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ORIGINAL RESEARCH PAPER

Attachment of *Acidithiobacillus ferrooxidans* onto different solid substrates and fitting through Langmuir and Freundlich equations

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Abstract Attachments of *Acidithiobacillus ferrooxidans* ATCC 23270 onto elemental sulfur, quartz and complex chalcopyrite were investigated by analysis of its extracellular polymeric substances as well as applying Langmuir and Freundlich equations. The two equations fitted the adsorption equilibrium data with significant correlation coefficient over 0.9. This indicated that bacterial attachment is complicated and involves Langmuir and Freundlich characterizations. Sulfur-grown cells showed the highest affinity for the three solid substrates. The investigated complex chalcopyrite possessed a higher maximum adsorption capacity for *A. ferrooxidans* than elemental sulfur or quartz. The Freundlich fitting parameters suggested that quartz had a weaker adsorption capacity and smaller adsorption areas than elemental sulfur or the complex chalcopyrite. It is not the content of total carbohydrates or proteins in EPS but their ratios that determine the affinity differences between cells and substrates.

Keywords *Acidithiobacillus ferrooxidans* · Attachment · Extracellular polymeric substances · Freundlich equation · Langmuir equation

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Introduction

Acidithiobacillus ferrooxidans is important in bioleaching as it can grow by oxidizing substrates such as ferrous salts elemental sulfur or sulfide minerals (Rohwerder and Sand 2007). Differences in physicochemical properties of substrates may induce physiological differences in chemotaxis as well as other responses including attachment behaviour, substrate recognition and induced-expression of function genes (Delgado et al. 1998, 1999). Bacterial attachment is a prerequisite for colonization and formation of biofilms on a mineral surface. As a result, a sulfide mineral is eroded by the attached cells and bacteria-like corrosion pits are formed (Rodriguez-Leiva and Tributsch 1988; Ostrowski and Skłodowska 1993). This is the basis of direct-bioleaching (Van Loosdrecht et al. 1990; Watling 2006). Also,

bacterial attachment onto mineral surfaces can release Fe^{3+} from minerals and H^+ from sulfur bio-oxidization (Eqs. 1, 2, 3) that enhance chemical leaching of some sulfide minerals such as chalcopyrite and pyrite (Ramírez et al. 2004). Therefore, bacterial attachment is significant for indirect bioleaching.

$$CuFeS_2 + 4Fe^{3+} \rightarrow Cu^{2+} + 5Fe^{2+} + 2S^0 \quad (1)$$

$$2S^0 + 3O_2 + 2H_2O \overset{\text{bacteria}}{\longrightarrow} 2SO_4^{2-} + 4H^+ \quad (2)$$

$$4Fe^{2+} + O_2 + 4H^+ \overset{\text{bacteria}}{\longrightarrow} 4Fe^{3+} + 2H_2O \quad (3)$$

Bacterial attachment onto a mineral surface in the early stages is considered as a transitory and reversible process (Africa et al. 2010; Coram-Uliana et al. 2006). This process is closely related to the structures and properties on mineral and cell surfaces such as extracellular polymeric substances (EPS), charge and hydrophobicity/hydrophilicity (Sharma et al. 2003). On the cell surfaces, bacteria produce a glycocalyx and EPS that bind Fe^{3+} in the bioleaching of sulfide minerals. EPS can mediate the attachment of *A. ferrooxidans* onto pyrite, chalcopyrite or quartz. The removal of EPS decreases the attachment of *A. ferrooxidans* onto minerals (Harneit et al. 2006; Kinzler et al. 2003; Sand et al. 1995). EPS-complexed Fe^{3+} leads to an increase of Fe^{3+} concentration around mineral particles by attached cells and contributes to dissolution of sulfide minerals (Sand and Gehrke 2006; Gehrke et al. 1998). Langmuir and Freundlich adsorption isotherms are widely-used to describe simple adsorptions such as metal ion adsorption onto bacteria or organic matter adsorption onto soil particles. These attachments are related to simple physical or chemical behaviours (Pagnanelli et al. 2003; Aksu and Akpinar 2001). Bacterial attachments onto sulfide minerals have also been investigated and correlate with Langmuir adsorption isotherms (Tan and Chen 2012; Song et al. 2010). However, a single adsorption isotherm was only used to fit the bacterial attachment in these researches. Bacterial attachments onto sulfide minerals in bioleaching systems are different from other usual attachments and involve biological interactions due to bacterial oxidization to growth substrates. Thus, a single model cannot clearly describe the microbial attachment occurred on mineral surfaces in bioleaching systems. Additionally, different growth substrates possess the various physicochemical properties and can induce different cell physiological behaviours including EPS generation on cell surfaces (Sharma et al. 2003). Consequently, these differences result in different bacterial attachment behaviours to various growth substrates. However, the understanding on their attachment differences and how to correlate with substrates and EPS is lacking in detail.

