsifying cells cultivated with the five different carbon sources were measured separately using a Zetasizer NanoZ analyzer (Malvern Instrument Ltd, Malvern, Worcestershire, UK). The bacterial cells were suspended in distilled water with an initial optical density at 580 nm of 0.8–1.0 (OD_{580 nm}). The bacterial suspension (12 mL) was injected into an electrophoresis cell, the original pH state of approximately 6.5 was altered to 2.0 in 0.5 pH unit intervals by adding 0.1 M HCl. The reported values are based on triplicate samples that were repeated three times. The pH value at the isoelectric point (pH_{IEP}) and the ZP in the original state were used to compare the demulsifying cells cultivated with the five carbon sources.

2.3. Contact angle measurements

The contact angles of the S-XJ-1 bacteria cultivated with the five carbon sources were measured following the procedures in the literature.[5,20] To prepare the bacterial lawns, 10 mL of an adequately dispersed bacterial suspension (8.0 g/L) was filtered through a 0.45 μm (pore size) cellulose acetate membrane (50 mm diameter) to deposit a uniform lawn. The membranes were transferred to Petri plates containing 1% agar and 10% glycerol, and then maintained at 35 °C for more than 2 h to standardize the humidity of the membranes. The membranes were cut into strips, mounted on glass slides with double-sided tape, and allowed to dry for 20–40 min at 35 °C. The contact angles between the droplet and the bacterial lawn were recorded by a contact angle meter SL200B (Shanghai Solon Technology Science Company, Ltd, Shanghai, China). The contact angle was read every second during the first 50 s, and obtained by a circular fitting analysis using CAST3.0 software (Solon Tech Co. Ltd, Shanghai, China). Distilled water (polar), formamide (polar), and diiodomethane (apolar) were used as the probe liquids. Each measurement was repeated for at least five droplets taken from different locations on each bacterial lawn for each of the three liquids.

2.4. Cell surface free energy and hydrophobicity calculations

The surface energy parameters of each demulsifying bacteria were calculated from the measured contact angles based on the acid–base theory proposed by van Oss et al.[6] The total surface energy (γ), its apolar (γ_{W}) and polar component (γ_{AB}), along with electron-donor
and electron-acceptor (γ⁺) parameters were estimated using the Young–Dupre equation. The components and parameters of the surface free energy for the probe liquids considered are shown in Table 1.

The CSH of the demulsifying bacteria can be directly estimated using the values of the water contact angle, semi-quantified using the electron-donor surface parameter (γ⁻), and quantified with the free energy of hydration (ΔGbw) or the free energy of interfacial interaction between two bacterial cells immersed in water (ΔGbwb). The ΔGbw and ΔGbwb values can be calculated using the surface free energy of the bacterial cells and water:

$$\Delta G_{bw} = -2 \left( \sqrt{\gamma^L_w \gamma^L_b} + \sqrt{\gamma^+ \gamma^-} + \sqrt{\gamma^- \gamma^+} \right)$$  \hspace{1cm} (1)

$$\Delta G_{bwb} = -2 \left( \sqrt{\gamma^L_w} - \sqrt{\gamma^L_b} \right) - 4 \times \left( \sqrt{\gamma^+ \gamma^-} + \sqrt{\gamma^+ \gamma^-} - \sqrt{\gamma^+ \gamma^-} - \sqrt{\gamma^+ \gamma^-} \right)$$  \hspace{1cm} (2)

### 2.5. Cell biphasic partitioning

The microbial adhesion to hydrocarbon (MATH) test was used to evaluate the biphasic partitioning of microbial cells in the kerosene–water system. The different demulsifying bacterial cells were suspended in distilled water with an initial optical density of 1.0 (OD_{580 nm}). Different test tubes containing 5 mL of this cell suspension were then vortex-mixed with 1 mL kerosene at 1800 rpm for 10, 20, 40, 60, and 90 s using a mini-shaker (MS3D, IKA, Staufen, Germany). The mixture was left undisturbed for 20 min and then the final OD_{580 nm} of the aqueous phase was measured. The MATH value (M) was calculated as follows:

$$M = \left[ 1 - \left( \frac{OD_{580\text{nm} \text{(final)}}}{OD_{580\text{nm} \text{(initial)}}} \right) \right] \times 100\%$$  \hspace{1cm} (3)

