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Effect of ambient conditions on simultaneous growth and bioaccumulation of mercuric ion by genetically engineered *E. coli JM109*

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Abstract

Genetically engineered *E. coli JM109*, namely M1, which expressed both Hg^{2+} transport system and metallothionein, was tested for its capability of simultaneous growth and bioaccumulation of Hg^{2+} under low nutritional circumstances. The influential factors of ambient conditions, e.g. initial concentrations of mercuric ion, ionic strength, the presence of metal chelators and other coexisting metal ions were investigated. Hg^{2+} bioaccumulation behavior of M1 proved to be well coupled with its growth. NaCl was essential to the growth of M1. Of all tested NaCl concentrations, 0.04 mol/L was optimal. The presence of 0.1 mol/L $CaCl_2$ or $MgCl_2$ could promote the growth of M1 and keep the Hg^{2+} removal ratio high, but the growth of M1 was inhibited seriously as the concentration of $CaCl_2$ or $MgCl_2$ reached 0.3 mol/L. Chelator EDTA had a significant influence on M1 growth and Hg^{2+} bioaccumulation, while the effect of citratio was little. The presence of other coexisting metal ions inhibited the growth of M1. The influential order was as follows: $Cd^{2+} > Zn^{2+} \geq Cu^{2+} > Pb^{2+} > Ni^{2+}$. However, only Cd^{2+} and Cu^{2+} posed obviously adverse effects on Hg^{2+} bioaccumulation during the SG&B process.

Keywords: Genetically engineered *E. coli*; Mercuric ion; Wastewater; Bioaccum ulation

1. Introduction

Traditional metal removal processes such as chemical oxidation–reduction, precipitation, adsorption, solidification, electrolytic recovery and ion exchange are some of the physico-chemical wastewater treatments. Application of such processes, however, is restricted because of technical or economical constrains [1]. Using microorganisms as biosorbents for heavy metals is an alternative to such methods mentioned above. Mercury is known to be one of the most toxic heavy metals in environment and removal of mercury from wastewater by microorganisms has widely been studied [2]. However, common bioprocesses lack specificity in metal binding, which may cause difficulties in the recovery and recycling of the desired metal(s) [3,4]. The developments of genetic engineering are bringing new means for metals cleanup. Through genetic engineering, microorganisms can enhance not only their specificity but also their ability to accumulate heavy metals [5–7].

merP and *merT* are two genes, which encode Hg^{2+} binding and transport protein respectively [8]. MTs (metallothioneins) are low molecular (6–7 kDa), cysteine-rich proteins, which can bind heavy metals and reduce their toxicity to living cells [9]. Genetically engineered *E. coli JM109* [10] expressing Hg^{2+} transport system (MerP and MerT) and GST–MT simultaneously can improve its Hg^{2+} resistance and bioaccumulation capacity compared to the original host strain. The bioaccumulation process of the recombinant strain showed resistance to ambient conditions such as pH, ionic strength, the presence of metal chelators (EDTA and citrate), and selectivity against other metals such as sodium, magnesium and cadmium [4]. Deng and Wilson used the recombinant strain to deal with actual electrolyte wastewater containing different metal ions [10].

Despite much research, few bioaccumulation processes with gene-modified microorganisms were applied into industrial use due to the difficulties in an attempt to realize continuous treatment. In order to make the treatment process continually operational, it might be a feasible and effective way to make the recombinant cells propagate without losing high specificity

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and affinity to the desired metal at the time when they are accumulating metal ions, considering the fact that heavy metals usually coexist in wastewater with other kinds of contaminants such as organic pollutants, which can possibly be used by microorganism as nutrients. Furthermore, using growing cells can avoid the need of a separate biomass production process such as cultivation, harvesting, etc. [11]. Therefore, it is meaningful to study the influential factors on the growth of microorganism as well as its heavy metal bioaccumulation.

The purpose of the investigation was to evaluate the process of Hg^{2+} bioaccumulation by growing cells of genetically engineered *E. coli JM109*, e.g. the process of simultaneous growth and bioaccumulation (SG&B). The influential factors on SG&B under various ambient conditions, e.g. concentrations of mercuric ion, ionic strength, the presence of metal chelators and other coexisting metal ions were tested in this paper.

In one of our previous studies, the behavior of simultaneous mercury bioaccumulation and cell propagation by the recombinant *E. coli* was preliminarily evaluated in LB + Hg^{2+} solutions [12]. However, no operational factors were investigated in that paper. Furthermore, considering in real heavy metal wastewater environments no abundant nutrient materials could be available generally, in this experiment we chose low nutrient 0.5 LB for the study.

2. Materials and methods

Genetically engineered *E. coli JM109* (namely M1) simultaneously harbors two compatible plasmids, pSUTP containing the *merP* and *merT* genes, and pGPMT containing the GST–PMT fusion gene [10].