Ghauri et al. (2007) reported the differences in the attachment rate of four strains of *A. ferrooxidans* to pyrite, glass and ferric hydroxysulfates. There are also strain differences in attachment capability, metabolic activity and the amount of EPS produced (Harneit et al. 2006). However, pure minerals were used in these researches but most minerals in industrial operations are complex with various components. Thus, these researches cannot provide information for an understanding on the bacterial attachment in industrial operations. The type strain of *A. ferrooxidans* ATCC 23270 is one of the most widely-studied and important bioleaching microorganisms. However, its attachment onto complex chalcopyrite has not been investigated to any depth. In this paper, both Langmuir and Freundlich equations were used to describe the attachment of *A. ferrooxidans* grown on Fe^{2+}, sulfur and complex chalcopyrite onto three solid adsorption substrates (sulfur, quartz and complex chalcopyrite). In each case, the amount of EPS was quantified to clarify differences in bacterial attachment.

Materials and methods

Minerals

Elemental sulfur, quartz and complex chalcopyrite were finely ground (<74 μm) for bacterial attachment experiments. The ground quartz and complex chalcopyrite were sterilized by the alternate treatments of cooling and heating (−80 °C for 24 h, 100 °C for 1 h) for five cycles. Preliminary experiments indicated that the treated minerals could be used for further study. Elemental sulfur was sterilized by dry-heat sterilization for five cycles (each cycle for 20 min) at 100 °C. Complex chalcopyrite was composed of chalcopyrite 61.7 %, sphalerite 29.7 %, PbS 3.9 %, $PbSO_4$ 4.63 % and $PbCuAsS_3$ 1.1 % (w/w).

Strains and culture conditions

Acidithiobacillus ferrooxidans^T (ATCC 23270-type strain) was inoculated into 100 ml 9 K medium in

Erlenmeyer flask (250 ml) with shaking (170 rpm) at an initial pH of 2 and 30 °C. 9 K medium containing 3 g $(NH_4)_2SO_4$ l^{-1}, 0.5 g $MgSO_4{\cdot}7H_2O$ l^{-1}, 0.5 g K_2HPO_4 l^{-1}, 0.1 g KCl l^{-1}, 0.01 g $Ca(NO_3)_2$ l^{-1} was used as a basal salt medium. Either 44.7 g $FeSO_4{\cdot}7H_2O$ l^{-1}, 10 g elemental sulfur l^{-1} or 10 g complex chalcopyrite l^{-1} were added into the basal salt medium as bacterial growth energy substrates. Cultures were filtered through 0.45 μm Whatman filter paper and then centrifuged at $\sim 10{,}000 \times g$ for 20 min at 4 °C. The cell pellets were washed for three times with sterile 9 K salt medium at pH 2 to prepare for bacterial attachment experiments and EPS extraction. All chemicals used were analytical grade reagents.

Bacterial attachment experiments

Attachment kinetics experiments were conducted on a rotary shaker (170 rpm) with 250 ml Erlenmeyer flasks at 30 °C. Bacteria were incubated in 100 ml basal salt medium (pH 2) with either 10 g elemental sulfur l^{-1}, 10 g chalcopyrite l^{-1} or 10 g quartz l^{-1}. The concentration of free cells (C_T) was determined microscopically using a blood cell counting chambers.

Equilibrium attachment time was determined as 1 h based on attachment kinetics data. The collected cells were inoculated into the above 9 K medium to generate a series of initial cell concentrations ranging from 10^7 to 5×10^8 cells ml^{-1}. The equilibrium adsorption capacity per unit mass of mineral (X_E, cells g^{-1}) was calculated on the basis of the initial concentration of free cells (C_0, cells ml^{-1}) and the cell equilibrium concentration in the liquid phase (C_E, cells ml^{-1}). Bacterial attachment experiments were carried out in duplicate.

