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Production of extracellular polymeric substances from *Rhodopseudomonas acidophila* in the presence of toxic substances

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Abstract A hydrogen-producing photosynthetic bacteria strain, *Rhodopseudomonas acidophila*, was used to investigate the production of extracellular polymeric substances (EPS) in the presence of toxic substances and the effect of toxicants on bacterial surface characteristics. Addition of the toxic substances including Cu(II), Cr(VI), Cd(II) and 2,4-dichlorophenol (2,4-DCP) stimulated the production of EPS but reduced the cell dry weight. At concentrations of 30 mg l^{-1} Cu(II), 40 mg l^{-1} Cr(VI), 5 mg l^{-1} Cd(II) and 100 mg l^{-1} 2,4-DCP, the EPS content increased by 5.5, 2.5, 4.0 and 1.4 times, respectively, than the control. These toxic substances also greatly influenced the proteins/carbohydrates ratio of EPS. The ratios in the presence of toxic substances were always higher than that of control. Furthermore, under toxic conditions, the increase in the protein content far exceeded than that of others in EPS, suggesting that extracellular proteins could protect cells against toxic substances. The toxic substances significantly changed the surface characteristics and flocculation ability of *R. acidophila*, such as surface energy, relative hydrophobicity and free energy of adhesion.

Introduction

In biological wastewater treatment, biomass generates extracellular polymeric substances (EPS) when consuming organic materials present in the wastewater. EPS are complex mixture of high molecular polymers (M_w>10,000) excreted by microorganisms, products from lysis and hydrolysis and adsorbed organic matters from wastewater. EPS of bacteria are involved in the formation of microbial aggregates, adhesion to surfaces and flocculation (Wingender et al. 1999). Furthermore, EPS are a major component of aggregates for keeping the floc together in a three-dimensional matrix due to bridging with multivalent cations and hydrophobic interactions (Frolund et al. 1996). Such a polymeric network has vast surface areas and is capable of adsorbing pollutants, nutrients and minerals. EPS play an important role in the flocculation of bacterial cells and provide with energy and carbon when substrate is in short supply. They also protect the cells from the harsh external environment (Wingender et al. 1999).

Biological hydrogen production from organic wastes by microorganisms, including photosynthetic bacteria (PSB), has attracted considerable attention as an efficient way of converting wastes to hydrogen (Barbosa et al. 2001). From application point of view, PSB is expected to be cultivated in a photo-bioreactor to continuously utilize short-chain organic acids in wastewater as electron donors to produce H_2 at the expense of solar energy. However, since the flocculation ability of PSB is poor, the PSB cells cannot be efficiently separated from supernatant, resulting in a low PSB cell concentration in a H_2-producing reactor (Watanabe et al. 1998). To solve this problem, it is essential to explore the flocculation and adhesion characteristics of PSB. Bacterial flocculation is highly related with EPS contents, components and bacterial surface characteristics. The contents of carbohydrates, proteins and nucleic acids in EPS have a substantial effect on the flocculation of bacterium (Watanabe et al. 1998). Large amount of work has been conducted on the relationship between EPS and the surface characteristics of activated sludge (Wilen et al. 2003). However, information on the production of EPS from PSB and relevant surface characteristics of bacterium is still sparse.

On the other hand, wastewaters used for H_2 production by PSB may contain many substances that are toxic to the bacteria. The cell wall is the first site of interaction between cell and its surrounding environment, and microorganisms would thus produce more EPS to cover the cell wall to protect themselves when they are exposed to unfavorable environment (Aquino and Stuckey 2004). Fukushi et al. (1996) reported that anionic ligands, e.g., proteins, lipids, polysaccharides, glycocalyxes, nucleic acids, found in biopolymers located at the cell membrane and cell wall could

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bind various metals. Santamaria et al. (2003) found that the EPS surrounding cells were able to chelate some metals and to bind the metal to bacterial surface. Some studies have demonstrated that EPS production is enhanced in the presence of toxic substances (Aquino and Stuckey 2004). It is not clear, however, how significant EPS production is under toxic conditions and which contents in EPS are more significantly influenced by the environmental stress. Simultaneously, the production of EPS under toxic conditions might change the surface characteristics and flocculation ability of PSB.

Therefore, the aim of this work was to investigate the growth of PSB and the production of EPS in the presence of toxic substances, such as Cu(II), Cr(VI), Cd(II) and 2,4-dichlorophenol (2,4-DCP). These toxic substances are frequently found to be present in various wastewaters. In this work, a hydrogen-producing PSB strain, *Rhodopseudomonas acidophila*, was used as the target bacterium. This strain can utilize acetate, propionate and butyrate as substrates to produce H_2. In addition, the surface characteristics of this bacterium in the presence of Cu(II) were also evaluated.