The recombinant strain was inoculated into LB (Soya peptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L) containing ampicillin (100 mg/L) and kanamycin (60 mg/L) overnight at 37 °C with rotary shaking (150–160 rev/min). For SG&B study, the above activated culture was reinoculated to 0.5 LB (Soya peptone 5 g/L, yeast extract 2.5 g/L and NaCl 2.5 g/L) containing ampicillin (100 mg/L) and kanamycin (60 mg/L), as well as the desired initial Hg^{2+} concentrations. The initial pH was about 6.5. Other influential factors to be studied such as coexisting metal ions, NaCl, $MgCl_2$, $CaCl_2$, EDTA and citrate were added into 0.5 LB according to study requirements. After shaking for several hours, isopropyl β-D-thiogalactoside (IPTG) was added to 1.0 mM concentration when the growth was within late-log phase. During culture the samples were taken out for cell density and Hg^{2+} concentration determination between whiles until the cells reach their stable-state phase.

The cell growth was determined by optical density measurements (at 600 nm). The dry weight of cells was determined from OD_{600} using the value of 0.35 g dry weight per liter of OD_{600} 1.0 [13]. The Hg^{2+} concentration was determined by intelligent cold vapor atom fluorescence mercuric ion measurement. To determine the Hg^{2+} concentration in the supernatant, sampled culture from 0.5 LB was centrifugalized (10,000 r/min, 10 min at room temperature) to remove cells, and then 5% HNO_3–0.05% $K_2Cr_2O_7$ was added to the supernatant to minimize Hg^{2+} lose due to glassware adsorption.

The specific Hg^{2+} bioaccumulation capacity was calculated from q (mg/g dry cell) = $V(C_i - C_t)/m_t$ and Hg^{2+} removal ratio was acquired from $(C_i - C_t)/C_i$, where V was the sample volume (L), C_i was the initial Hg^{2+} concentration (mg/L), C_t and m_t were the residual Hg^{2+} concentration (mg/L) and the weight (g) of dry cell at different times, respectively.

To prevent metal contamination, all glassware were soaked in 20% nitric acid overnight and rinsed three times with deionized water before complete drying.

3. Result and discussion

3.1. Effect of Hg^{2+} concentration on the SG&B

In previous studies [10] no data showed how the presence of Hg^{2+} affected M1 growth, though the Hg^{2+} resistance of M1 was highly improved due to the introduction of plasmids pSUTP and pGPMT. Therefore, the effect of different initial Hg^{2+} concentrations on M1 growth as well as Hg^{2+} bioaccumulation was evaluated. In 0.5 LB, the inhibition effect on cell growth was elevated with the increase of Hg^{2+} (Fig. 1a). Through the analysis of Fig. 1(a) and (b), 1 mg/L Hg^{2+} had no obvious inhibition effect on M1 growth (OD_{600} reached 1.90 or so) and Hg^{2+} removal ratio was more than 90%. The growth of M1 was inhibited obviously when Hg^{2+} concentration was 2 mg/L (OD_{600} reached only 0.73, much lower than 1.9) while Hg^{2+} removal ratio was still quite high and more than 85%. As Hg^{2+} concentration was increased up to 4 mg/L, the growth of M1 was strongly inhibited (only about 0.25 of OD_{600} was achieved after more than 10 h incubation) and Hg^{2+} removal ratio dropped to 37%. By comparison, in Zhao's study [12], M1 proved to be able to propagate itself in LB with Hg^{2+} concentration up to 7.4 mg/L (OD_{600} close to 7.0), although cell reproduction was delayed with increasing Hg^{2+} concentration. The difference between these two growth conditions might be based on the fact that LB medium is more nutritional than 0.5 LB, possibly making the cells more resistant to the toxic effect of mercury in the solution.

Although the inhibition effect increased and Hg^{2+} removal ratio decreased with the increase of Hg^{2+} concentration in 0.5 LB, the specific Hg^{2+} bioaccumulation capacity, which can be calculated through Fig. 1(a) and (b), increased from 1.5 to 25 mg/g dry cell as Hg^{2+} concentration varied from 1 to 4 mg/L Hg^{2+} in 0.5 LB. The results were easily understood and consistent with previous studies that the decrease of biomass loading and the increase of initial metal concentration [14], which enhanced the metal/biomass ratio, led to improved specific adsorption capacity.

As shown in Fig. 1(a) and (b), the cell growth and Hg^{2+} bioaccumulation in 0.5 LB were well-coupled, suggesting the potential use in dealing with low-nutrient heavy metal contaminated wastewater, from which cells may utilize the nutrition to grow and bioaccumulate Hg^{2+} at the same time.

To study other influential factors on SG&B, Hg^{2+} concentration of 1 mg/L in 0.5 LB was chosen in the following tests.