Langmuir and Freundlich adsorption models

The application of Langmuir model is based on three assumptions: (1) monomolecular adsorption; (2) uniform adsorbent surface; (3) no interaction between any two cells. The Langmuir isotherm equation (Langmuir 1918; Song et al. 2010; Tan and Chen 2012) is described as follows:

$$X_E = \frac{X_M \cdot K_L \cdot C_E}{1 + K_L \cdot C_E} \tag{4}$$

Equation 4 can be easily linearized as the reciprocals as follows:

$$\frac{1}{X_E} = \frac{1}{X_M \cdot K_L} \cdot \frac{1}{C_E} + \frac{1}{X_M} \tag{5}$$

where X_M is the maximum adsorption capacity per unit mass of adsorbent (cell g^{-1}), and K_L is the Langmuir adsorption equilibrium constant (g $cells^{-1}$). X_M and K_L are determined by the plots of $1/X_E$ versus $1/C_E$.

Given the heterogeneous surface of solid absorbent and multilayer adsorption, Freundlich model is usually used to illustrate bacterial attachment it presents as:

$$X_E = K_F \cdot C_E^{1/n} \tag{6}$$

Freundlich equation can be written in the logarithmic form as follows:

$$\log X_E = \log K_F + \frac{1}{n} \log C_E \tag{7}$$

where K_F is a rough measure of the attachment surface area of solid substrates, reflecting the adsorption capacity. And n is an indicator of adsorption effectiveness, and represents the inherent properties of adsorbent.

Extraction and chemical analysis of EPS

The collected cells, grown with various growth substrates, were suspended in 10 ml sterile water (pH 2), followed by holding in an ice bath for 1 h. The cell suspension was used for the extraction of EPS using an ultrasonic method (Yu et al. 2011). The concentrations of total EPS and proteins were determined by the phenol/sulfuric acid and Bradford methods, respectively. The quality of extracted EPS was evaluated by analysis of 2-keto-3-deoxyoctonate and DNA (Adav et al. 2009).

Results and discussion

Bacterial attachment kinetics

As shown in Fig. 1, the attachment equilibrium time of *A. ferrooxidans*^{T} in all experiments was different but no more than 60 min. Free cells (C_T) decreased and attached cells increased over time. Bacterial attachment onto a solid substrate is a dynamic process. In the first 10 min of bacteria-adsorbent contact, the attachment rate was considerably greater than the detachment

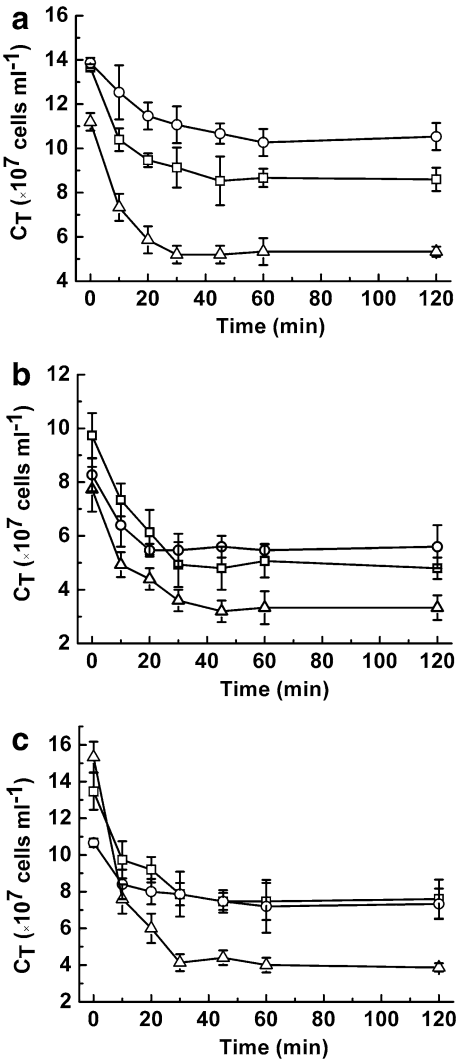

Fig. 1 Changes of the number of free cells (C_T) of *A. ferrooxidans*^{T} grown with Fe^{2+} (**a**), elemental sulfur (**b**) and complex chalcopyrite (**c**) as growth substrates over time. Each kind of adsorbent, 10 g l^{-1} elemental sulfur (*open squares*), 10 g l^{-1} quartz (*open circles*), 10 g l^{-1} complex chalcopyrite (*open triangles*), was inoculated into bacterial suspension. The mixture was incubated for 10, 20, 30, 45, 60, 120 min on a rotary shaker (170 rpm), respectively