Materials and methods

Photosynthetic bacterium and growth condition

R. acidophila was obtained from the East Sea Fisheries Research Institute, China. At 4,000 lx and 30°C, 494 ml H_2 was produced with a maximum production rate of 21.9 ml l^{-1} h^{-1} from a mixture of 1.8 g l^{-1} acetate, 1.0 g l^{-1} propionate and 0.4 g l^{-1} butyrate within 208 h. The strain was anaerobically grown in a modified aSy medium at pH 7.0. This medium was composed of a basal solution per liter: KH_2PO_4 0.5 g, K_2HPO_4 0.6 g, NaCl 0.4 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, $CaCl_2{\cdot}2H_2O$ 0.05 g, $(NH_4)_2SO_4$ 1.25 g, $(CH_2COONa)_2{\cdot}6H_2O$ 9.8 g, $FeSO_4{\cdot}7H_2O$ 1 mg, $(NH_4)_6Mo_7O_{24}$ 0.5 mg, $CoCl_2{\cdot}6H_2O$ 0.01 mg, $ZnCl_2$ 0.1 mg, $CuCl_2$ 0.01 mg, H_3BO_3 2 mg, EDTA-2Na 2 mg, vitamin B_1 1 mg, biotin 15 μg. The culture was grown in 300-ml rubber-stopper vials at 30°C and 3,000 lx for 70 h. The vials were purged with argon to create anaerobic conditions. The toxic substances, including Cu(II), Cr(VI) and Cd(II), as the form of $CuSO_4$, K_2CrO_7, $CdSO_4$, and 2,4-DCP, were respectively dosed to medium to desired concentrations prior inoculation. The vial without dosage of the toxic substances was run as the control.

Extraction of EPS

The EPS of *R. acidophila* was extracted by using ethylenediaminetetraacetic acid disodium (EDTA). This method was better than other extraction methods including heating, alkaline, sulphuric acid and high-speed centrifugation because of its higher extraction efficiency and lower cell lysis (Sheng et al. 2005). Since *R. acidophila* had poor flocculation ability, a centrifugation time as long as 10 min and a centrifugation speed as high as 12,000 rpm were selected for the efficient separation of the cell from the solutions (Sheng et al. 2005). After 70-h cultivation, 20-ml PSB solution was harvested by centrifugation at 12,000 rpm and 4°C for 10 min immediately, and then the pellets were washed twice with 0.9% NaCl solution to minimize the desorption of bound EPS and the cell lysis. Later, the cell pellets were transferred to 10-ml double-distilled water. Thereafter, 5 ml EDTA (2%) was added and placed at 4°C for 3 h. The dosage of EDTA and the extraction time were chosen based on the preliminary tests (Sheng et al. 2005). After that, the supernatant was centrifuged at 12,000 rpm and 4°C for 30 min in order to remove remaining cells. The supernatant was dialyzed against double-distilled water for 24 h and was then filtrated through 0.45-μm cellulose acetate membrane. The extraction was performed in duplicate. The supernatant was used as the EPS fraction for chemical analyses.

Chemical analysis

All chemicals used in this work were of analytical grade. Cell growth was measured by the dry cell weight. After 10-min centrifugation at 12,000 rpm, the cell pellets were washed twice with double-distilled water to remove the remaining components of medium and then were dried at 105°C for 2 h. The pellets were placed in a desiccator and cooled for 30 min. Thereafter, the dry cells were weighted. The contents of carbohydrates and proteins were determined, respectively, by the anthrone method using glucose as a standard and the Lowry method with egg albumin as a standard (Frolund et al. 1996). The content of nucleic acids was determined according to Boonaert et al. (2001) using a UV spectrophotometer (UV751GD, Analytical Instrument Co., Shanghai). The total content of EPS was measured as the sum of the three components.

The contents of Cu, Cd and Cr were measured using atomic adsorption spectrometry (AAS Vario 6, Analytikjena Co., Germany). Samples were diluted 1:1 in concentrated nitric acid and heated at 120°C for 1 h and were then diluted in 5% HCl solution for analysis.