For the kinetic MATH test, an equal number of test tubes to the number of time points were prepared and subjected to the MATH test, as described above. The MATH values obtained for each time point were plotted against vortex time (t), and fitted as follows [21,22]:

$$M = \left( M_{\text{max}} \times t^N \right) / \left( K^N + t^N \right)$$  \hspace{1cm} (4)

where $M_{\text{max}}$ is the cell adhesion percentage at a steady-state plateau, K is the time to half-maximal adhesion, and N is an exponential term related to the steepness of the cell number increase over time.
2.6. Cell aggregation

The aggregation of demulsifying cells was determined by the turbidity variation of the bacterial suspension. The demulsifying cells cultivated with the five carbon sources were resuspended in distilled water to turbidity at an \( OD_{580} \) of 1.0. The suspension was then transferred into a cuvette. The suspension turbidity in the middle of the cuvette was periodically determined at 580 nm by a spectrophotometer. As the cells aggregated, they settled to the bottom of the cuvette and the percentage aggregation was calculated as follows:

\[
\% \text{Aggregation} = \left[ 1 - \left( \frac{OD_t}{OD_0} \right) \right] \times 100\%
\]

(5)

where \( OD_t \) and \( OD_0 \) represent the turbidities of the bacterial suspension at time \( t \) and 0, respectively.

2.7. Thermodynamic and XDLVO analysis

Partitioning of the demulsifying cells in an emulsion is an important process of demulsification. Variations in the system Gibbs energy, due to bacterial translocation in the kerosene–water biphasic system, are closely related to interfacial surface energies [23]:

\[
\Delta G_{\text{water to oil}} = \gamma_{bo} - \gamma_{bw}
\]

(6)

\[
\Delta G_{\text{water to interface}} = \gamma_{bo} - \gamma_{bw} - \gamma_{wo}
\]

(7)

\[
\Delta G_{\text{oil to interface}} = \gamma_{bw} - \gamma_{bo} - \gamma_{wo}
\]

(8)

where \( \gamma_{bo}, \gamma_{bw}, \) and \( \gamma_{wo} \) are the bacteria–oil, bacteria–water, and water–oil interfacial energies, respectively. The interfacial energies can be calculated according to the bacterial, oil, and water surface free energies using the LW–AB approach [24]:

\[
\gamma_{bi} = \left( \sqrt{\gamma_{bo}^{\text{LW}}} - \sqrt{\gamma_{bi}^{\text{LW}}} \right)^2 + 2 \times \left( \sqrt{\gamma_{bo}^{+}} - \sqrt{\gamma_{bi}^{+}} \right) \times \left( \sqrt{\gamma_{bo}^{-}} - \sqrt{\gamma_{bi}^{-}} \right)
\]

(9)

where \( i \) denotes either the water or oil phase. For kerosene oil, we determined the surface energy components and parameters to be \( \gamma_{bo}^{\text{LW}} = 27.7 \), \( \gamma^{+} = 0 \), and \( \gamma^{-} = 0 \).

The XDLVO theory was employed to determine the correlation between demulsifying cell aggregation and cell surface properties. To ascertain the value of the interaction energy of two cells in water (\( \Delta G_i \)) as a function of the interparticle distance (\( l \)), \( \Delta G_i \) can be separately plotted against \( l \) for apolar (LW), polar (AB), and electrostatic (EL) interactions [6]:

\[
\Delta G_i = \Delta G_i^{\text{LW}} + \Delta G_i^{\text{AB}} + \Delta G_i^{\text{EL}}
\]

(10)

\[
\Delta G_i^{\text{LW}} = -(A \times R)/(12 \times l)
\]

(11)

\[
\Delta G_i^{\text{AB}} = \pi R \lambda \Delta G_i^{\text{AB}^*} \exp \left[ (l_0 - l)/\lambda \right]
\]

(12)
where $A$ is the Hamaker constant: $A = 24/\lambda_0^3 l_0^2 \ln \left[1 + \exp(-\kappa l_0)\right]$, and $l_0 = 0.157$ nm. $R$ is the radius of the bacterial cells, which were estimated to be spherical particles with a 0.36 μm radius (the cells were the shape of short rods about 1.0 μm long and 0.5 μm wide [25]). $\lambda$ is the decay length, which is approximately 1.0 nm in aqueous systems. $\Delta G_{AB}^{\text{EL}} l_0$ is the interaction energy between two bacterial cells at the minimum equilibrium distance $l_0$,

$$\Delta G_{lb}^{\text{EL}} = -4 \times \sqrt{\gamma_b^+ - \gamma_w^+} \times (\sqrt{\gamma_b^-} - \sqrt{\gamma_w^-}).$$

$\varepsilon$ is the dielectric constant of the liquid medium (for water, $\varepsilon = 80$), $\psi_0$ is the potential of the cell itself, estimated from the ZP of the cells, and $\kappa$ is the inverse of Debye length.