rate, then the attachment rate decreased and the detachment rate increased till the occurrence of attachment equilibrium.

Linear fitting of Langmuir isotherm

The linear fitting of Langmuir equation revealed the significant correlation with the attachments between cells and the three adsorption substrates in all systems (Fig. 2). But the fitting parameters were obviously different (Table 1). The parameter K_L indicates the bacteria-adsorbent affinity. Sulfur-grown *A. ferrooxidans*^{T} exhibited higher affinity for the three adsorption substrates due to its higher K_L value than Fe^{2+}- or complex chalcopyrite-grown *A. ferrooxidans*^{T}, which could be contributed to the stronger hydrophobicity of sulfur-grown cells than other cells (hydrophobicity is responsible for bacterial attachment onto hydrophobic substrate). Porro et al. (1997) measured the hydrophobicity of *A. ferrooxidans* and *Acidithiobacillus thiooxidans* grown on various substrates by liquid–liquid partition in aqueous and organic phase. Their result also showed that sulfur-grown cells possessed a stronger hydrophobicity than those grown on other growth substrates. Complex chalcopyrite surface showed a higher maximum attachment capacity (X_M) for *A. ferrooxidans*^{T} than elemental sulfur or quartz surfaces (Table 1). The BET surface areas of chalcopyrite and pyrite were larger than that of quartz, the X_M of *A. ferrooxidans* cells was larger on sulfide minerals than that on quartz (Tan and Chen 2012). Harneit et al. (2006) revealed that the amounts of *A. ferrooxidans* attached on chalcopyrite and sphalerite surfaces were significantly larger than that on quartz surfaces. Chalcopyrite in the experiments is a complex chemical composition (chalcopyrite 61.7 % and sphalerite 29.7 %, w/w). Thus, we proposed that the complexity of chemical components leaded to more binding sites for bacterial attachment than a single chemical component of quartz or elemental sulfur.

Various metal ions from the dissolution of sulfide minerals play important roles in regulating the chemotaxis response of *A. ferrooxidans* and *Leptospirillum ferrooxidans* (Jerez 2001). This chemotaxis response is closely related to bacteria-adsorbent contact and the change of specific proteins on cell surfaces (Delgado et al. 1998; Jerez 2001). Therefore, the cellular surface structures of complex chalcopyrite-grown cells are more complicated than that of other substrates-grown cells due to the complex components released from complex chalcopyrite (containing sulfur, iron, jarosite, chalcopyrite, sphalerite and so on). Thus, a part of cells from complex chalcopyrite system were grown by oxidizing iron and/or sulfur, which led to the higher affinity of complex chalcopyrite-grown cells than iron-grown cells and lower affinity than sulfur-grown cells. Additionally, Langmuir fitting parameters indicated