The contents of 2,4-DCP in medium and EPS were determined by HPLC (HP1100, Agilent Co., USA) equipped with a 5 μm×4 mm×250 mm Hypersil ODS column and a UV detector at wavelength of 284 nm. The mobile phase was a mixture of 1% acetic acid solution/methanol in the proportion of 23:77 (v/v) at a flow rate of 1 ml/min. The column temperature was set at 30°C.

Contact angle and surface thermodynamic characteristics

Microbial surface thermodynamic properties and bacterial hydrophobicity were evaluated by using the contact angle measurement (JC2000A, Powereach Co., Shanghai). Homogenous cellular layers were prepared by collecting bacterial cells on 0.45-μm cellulose acetate membranes, which were washed twice with distilled water, and were then

placed on 1% agar plate. Before measurement, the membranes were mounted on glass slides and air-dried for 20 min, and so-called plateau contact angles were measured by the sessile drop technique using water and 1-bromonaphthalene (Busscher et al. 1984). All contact angle values are based on arithmetic means of at least ten independent measurements.

According to the geometric-mean equation, the surface free energies can be separated into two components, i.e., an apolar or Lifshitz–van der Waals, γ^{LW}, and a polar or acid–base, γ^{AB} (Bos et al.1999). The pure liquid (L) contact angles (θ) can be expressed as:

$$\cos(\theta) = -1 + 2\left(\gamma_B^{LW}\gamma_L^{LW}\right)^{1/2}\gamma_L^{-1} + 2\left(\gamma_B^{AB}\gamma_L^{AB}\right)^{1/2}\gamma_L^{-1} \quad (1)$$

where bacteria γ_B and the liquid γ_L are the bacterial and the liquid surface free energies, respectively. The values of γ_B^{LW} and γ_B^{AB} could be estimated from Eq. 1 with the contact angle data. The surface energy of bacterium was expressed as:

$$\gamma_B = \gamma_B^{LW} + \gamma_B^{AB} \quad (2)$$

Adhesion of bacteria is driven by decreases in free energy, which are due to change in free energy at interfaces. According to thermodynamic principles, an adhesion process is favored when the process decreases the free energy (Busscher et al. 1984). In such systems, the change in the free energy per unit of area (ΔG_{adh}) can be expressed as follows:

$$\Delta G_{adh} = \gamma_{BS} - \gamma_{BL} - \gamma_{SL} \quad (3)$$

where γ_{BS}, γ_{BL} and γ_{SL} denote the interfacial energy of the bacterium–substratum, bacterium–liquid and substratum–liquid interfaces, respectively.

For the sake of simplicity, when only flocculation of two identical bacteria is considered, γ_{BS}=0 and γ_{BL}=γ_{SL}, thus, Eq. 3 is changed into:

$$\Delta G_{adh} = -2\gamma_{BL} \quad (4)$$

The interfacial free energy between any two surfaces 1 and 2 can be expressed in its apolar and polar components by:

$$\gamma_{12} = \left(\sqrt{\gamma_1^{LW}} - \sqrt{\gamma_2^{LW}}\right)^2 + \left(\sqrt{\gamma_1^{AB}} - \sqrt{\gamma_2^{AB}}\right)^2 \quad (5)$$

Thus, Eq. 4 becomes:

$$\Delta G_{adh} = -2\left(\sqrt{\gamma_B^{LW}} - \sqrt{\gamma_L^{LW}}\right)^2 - 2\left(\sqrt{\gamma_B^{AB}} - \sqrt{\gamma_L^{AB}}\right)^2 \quad (6)$$

The values of the first term (ΔG_{adh}^{LW}) are nearly always negative, indicating that the Lifshitz–van der Waals forces are predominantly attractive; the values of the second term (ΔG_{adh}^{AB}) accord to the acid–base interactions.

Results

Effect of toxic substances on EPS production

Figure 1 illustrates the effect of Cu(II) concentration on the cell growth, production of EPS and ratio of proteins to carbohydrates. As shown in Fig. 1a, the cell dry weight per liter decreased rapidly when Cu(II) was present, indicating that the bacterium did not grow well in the presence of Cu (II). For example, the cell level was 2.69 g l^{-1} for the control, whereas it was 0.39 g l^{-1} only for 30 mg l^{-1} Cu(II). Figure 1b illustrates the production of EPS at different Cu (II) concentrations. The protein content in EPS increased sharply from 17.8 to 150.1 mg g^{-1} dry cell with an increase in Cu(II) concentration from 0 to 30 mg l^{-1}, but it decreased to 54.2 mg g^{-1} dry cell at 50 mg l^{-1} Cu(II). Similarly, the contents of carbohydrates and nucleic acids both increased with an increase in Cu(II) concentration from 0 to 30 mg l^{-1} and then decreased with a further increase in Cu(II) concentration to 50 mg l^{-1}. Cu(II) concentration also affected the proteins/carbohydrates ratio in EPS. These ratios varied from 2.4 to 4.4 as the Cu(II) concentration increased and peaked at Cu(II) of 30 mg l^{-1} (Fig. 1a).