### 3. Results

#### 3.1. Cell surface properties

**3.1.1. Surface charge**

The surface charge of the demulsifying cells can affect cell behavior in the emulsion by electrostatic interactions. The ZPs of S-XJ-1 cells cultivated with the five different carbon sources are plotted against pH in Figure 1. The ZPs of the S-XJ-1 were negative at the initial pH (pH approximately 6.5), ranged from $-35$ to $-60$ V, and decreased from high to low in cells cultivated using paraffin, octadecane, rape oil, olein, and glucose. The isoelectric points were at pH 2–3, similar to other bacterial cells.[26]

#### 3.1.2. Contact angles

Table 2 presents the mean contact angles of water, formamide, and diiodomethane, the components of the surface free energies, and the interaction Gibbs free energies of S-XJ-1 cultivated with the five carbon sources. The S-XJ-1 cells cultivated with paraffin exhibited the highest water contact angle of 91.1°, whereas the cells cultivated with glucose only...
represented a water contact angle of 40.4°. The contact angles for the different demulsifying cells varied from 54.8° to 83.7° for formamide, and from 67.4° to 76.8° for diiodomethane. The majority of the contact angles of the apolar liquid (diiodomethane) for bacterial isolates fall within a very narrow band.[7] This means that the S-XJ-1 cells cultivated with the five carbon sources have very similar interactions with apolar liquids.

### 3.1.3. Surface free energies

The surface free energy components of the S-XJ-1 cells cultivated with the five carbon sources in Table 2 were calculated from the contact angles using the Young–Dupre equation (Equation 3). The total free energy (γ\text{Total}) values for S-XJ-1 ranged from 19 to 35 mJ/m². Cells cultivated using paraffin exhibited the lowest value. The surface free energy is considered to be the sum of the LW and AB components. The LW component, which is apolar and generally attractive, was not significantly different from the cells cultivated with the five carbon sources. The AB component is polar, comprising of electron-donor (γ\text{−}) and electron-acceptor (γ\text{+}) contributions. All of the S-XJ-1 cells cultivated with different carbon sources were predominantly electron donors (γ\text{−}), with low electron-acceptor (γ\text{+}) parameters. This is consistent with most biological surfaces as a consequence of the presence of oxygen in the atmosphere and the hydration of microbial cells.[3,25,27,28] The γ\text{−} component can be used as a semiquantitative measure of hydrophobicity.[3,28] The cells cultivated with hydrophobic carbon sources had γ\text{−} values (8.8–18.0 mJ/m²) lower than 25.5 mJ/m², which are typical values of hydrophobic bacteria. The γ\text{−} value for the glucose cultivated cells was 57 mJ/m², which is much higher than 35 mJ/m² and means that the cells exhibit hydrophilicity.

### 3.1.4. Cell surface hydrophobicity

The free energy of hydration (Δ\text{G}_\text{bw}), or the total free energy of the interaction between cells in water (Δ\text{G}_\text{bwb}), is a quantitative expression of the CSH. When Δ\text{G}_\text{bw} > −113 mJ/m² or Δ\text{G}_\text{bwb} < 0 mJ/m², the bacteria are considered hydrophobic, conversely, the bacteria are considered hydrophilic.[6] However, Δ\text{G}_\text{bw} is largely of apolar (LW) in nature, especially

![Figure 2. Kinetics of adhesion of S-XJ-1 to the oil phase or the interface from water, cultivated with five different carbon sources.](image-url)
For hydrophobic cells, while the $\Delta G_{bw}$ is largely (90%) due to polar forces (see Table 2). Because hydrophobic interactions are mostly polar (AB) driven, rather than driven by van der Waals attractions between hydrophobic particles,[28] $\Delta G_{bw}$ is the standard quantitative measure of the degree of hydrophobicity.[27] From the results in this study, the S-XJ-1 cells cultivated with the five different carbon sources can be arranged in order of decreasing hydrophobicity from paraffin > octadecane > rape oil > olein > glucose.