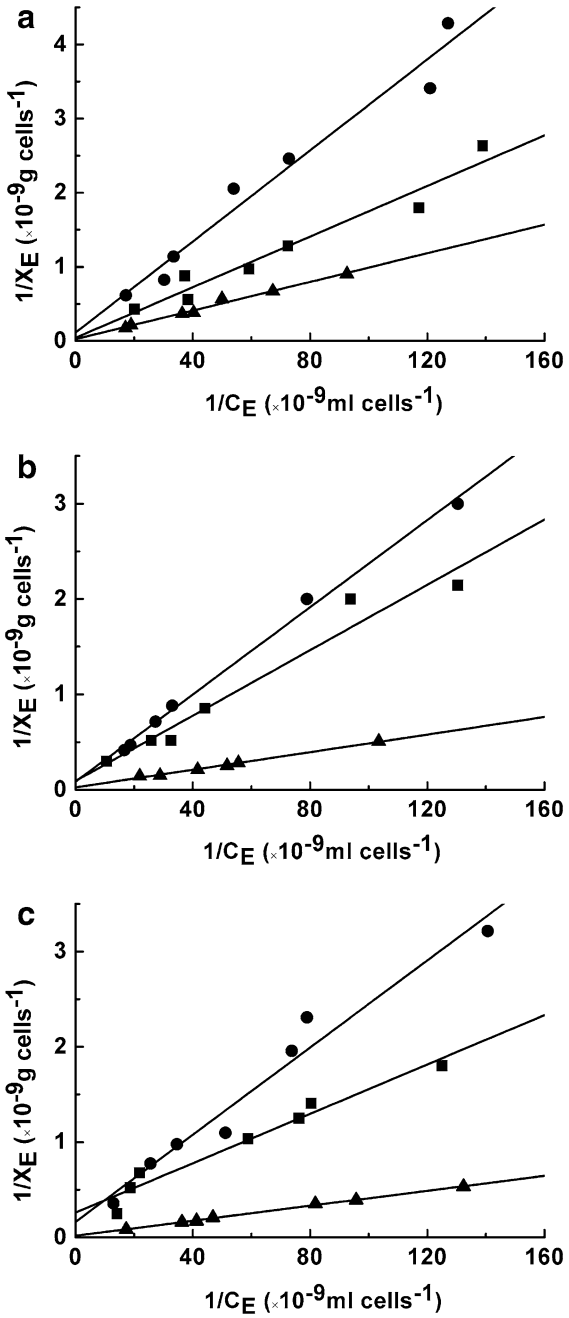

Fig. 2 Langmuir linear fitting of attachment equilibrium data of *A. ferrooxidans*^{T} grown with Fe^{2+} (**a**), elemental sulfur (**b**) and complex chalcopyrite (**c**) as growth substrates. 10 g l^{-1} Elemental sulfur (*closed squares*), 10 g l^{-1} quartz (*closed circles*), 10 g l^{-1} complex chalcopyrite (*closed triangles*) were determined as adsorption substrates for bacterial attachment

that the affinity of cells for solid substrates is not related to maximum adsorption capacity. This is because the bacteria-mineral affinity is dependent on the surface properties of cells and solid adsorption substrates, as well as their interaction such as electrostatic forces and hydrophobicity. But the maximum adsorption capacity is determined by valid surface areas of solid substrates for bacterial attachment.

Linear fitting of Freundlich isotherm

The linear fitting of Freundlich equation exhibited a significant correlation (Fig. 3). The Freundlich parameter K_F indicates the adsorption capacity of adsorbent (Deepatana et al. 2006). The K_F of *A. ferrooxidans*^{T} attached onto quartz was lower than those on elemental sulfur or chalcopyrite (Table 1). This result indicated that quartz as adsorbent had the lowest adsorption capacity of cells, which was in agreement with experimental data from the Langmuir equation fitting. *A. ferrooxidans* has no physiological tendency to quartz because bacteria cannot oxidize quartz for its growth. The attachment of *A. ferrooxidans* onto adsorption substrates is defined as a selective attachment (Harneit et al. 2006). A lot of *A. ferrooxidans* cells attached onto pyrite or chalcopyrite, but few attached onto galena or quartz (Ohmura et al. 1993; Tan and Chen 2012). The analysis of Freundlich fitting parameters showed that complex chalcopyrite-grown *A. ferrooxidans*^{T} has a relatively larger K_F value which represents a higher adsorption capacity. As the former segment mentioned, Fe^{2+}, thiosulfate and sulfur would be generated from the dissolved complex chalcopyrite, thus, *A. ferrooxidans* can grow not only with soluble Fe^{2+} or thiosulfate, but also with sulfur or complex chalcopyrite as growth substrates in the bioleaching of complex chalcopyrite. These new substrates from the dissolution of sulfide minerals induced the secretion of specific proteins on cell surfaces, in response to chemotactic interaction between cells and minerals (Delgado et al. 1999; Zhulin 2001). Therefore, the cellular surfaces of complex chalcopyrite-grown *A. ferrooxidans*^{T} exhibited various chemical components that resulted from both soluble substrates (Fe^{2+}, thiosulfate) and insoluble substrates (sulfur, chalcopyrite). The complex chemical components contribute to the higher adsorption capacity of complex chalcopyrite-grown *A. ferrooxidans*^{T} onto the three solid substrates.