The production of EPS was also measured in the presence of Cr(VI) at concentrations 0–50 mg l^{-1}. As shown in Fig. 2, the cell level decreased rapidly as Cr(VI) concentration increased from 0 to 50 mg l^{-1}. However, the contents of EPS increased with increasing Cr(VI) concentration and peaked

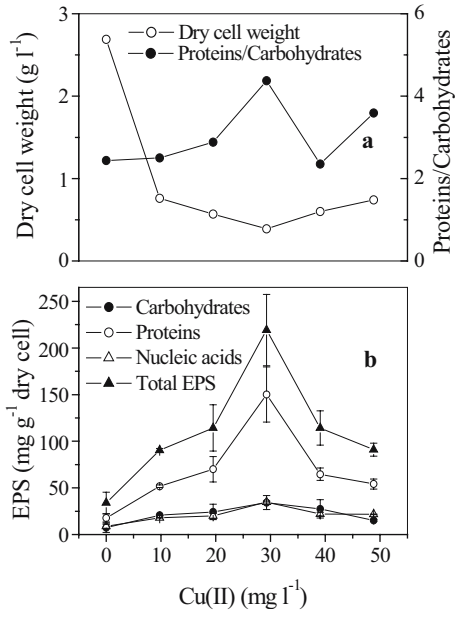

Fig. 1 Effect of Cu(II) concentration on **a** cell growth and ratio of proteins to carbohydrates and **b** EPS content

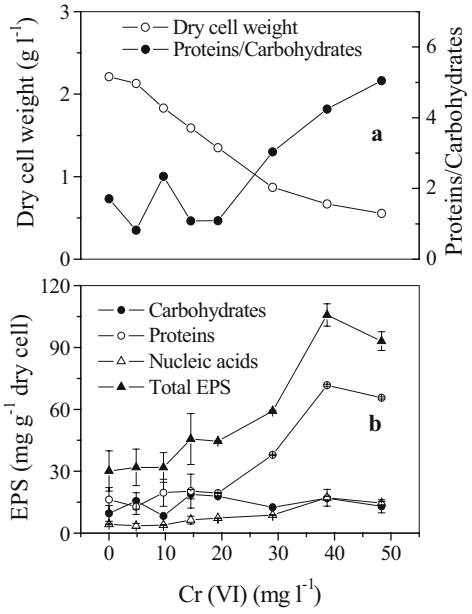

Fig. 2 Effect of Cr(VI) concentration on **a** cell growth and ratio of proteins to carbohydrates and **b** EPS content

at Cr(VI) concentration of 40 mg l^{-1}, then decreased with a further increase in Cr(VI) concentration. The contents of carbohydrates, proteins and nucleic acids were 16.9, 71.7 and 17.2 mg g^{-1} dry cell, respectively, at 40 mg l^{-1} Cr(VI). The proteins/carbohydrates ratios varied from 0.8 to 5.1 at Cr(VI) of 0–50 mg l^{-1} (Fig. 2a).

Another heavy metal, Cd(II), with concentrations of 0 and 100 mg l^{-1}, was also added to the cultivation medium of PSB in the form of $CdSO_4$. As illustrated in Fig. 3, the cells did not grow well, and the dry cell weight decreased sharply to a minimum value of 0.57 g l^{-1} at Cd(II) of 5 mg l^{-1}. However, the content of proteins in EPS increased rapidly to a peak of 100.0 mg g^{-1} dry cell at Cd(II) of 5 mg l^{-1}; then it decreased to a lower level rapidly with increasing Cd(II) concentration from 5 to 30 mg l^{-1}; but it kept almost unchanged at a level of 47.6 mg g^{-1} dry cell thereafter. The contents of carbohydrates and nucleic acids had a similar changing pattern with proteins, but the variation was much less than that of proteins. As shown in Fig. 3a, the ratios of proteins to carbohydrates varied between 1.7 and 9.9 and peaked at 40 mg l^{-1} Cd(II) presence.