### 3.2. Cell biphasic partitioning

To determine the difference in the biphasic partitioning of the S-XJ-1 cells cultivated with different carbon sources, we compared the changes in the MATH value as a function of vortex time (Figure 2). With the exception of the curve for glucose, the kinetic curves increased from zero to a steady-state plateau. The cells cultivated with glucose demonstrated a negative partitioning fraction during the MATH assay, a similar result has also been reported elsewhere.[29] The cause of the negative values may be because the hydrophilic cells did not rise to the oil phase or oil–water interface, but emulsified the oil droplets within the bacterial suspension. This was observed by optical microscope and led to an overestimation of the final absorbance values. These kinetic MATH curves were fitted to the logistic function (Equation 7), which describes a sigmoidal curve,[21] and the kinetic parameters are shown in Table 3. The $%M_{max}$ values of the cells grown with paraffin, octadecane, and

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**Table 2.** Contact angles ($\theta$), surface free energy ($\gamma$) components, and interaction Gibbs free energies of Alcaligenes sp. S-XJ-1 cultured with different carbon sources.

| Carbon source   | Paraffin | Octadecane | Rape oil | Olein | Glucose |
|-----------------|----------|------------|----------|-------|---------|
| Contact angles  |          |            |          |       |         |
| $\theta_W$      | 91.1 ± 1.0 | 79.0 ± 4.9 | 74.6 ± 1.1 | 71.7 ± 1.5 | 40.4 ± 1.4 |
| $\theta_f$      | 83.7 ± 2.2 | 67.5 ± 2.9 | 67.1 ± 2.5 | 64.6 ± 0.8 | 54.8 ± 1.0 |
| $\theta_d$      | 76.6 ± 1.2 | 76.8 ± 0.7 | 71.3 ± 4.0 | 67.4 ± 0.9 | 75.1 ± 1.7 |
| Surface free energies (mJ/m$^2$) |          |            |          |       |         |
| $\gamma_{Total}$ | 19.54 ± 1.43 | 27.32 ± 1.90 | 28.23 ± 4.19 | 30.00 ± 0.97 | 35.25 ± 2.91 |
| $\gamma_{lW}$   | 19.27 ± 0.64 | 19.19 ± 0.38 | 22.15 ± 2.22 | 24.34 ± 0.50 | 20.05 ± 0.91 |
| $\gamma_{AB}$   | 0.27 ± 1.28 | 8.13 ± 1.86 | 6.17 ± 3.55 | 5.66 ± 0.83 | 15.20 ± 2.76 |
| $\gamma^+$      | 0.002 ± 0.03 | 1.51 ± 0.22 | 0.60 ± 0.78 | 0.44 ± 0.22 | 1.01 ± 0.40 |
| $\gamma^-$      | 8.82 ± 1.11 | 10.97 ± 3.50 | 16.00 ± 2.84 | 18.04 ± 2.59 | 57.00 ± 3.54 |
| Interaction Gibbs free energies (mJ/m$^2$) |          |            |          |       |         |
| $\Delta G_{bw}$ | -71.44 ± 0.86 | -86.75 ± 6.10 | -92.13 ± 1.38 | -95.69 ± 1.86 | -128.23 ± 1.52 |
| $\Delta G_{bw,1W}$ | -40.99 ± 0.69 | -40.90 ± 0.40 | -43.95 ± 2.20 | -46.07 ± 0.48 | -41.81 ± 0.95 |
| $\Delta G_{bw,AB}$ | -30.45 ± 0.74 | -45.85 ± 5.98 | -48.18 ± 2.29 | -49.62 ± 1.64 | -86.42 ± 0.23 |
| $\Delta G_{bw}$ | -41.78 ± 2.63 | -26.73 ± 8.27 | -17.98 ± 4.39 | -14.21 ± 4.84 | 40.36 ± 6.04 |
| $\Delta G_{bw,1W}$ | -0.15 ± 0.08 | -0.16 ± 0.05 | -0.002 ± 0.07 | -0.14 ± 0.06 | -0.07 ± 0.07 |
| $\Delta G_{bw,AB}$ | -41.63 ± 2.68 | -26.57 ± 8.25 | -17.97 ± 4.45 | -14.07 ± 4.86 | 40.43 ± 5.98 |