Theoretically, Langmuir isotherm reflects a monolayer adsorption and Freundlich isotherm reflects a multi-layer or mono-layer adsorption. Here, the data

Table 1 Langmuir and Freundlich fitting adsorption parameters under different conditions

Growth substrate	Adsorption substrate	Langmuir fitting parameters			Freundlich fitting parameters		
		$K_L \times 10^{-9}$ (ml cells^{-1})	$X_M \times 10^{10}$ (cells g^{-1})	R^2	K_F	n	R^2
Fe^{2+}	Sulfur	2.4	2.4	0.94	303.2	1.1	0.92
	Quartz	3.7	0.9	0.91	87.2	1.1	0.95
	Complex chalcopyrite	2.5	4.2	0.98	104.2	1.0	0.99
Sulfur	Sulfur	5.3	1.1	0.95	606.8	1.2	0.95
	Quartz	5.9	0.7	0.98	55.5	1.0	0.98
	Complex chalcopyrite	5.7	3.9	0.98	4170.9	1.2	0.96
Complex chalcopyrite	Sulfur	0.2	0.4	0.95	2734.8	1.3	0.89
	Quartz	7.1	0.6	0.96	133.4	1.1	0.97
	Complex chalcopyrite	4.6	5.5	0.99	884.6	1.1	0.99

Adsorption equilibrium parameters were determined by linear fitting of adsorption equilibrium data with Eqs. 5 and 7

from bacterial attachment experiments were well fitted by both Langmuir and Freundlich equations. This revealed that bacterial attachments onto sulfide minerals were complicated in bioleaching and different from other usual attachments.

Analysis of extracellular polysaccharides and proteins

The contents of total extracellular polysaccharides (C_{TS}) and proteins (C_P) and the ratio of total polysaccharides to proteins are shown in Table 2. The low amounts of KDO and DNA indicated that the trace contamination from damaged cells could be negligible when the ultrasound method was used to extract EPS (data not shown). The protein content of sulfur-grown cells was similar to Fe^{2+}-grown cells, and C_{TS} of chalcopyrite-grown cells was similar to that of sulfur-grown cells. But there were significant differences in the ratio of total polysaccharides to proteins. The ratio (C_{TS}/C_P) for Fe^{2+}-grown cells was the greatest but was the lowest for sulfur-grown cells. Considering the adsorption affinities of cells grown with the three different growth substrates, the adsorption affinity differences correlated better with C_{TS}/C_P than to C_{TS} or C_P. It is not difficult to understand this relationship: bacterial attachment onto hydrophobic substrate surfaces is not mainly dependent on hydrophilic interaction but hydrophobic interaction. Moreover, hydrophobicity of cell surfaces is mostly determined by proteins in EPS, and hydrophilicity is mostly determined by extracellular polysaccharides (because of lots of hydroxyl groups) in EPS (Zhang et al. 2007). Additionally, extracellular polysaccharides and uronic acid in EPS are hydrophilic by binding irons from medium (Sand and Gehrke 2006). The above results suggested that elemental sulfur-grown cells exhibited the highest hydrophobicity and Fe^{2+}-grown cells had the lowest hydrophobicity. Likewise, *A. ferrooxidans*^{T} grown on solid-substrate exhibited higher hydrophobicity than those grown in the soluble iron. The result was in agreement with the previous report (Sharma et al. 2003).

Conclusions

Both Langmuir and Freundlich equations could be used to fit the adsorption equilibrium data of *A. ferrooxidans*^{T} on the three solid adsorption substrates with significant correlation coefficient. This indicated that bacterial attachments onto the three adsorption substrates were complicated and followed Langmuir and Freundlich models including monolayer homogeneous attachment and multilayer uneven attachment.