Figure 4 shows the dry cell weight, the ratios of proteins to carbohydrates and the contents of EPS profiles in the presence of 2,4-DCP at different concentrations. 2,4-DCP is an organic pesticide and toxic to microorganisms. As shown in Fig. 4a, the growth of *R. acidophila* was negatively affected by the addition of 2,4-DCP. Compared with the heavy metals, the toxicity of 2,4-DCP was lower. At 2,4-DCP concentration of 100 mg l^{-1}, the cell level was 44% of that for the control. The contents of EPS were not significantly influenced by the increase in 2,4-DCP concentration from 0 to 80 mg l^{-1}. However, at 100 mg l^{-1} of 2,4-DCP, the EPS contents were 2.4 times of that for the control. As shown in Fig. 4b, the production of carbohydrates and nucleic acids was not significantly influenced by 2,4-DCP, and their contents remained at a low level.

Table 1 summarizes the ratio of EPS contents in the presence of toxic substances to those of the control. The production of EPS was greatly promoted in the presence of toxic substances. However, no quantitative relationship between the EPS production and dosage of the chemicals could be found, and no significant correlation between the EPS production and the relative toxicity was observed as well. At 30 mg l^{-1} Cu(II), 40 mg l^{-1} Cr(VI), 5 mg l^{-1} Cd(II)

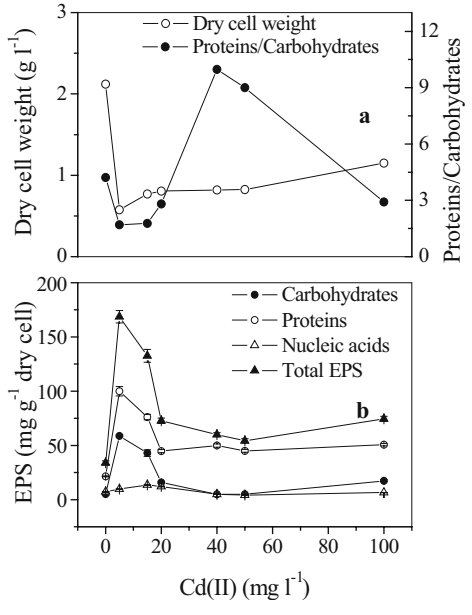

Fig. 3 Effect of Cd(II) concentration on **a** cell growth and ratio of proteins to carbohydrates and **b** EPS content

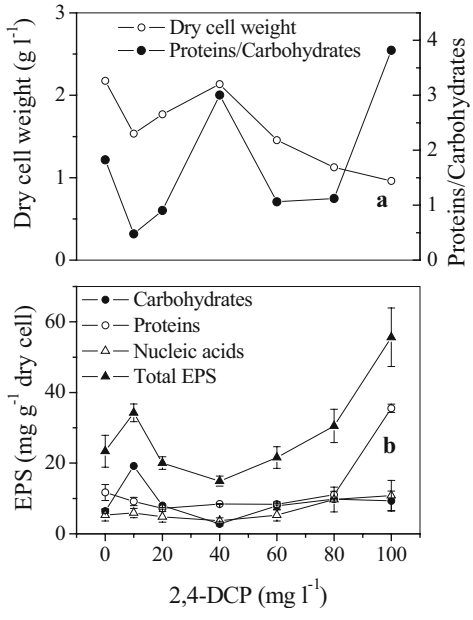

Fig. 4 Effect of 2,4-DCP concentration on **a** cell growth and ratio of proteins to carbohydrates and **b** EPS content

Table 1 The ratios of EPS contents in the presence of toxic substances to those of the control

	Carbohydrates	Proteins	Nucleic acids	Total EPS
Cu(II) (30 mg l^{-1})	4.7	8.4	4.0	6.5
Cr(VI) (40 mg l^{-1})	1.8	4.4	4.0	3.5
Cd(II) (5 mg l^{-1})	11.5	4.6	1.3	5.0
2,4-DCP (100 mg l^{-1})	1.5	3.0	2.0	2.4

plus 100 mg l^{-1} 2,4-DCP, the production of EPS peaked, and the EPS contents were 6.5, 3.5, 5.0 and 2.4 times of those for the control, respectively (Table 1), whereas the cell concentration was only 14.5, 30.3, 27.1 and 44% of the control, respectively.

Table 2 summarizes the partition of Cu, Cd, Cr and 2,4-DCP in the medium, microbial cells and EPS. Most of Cu and Cd were combined by EPS, and much less metals were combined by the cells. Most of Cr was in the medium, but the content of Cr combined by EPS was 1.5 times of that combined by cells. Approximately 63.3% of 2,4-DCP remained in medium even after 70-h cultivation, and only 3.2% of 2,4-DCP was adsorbed onto EPS.