**Table 3.** Adhesion kinetics parameter of the Alcaligenes sp. S-XJ-1 cultivated with different carbon sources measured by the MATH.

| Carbon source | $M_{max}$ (%) | $K$ (s) | $N$ | $\sigma$ |
|---------------|---------------|--------|-----|---------|
| Paraffin      | 88.1 ± 6.5    | 4.1 ± 0.6 | 0.7 ± 0.3 | 0.978 |
| Octadecane    | 90.7 ± 9.2    | 20.1 ± 3.8 | 1.4 ± 0.3 | 0.980 |
| Rape oil      | 85.9 ± 8.7    | 8.2 ± 1.4 | 1.0 ± 0.3 | 0.965 |
| Olein         | 74.9 ± 2.3    | 10.3 ± 0.6 | 1.6 ± 0.3 | 0.987 |
rape oil were very high and reached 85–90%, while the \( K \) value of cells grown with paraffin were lower than others (4.1 s). This indicates that the cells cultivated with paraffin rapidly adhered to the oil phase or the oil–water interface.

### 3.3. Cell aggregation

The demulsifying cell-to-cell interaction in an emulsion is of great significance during the demulsification process. Bacterial aggregation can be used as an indicator to evaluate its demulsifying capability. The aggregation ability of the S-XJ-1 cells cultivated with the five carbon sources is shown in Figure 3. The cells cultivated with paraffin displayed the greatest aggregation potential, where the aggregation percentage dramatically increased to 43.2% in 3 h and then remained relatively constant (47.4%). The cells cultivated with octadecane, rape oil, and olein also exhibited good aggregation potentials, where the aggregation percentage gradually increased to 39.7, 34.2, and 19.0% after 24 h, respectively. However, the aggregation percentage of cells cultivated with glucose was less than 10% after 24 h.

![Figure 3. Plots of % aggregation against standing time of S-XJ-1 suspensions cultivated with five different carbon sources.](image)

**Figure 3.** Plots of % aggregation against standing time of S-XJ-1 suspensions cultivated with five different carbon sources.

### 4. Discussion

The demulsifying strains cultivated with various carbon sources exhibited clear differences in their cell surface composition and demulsifying capability.[19,30] The surface properties of demulsifying cells govern bacterial behaviors in oil–water systems and this is correlated with their demulsifying capability. The demulsifying bacteria, *Alcaligenes* sp. S-XJ-1, with various surface properties were obtained using different carbon sources. However, it is unclear which cell surface properties are governing this process. This work focused on revealing the influence of the demulsifying cell surface properties on bacterial behaviors according to thermodynamic and XDLVO theories, and aimed to determine the main surface properties that contributed to its demulsifying capability.
**4.1. Carbon source influence on cell surface properties**

Bacteria cultivated with various hydrophobic or hydrophilic carbon sources had a significant influence on their cell surface properties.\[16,17\] According to the cell surface charge, the ZP was pH independent at pH > 5, suggesting full deprotonation of surface carboxylate (R-COO−) groups, which means bacterial cell surfaces possess the net negative electrostatic charge by virtue of an ionized carboxylate substituent.\[31\] In our previous study, the potentiometric titration data showed that the proportion of carboxylate groups in the cell surface increased from low to high for S-XJ-1 cultivated with paraffin, n-octadecane, rape oil, olein, and glucose.\[19\] This indicates that the net negative charge of S-XJ-1 cultivated with glucose was higher than other carbon sources, which were dominated by the proportion of carboxylate groups.