Langmuir fitting parameters indicated that there are significant differences in the early attachments of *A. ferrooxidans*^{T} grown on different growth substrates on the three adsorption substrates. Sulfur-grown *A. ferrooxidans*^{T} exhibited the highest affinity for the three adsorption substrates. There are more specific binding sites for the attachment of *A. ferrooxidans*^{T} cells on complex chalcopyrite surface than quartz or

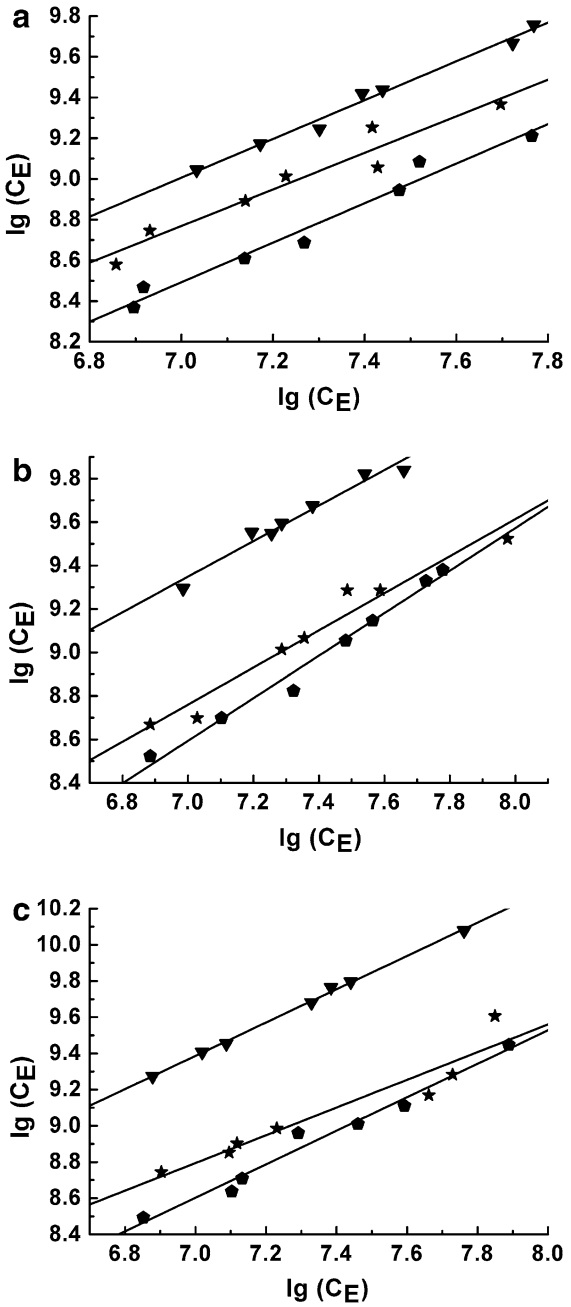

Fig. 3 Freundlich linear fitting of attachment equilibrium data of *A. ferrooxidans*^{T} grown with Fe^{2+} (**a**), elemental sulfur (**b**) and complex chalcopyrite (**c**) as growth substrates. 10 g l^{-1} Elemental sulfur (*closed pentangles*), 10 g l^{-1} quartz (*closed pentagons*), 10 g l^{-1} complex chalcopyrite (*closed triangles*) were determined as adsorption substrates for bacterial attachment

elemental sulfur. Freundlich fitting parameters indicated that quartz had the lowest adsorption capacity of *A. ferrooxidans*^{T} cells.

Table 2 The content of total polysaccharides and proteins per gram of dry cells

Growth substrate	C_{TS} (mg g^{-1})	C_P (mg g^{-1})	C_{TS}/C_P
Fe^{2+}	72 ± 5.7	21 ± 2.9	3.5
Elemental sulfur	51 ± 3	21 ± 1.2	2.5
Complex chalcopyrite	52 ± 1.5	18.4 ± 1.1	2.8

mg g^{-1} is the unit of content and it indicates the quality of total polysaccharides and proteins per gram of dry cells. The ultrasonic extraction of EPS was carried out on 80 W for 12 min. After ultrasonic extraction and high-speed centrifugation (10,625×*g*) for 20 min, the supernatant was defined as EPS solution without cells, and stored at 20 °C for chemical analysis of total polysaccharides and proteins

The quantification result of EPS revealed that the various ratios of total polysaccharides to proteins are responsible for the differences in the attachments of *A. ferrooxidans*^{T} onto three adsorption substrates.

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