Effect on surface characteristics of *R. acidophila* and free energy of adhesion

Presence of toxicant affected the bacterial surface characteristics such as contact angle, hydrophobicity and surface energy. As shown in Fig. 5, the influence of Cu(II) on the water contact angle of *R. acidophila* was not significant, whereas the corresponding effect on the 1-bromonaphthalene contact angle was substantial. As Cu(II) concentration increased, the water contact angle changed little and varied slightly between 43.2 and 57.2°. However, the changing tendency of the 1-bromonaphthalene contact angle was not the same as that of water because 1-bromonaphthalene was an apolar liquid while water was a polar liquid. When the Cu (II) concentration increased from 0 to 10 mg l^{-1}, the 1-bromonaphthalene contact angle increased from 41.6 to 75.6° but decreased slightly thereafter. Figure 6 shows the effect of Cu(II) on the surface energy of *R. acidophila*. As illustrated in Fig. 6a, with Cu(II) concentration increasing from 0 to 10 mg l^{-1}, the polar component (γ^{AB}) increased

Table 2 The partition of the toxic substances in the medium, cell and EPS after 70-h cultivation

Toxic substances	Medium (%)	Cell (%)	EPS (%)
Cu	8.2±0.2	2.6±0.1	89.2±2.7
Cd	18.8±0.7	2.7±0.2	78.5±3.3
Cr	81.0±1.2	7.6±0.1	11.4±0.7
2,4-DCP	63.3±1.0	nd	3.2±1.1

The dosages of Cu(II), Cd(II), Cr(IV) and 2,4-DCP were 30, 5, 40 and 100 mg/l, respectively
nd Not determined

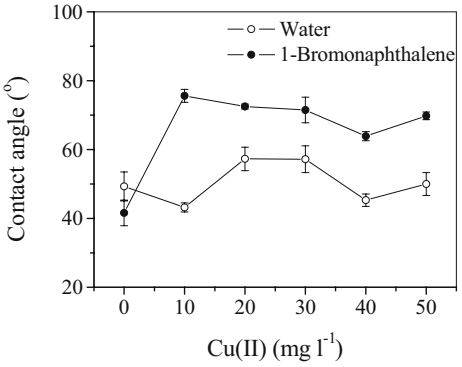

Fig. 5 Contact angles of *R. acidophila* at various Cu(II) concentrations

from 21.3 to 37.1 mJ m^{-2}, and then slightly decreased. The corresponding apolar component (γ^{LW}) decreased from 33.9 to 20.0 mJ m^{-2} and thereafter remained almost unchanged.

The effect of Cu(II) on the ratio of the apolar to polar component of surface energy ($\gamma_B^{LW}/\gamma_B^{AB}$) is shown in Fig. 6b. The value of these ratios decreased dramatically from 1.6 to 0.5 as Cu(II) concentration increased from 0 to 10 mg l^{-1}; after that, it increased slightly until leveling off at a value of 0.76.

The effect of Cu(II) on ΔG_{adh} calculated from Eq. 6 is illustrated in Fig. 7. The ΔG_{adh} value increased when *R. acidophila* was exposed to toxic substances, but then decreased when the chemical dosage increased furthermore. As Cu(II) concentration increased, the free energy of adhesion increased from initial −15.4 to −2.7 mJ m^{-2} at 10 mg l^{-1} of Cu(II). After that, ΔG_{adh} decreased slightly to −10.2 mJ m^{-2} when Cu(II) concentration increased to 30 mg l^{-1}, corresponding to the maximum EPS content. Thereafter, ΔG_{adh} increased slightly.

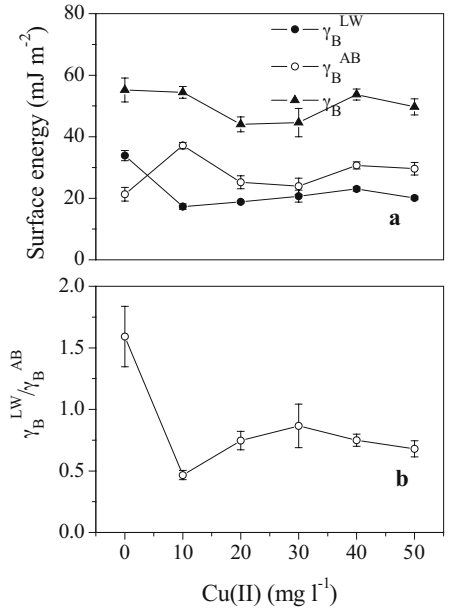

Fig. 6 Effect of Cu(II) concentration on **a** bacterial surface energy and **b** ratio of apolar to polar component of surface energy ($\gamma_B^{LW}/\gamma_B^{AB}$)