According to CSH, the results showed that the S-XJ-1 cells cultivated with hydrophobic carbon sources were hydrophobic, while the glucose cultivated cells were hydrophilic. This corresponds well with many researchers that carbon source-induced changes in CSH occur.\[16,17\] It was inferred that the hydrophobicity of S-XJ-1 cultivated with hydrophobic carbon sources promoted the attachment of cells to oil droplets, allowing an enhanced uptake of water immiscible substrates. Moreover, we found that the CSH of the S-XJ-1 cells cultivated with alkanes (paraffin or octadecane) were significantly higher than cells cultivated with fatty acid esters (rape oil or olein). The results could be attributed to the characteristic cell surface compounds. It has been reported that CSH shows a positive correlation with the nitrogen-to-carbon ratio (N/C) of the bacterial surfaces and a negative correlation with the oxygen-to-carbon ratio (O/C).\[32,33\] For S-XJ-1, the CSH (water contact angle, γ, and ΔG_{bwb}) exhibited a well negative linear correlation with the O/C and a poor, positive correlation with the N/C (data presented in a previous study\[19\]). According to cell surface compounds, oxygen is mainly contributed by polysaccharides, while nitrogen is contributed by proteins. The S-XJ-1 cells cultivated with alkane exhibited an excess of protein, while the cells cultivated with fatty acid esters exhibited an abundant lipid content.\[19\] It could be that the high levels of polysaccharides on the cell surface decreased the hydrophobicity. In contrast, the excess of cell surface protein or lipids increased the hydrophobicity, and the role of surface proteins was more important.

Thus, the demulsifying bacteria S-XJ-1 cultivated with different carbon sources such as alkane, fatty acid ester, or carbohydrate demonstrated various cell surface properties. These changes could be attributed to differences in the cell surface composition.

**4.2. Influence of cell surface properties on cell behaviors**

**4.2.1. Cell biphasic partitioning**

An important step in biological demulsification is the partitioning of demulsifying cells in an oil–water system. Using a thermodynamic approach, the free energies of adhesion of S-XJ-1 cells to the oil–water interface and oil phase were determined from the surface free energies of the bacterial cells, water, and oil (Figure 4). The S-XJ-1 cells cultivated with hydrophobic carbon sources had a favorable adhesion to the oil–water interface because of the large negative free energy changes. The largest negative free energy change for adhesion to the oil–water interface was for paraffin-cultivated cells (−53.8 mJ/m²), followed by cells cultivated with octadecane, rape oil, and olein. The free energy changes for the adhesion
of glucose cultivated cells to the oil–water interface and oil phase were positive (35.9 and 2.1 mJ/m², respectively). This indicates that it is impossible for the cells to move from the water phase to the oil phase or oil–water interface. The biphasic partitioning results for S-XJ-1 obtained from the MATH kinetic assay roughly agreed with the free energy change from the thermodynamic analysis. The paraffin cultivated cells, which had the most negative \( \Delta G \)_{water to interface}, exhibited the highest density and rate of cell adhesion to the oil phase or interface in the experiments, while the glucose cultivated cells were opposite. Therefore, the adhesion density and rate of adhesion of the demulsifying cells to the oil–water interface were dominant in the cell surface free energies.

### 4.2.2. Cell aggregation

Aggregation mechanisms are complex and generally governed by the XDLVO theory by calculating the interaction energy as a function of separation distance (Figure 5). The calculated interaction Gibbs free energy for the glucose cultivated S-XJ-1 cells remained positive for all separation distances, which meant that the cells were always in a repulsion state and the suspension was stable. For the S-XJ-1 cell suspension cultivated with hydrophobic carbon sources, a secondary maximum repulsion of 10 kT was sufficient to maintain stability for some time (Figure 5). Ultimately, gradual destabilization must prevail because of the strong primary minimum of attraction, which is in the order of more than \(-10^3\) kT.\[6\] This means that the bacterial suspension exhibited a certain degree of stability (metastability), and cell aggregation would occur with increasing standing time. The distance at which the value of the interaction energy changed from positive (repulsion) to negative (attraction) gradually shortened from 6.3 to 4.6 nm for cell suspensions cultivated with paraffin, octadecane, rape oil, and olein. Accordingly, the interspace where the cells were in an attractive state narrowed, and the degree of cell aggregation increased. The XDLVO interaction energy profiles are in good agreement with the experiment results of the aggregation behavior of S-XJ-1. To ascertain which components were dominant, \( \Delta G \) was separately plotted against interparticle distance for the three different functions (LW, AB, and EL), and then combined into a \( \Delta G \) vs. distance plot, as shown in supplemental figure, Figure S1. These results suggested that the interaction between the cells was driven by repulsive EL interactions at long range, and attractive or repulsive AB interactions became pivotal at close range, which determines cell aggregation.