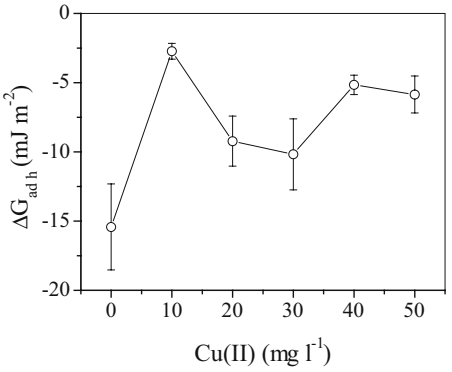

Fig. 7 Bacterial free energy of adhesion (ΔG_{adh}) at various Cu(II) concentrations

Discussion

One natural response of microbes upon exposure to a toxic environment is to increase the production of EPS. EPS could form a protective shield for the cells against the adverse influences from the external environment. Experimental results show that productions of carbohydrates, proteins and nucleic acids all increased considerably when bacterium was exposed to the toxic chemicals (Table 1). This increase might be due to an increase in the EPS production rate, or a reduction in the EPS degradation rate or both (Aquino and Stuckey 2004). There are many ways for microorganisms to cope with a high level of toxic substances. Urrutia and Beveridge (2003) found that the cell surface is the place where heavy metals are accumulated. As EPS is one gel-like layer out of the cell wall, EPS either delay or prevent toxicants from reaching microbes by diffusion limitation and/or by chemical reactions to reduce the harmfulness of the toxic substances (Wingender et al. 1999). Furthermore, since the EPS were mainly composed of proteins, carbohydrates and only a small amount of nucleic acids, the EPS also contained numerous potential binding sites for metals including carboxyl, phosphoryl and sulphate groups (Santamaria et al. 2003). Results of the present study showed that the combination of heavy metals by EPS was greater than that by microbial cells for *R. acidophila* (Table 2). Wuertz et al. (2000) suggested that in addition to cellular sorption, metals were bound extracellularly by EPS in intact and undisturbed microbial flocs. Urrutia and Beveridge (2003) reported that only part of heavy metals was combined by out of cell wall, as evidenced by the fact that most of heavy metals were remobilized again after EDTA treatment. These show that the toxicity of metal is related to free ion activity, and metal becomes not toxic to bacteria themselves when it is adsorbed or combined with inorganic or organic ligand (Hsieh et al. 1994). Thus, under toxic conditions, bacteria should produce more EPS to form a protective layer and bind or adsorb those toxic substances to the cell surfaces. Previous studies have demonstrated that more EPS are produced under toxic conditions (Rudd et al. 1984; Aquino and Stuckey 2004). However, the results from the present work show that toxic substances at a high concentration might not promote the EPS production more significantly. As shown in Figs. 1, 2, 3 and 4, the most EPS were produced at a certain concentration of toxic substances. Heavy metals at a high concentration might decrease the bacterial metabolism activity and, accordingly, reduce the production of EPS (Hsieh et al. 1994).

For *R. acidophila*, carbohydrates and proteins were found as the two major EPS components. Table 1 also shows that the effect of toxic substances on the production of different components in EPS was not of the same level. Toxic substances did not only result in an increase in EPS production, but also led to a change of the ratio among the EPS components, e.g., the ratios of proteins to carbohydrates. Furthermore, the proteins/carbohydrates ratios were dependent upon the concentration of toxic substances. For example, these ratios ranged from 2.4 to 4.4 at Cu(II) from 0 to 50 mg l^{-1}. These results were in good agreement with those of a previous study (Aquino and Stuckey 2004). As shown in Figs. 1, 2, 3 and 4, compared with proteins, less nucleic acids and carbohydrates were excreted under toxic stress. In general, the presence of toxic substances resulted in more production of proteins than that of carbohydrates, as shown in Table 1. This phenomenon implies that the production of proteins would be enhanced significantly under the toxic conditions. Under neutral conditions, both proteins and carbohydrates in EPS have plenty of ionic negative functional groups, such as carboxyl, phosphoryl and sulphate groups, which could bind with metals. However, for the EPS of *R. acidophila*, the content of proteins was greatly higher than that of carbohydrates. Our previous study also showed that with the increase in the removal of divalent ions, the content of proteins increased, whereas the contents of carbohydrates almost kept unchanged (Sheng et al. 2005). Thus, for *R. acidophila*, the proteins in EPS might be the major components to protect cell from the harmfulness by diffusion limitation and/or by chemical binding.

The ratio of the apolar to polar component of surface energy, indicating the relative hydrophobicity of bacteria, decreased rapidly when *R. acidophila* was growing in the presence of Cu(II). Under neutral conditions, the function groups of EPS are almost ionized and negatively charged. Furthermore, the total content of EPS showed a negative effect on the relative hydrophobicity and had a positive correlation with negative surface charge (Wilen et al. 2003). As the EPS content of *R. acidophila* increased, the cell became less hydrophobic, and thus the ratio of the apolar to polar component of surface energy of bacterium decreased. However, when the Cu(II) concentration increased thereafter, the bacterial hydrophobicity did not change substantially. This might be due to the combination of EPS and added heavy metal ions, which changed the hydrophobicity/hydrophilicity characteristics of EPS.

The poor flocculation ability of PSB has limited their utilization as a culture for a continuously flow bioreactor. Evaluation on the EPS of PSB is able to provide useful information for designing highly efficient phototrophic H_2-producing reactors. Bacteria self-flocculation is a very complex physicochemical process (Kos et al. 2003) and is always governed by electrostatic force, van der Waals interaction, hydrophobic forces, ion bridging and polymer

entanglement in the presence of EPS (Chen and Stewart 2002). ΔG_{adh} for identical bacteria could reflect the attractive force between two cells and the adhesion ability of bacterium. As shown in Fig. 7, the addition of heavy metals could alter the adhesion ability of *R. acidophila* significantly. The valley point of ΔG_{adh} was approximately consistent with the peak point of the contents of EPS production under Cu(II) presence (e.g., 30 mg l^{-1}), implying that the content of EPS could affect the adhesion ability substantially. As the Cu(II) concentration increased from 0 to 10 mg l^{-1}, the EPS contents were greatly promoted from 33.9 to 90.4 mg g^{-1} dry cell, resulting in an increase in ΔG_{adh} (Figs. 1 and 7). The increase in EPS contents would lead to a significant decrease in hydrophobicity of bacterium and an increase in the negative surface charge (Wilen et al. 2003), corresponding to a decrease in attractive hydrophobicity interaction and an increase in electrostatic repulsive interaction. The calculation of ΔG_{adh} with the thermodynamic method concerns the hydrophobic interaction, van der Waal's interaction and electrostatic repulsive interaction only (Bos et al. 1999). However, polymer entanglement through physical or chemical approaches might be key ones. Unfortunately, it was not considered in the thermodynamic calculations. In the presence of Cu, more EPS were produced than in the absence of Cu; accordingly, the hydrophobic interaction decreased and the electrostatic repulsive interaction increased. This in turn caused an increased in ΔG_{adh}. However, this did not mean that the best cell adhesion ability occurred when no Cu was added, as the ion bridging through EPS and the polymer entanglement increased significantly and would enhance the adhesion ability, but these interactions would not be involved in the calculation of ΔG_{adh}. After cells are attaching each other initially, positively charged ions could act as bridging agents between two negatively charged surfaces, and EPS might act as an adhesive binding and thus further enhance the aggregation of bacteria during cluster formation (Cheung et al. 2000). Thus, the toxic substances could affect the physicochemical properties and flocculation ability of *R. acidophila* due to the promotion of EPS production.

The difference in the EPS contents and composition might be responsible for the significant difference in the flocculation of bacterium. Exposure of bacterium to toxic conditions could alter the relative content of different compounds in EPS and the flocculation ability of bacterium. However, the precise roles of EPS compounds in the flocculation were not clear. Therefore, the conditions for cultivating *R. acidophila* should be adapted to influence the excretion of different compounds of EPS in order to enhance the flocculation ability of *R. acidophila*. The acclimated microorganisms with good flocculation ability would benefit for retaining high-cell density of PSB in a photobioreactor. As a result, the PSB cells could be efficiently separated from supernatant, leading to a high PSB cell concentration in a hydrogen-producing reactor for hydrogen generation. This self-immobilizing method regulating EPS contents and composition might avoid the shortcomings of immobilizing methods, such as addition of a bacterial-immobilizing carrier, poor stability or low efficiency.

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