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**Figure 4.** Diagram showing of Gibbs free energy change for adhesion of S-XJ-1 from water to the kerosene phase and the kerosene–water interface, cultivated with five different carbon sources. The kerosene–water interface is shown by the red line.
The demulsifying capability of S-XJ-1 was directly correlated with the demulsifying cells’ biphasic partitioning and aggregation behavior. Thus, understanding the relationship between demulsifying bacterial behaviors and cell surface properties is of great interest. Bacterial behaviors are governed, not only by LW, AB, and EL interactions, but also by steric repulsion and bridging attraction, because bacterial cell surfaces are structurally and chemically heterogeneous.[34–37] In this study, the behavior of S-XJ-1 was mainly dependent on interaction forces (particularly for the AB interaction), pointing to the possible influence of cell surface properties. Accurate quantification of S-XJ-1 cell surface properties and information about the forces governing the cell behaviors at the molecular scale will be studied in the future.

4.3. Influence of cell surface properties on demulsifying capability

CSH and surface charges can be used to evaluate the influence of cell surface properties on demulsification.[9,10] It has been shown that the ability of bacterial demulsifiers to break emulsions has a positive correlation with CSH.[11–13] Most bacterial cells are negatively charged, although the effect on demulsification is not significant. Contact angle measurements, MATH, and adhesion to polystyrene appear to be most commonly used to characterize CSH.[38] The MATH test, a simple and easy hydrophobicity assay, has promoted researchers to consider the hydrophobic effect.[39,40] However, the MATH test also measures the combination of electrostatic and other ‘hydrophobic’ interactions as well as hydrophobicity.[41] The MATH assay is appropriate for evaluating bacterial adhesion to a hydrocarbon phase. The contact angle measurements are less affected by electrostatic and other interactions and has a solid theoretical basis. The hydrophobic interaction free energy of bacterial cells in water ($\Delta G_{\text{bwb}}$) calculated from the contact angle data can be used as a quantitative measure of CSH.[6]
In previous studies, the correlation between the demulsification ratios and MATH values were positive for S-XJ-1 cultivated with various pH mediums ($R^2 = 0.77$) [11] and different fatty acid esters (vegetable oils) as carbon sources ($R^2 = 0.88$).[12] In this study, S-XJ-1 was cultivated with various types of hydrocarbons, fatty acid esters, and glucose carbon sources, and the positive linear relationship between the demulsification ratio and the MATH value was not good. Generally, the higher the amount and speed of demulsifying cell adhesion to a hydrocarbon, the better the demulsifying efficiency of the demulsifying cells. When the $\Delta G_{bwb}$ value was used to characterize CSH, the $\Delta G_{bwb}$ value for S-XJ-1 cultivated with glucose was 40.36 mJ/m$^2$, which indicated a strong hydrophilicity and low demulsifying efficiency. In contrast, the $\Delta G_{bwb}$ values of S-XJ-1 cultivated with the different hydrophobic carbon sources were negative, which indicated that the cells were hydrophobic. The plot of $\Delta G_{bwb}$ values against the demulsification ratio of S-XJ-1 (data provided in a previous study [19]) exhibited a significant negative linear correlation ($R^2 = 0.97$), as shown in Figure 6. This indicates that the hydrophobic interaction energy ($\Delta G_{bwb}$) can accurately quantify the CSH of demulsifying cells and shows a good relationship with demulsifying efficiency. In addition, the magnitude of the electron-donor character ($\gamma^-$) was well correlated with the hydrophobic interaction energy ($\Delta G_{bwb}$) of the demulsifying cells ($R^2 = 0.97$), similar to results reported by Ozkan. [42] Thus, the lower the electron-donor character ($\gamma^-$) and the higher the CSH, the greater the demulsifying efficiency of S-XJ-1.

5. Conclusions

The demulsifying strain Alcaligenes sp. S-XJ-1 cell biphasic partitioning and aggregation behavior are correlated with the LW, AB, and EL interfacial energy components, which are mainly governed by the cell surface free energy and charge. When comparing the different cell surface properties of S-XJ-1 cultivated with five different carbon sources, the electron-donor character ($\gamma^-$), one of the polar free energy components of the cells, was found to be crucial for controlling cell behavior. The lower the value of $\gamma^-$, which equates to a higher CSH ($\Delta G_{bwb}$), the more cells adhere to the oil–water interface and the higher
the cell aggregation percentage, which eventually leads to demulsification. Determining the cell surface properties that contribute to cell behavior in oil–water biphasic systems helps to elucidate microbial demulsification mechanisms. These results and analyses are a useful reference when investigating microorganism behavior at the oil–water interface.

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