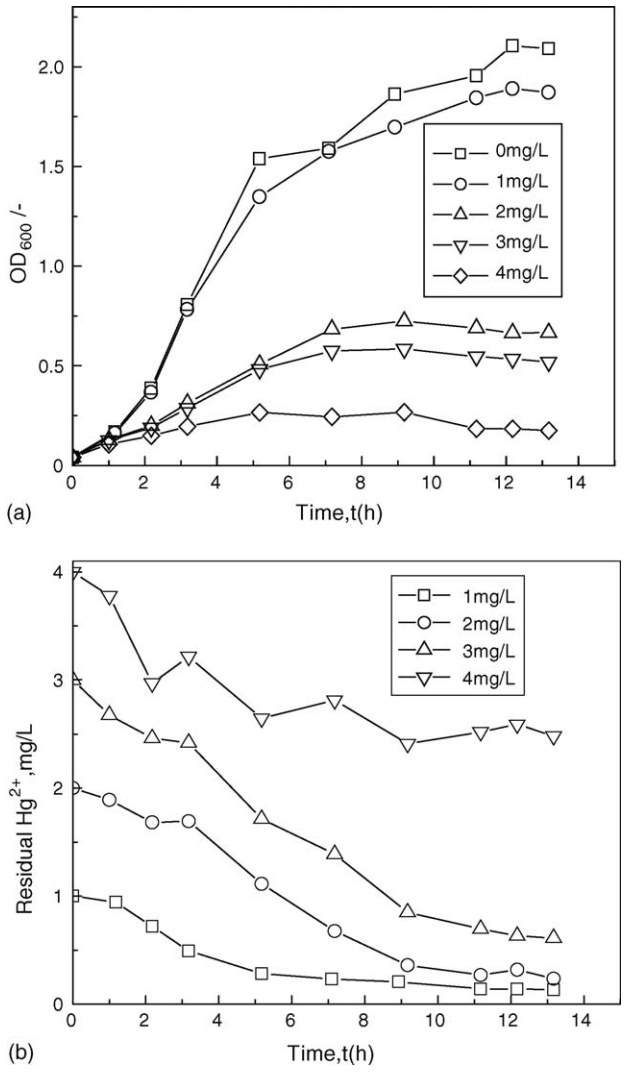

Fig. 1. (a) Effect of Hg^{2+} on the growth of M1 in 0.5 LB. (b) Effect of initial Hg^{2+} concentrations on Hg^{2+} bioaccumulation by M1 in 0.5 LB.

3.2. Effect of ionic strength on SG&B

Alkaline metal ions and alkaline-earth metal ions such as sodium, magnesium and calcium are often found in wastewater. These ions may reduce the efficiency of ion exchange resins or biosorbents due to ionic strength or competing effect. Chang and Hong reported that the Hg^{2+} adsorption by Hg^{2+} resistant strain *Pseudomonas aeruginosa* PU21 (Rip64) was reduced at low Hg^{2+} concentration by the presence of Na^+ [15]. Chen and Wilson demonstrated that Na^+ concentration up to 400 mM and Mg^{2+} concentration up to 200 mM did not affect Hg^{2+} bioaccumulation by genetically engineered *E. coli*, indicating the intracellular Hg^{2+} bioaccumulation process was resistant to elevated ionic strength caused by the presence of alkaline and alkaline-earth metal ions [4]. In another paper, Deng and Wilson found mercury uptake by genetically engineered *E. coli* was faster from the wastewater containing other metal ions than from distilled water [10]. In the present investigation, the effect of ionic strength caused by these alkaline and alkaline-earth metal ions on SG&B was determined.

3.2.1. Effect of Na^+ on SG&B

In 0.5 LB medium, 2.5 g/L NaCl is included, meaning about 0.04 mol/L Na^+ already existing in the medium. In our experiment, to evaluate the effect of Na^+ on SG&B, we tested five Na^+ levels, e.g. 0, 0.04, 0.47, 0.73 and 0.90 mol/L, respectively. 0 mol/L Na^+ meant no NaCl was added in the solution. As shown in Fig. 2(a), moderate Na^+ concentration was helpful to M1 growth. Of all tested concentrations of Na^+, 0.04 mol/L Na^+ concentration in the solution, e.g. original 0.5 LB was optimal. Cells without Na^+ grew slowly, indicating Na^+ was essential to M1 growth. Under the presence of 0.47 mol/L Na^+ M1 growth was slightly inhibited, while as Na^+ concentration reached 0.90 mol/L the inhibition effect on M1 growth was much stronger than that without Na^+ and OD_{600} reached only about 0.40.

Fig. 2(b) displayed the Hg^{2+} removal ratio was little influenced by Na^+. As Na^+ concentration was 0.90 mol/L, where the growth of M1 was inhibited strongly, the Hg^{2+} removal ratio from the solution still reached more than 80%. Although the inhibition effect on the growth of M1 increased as Na^+

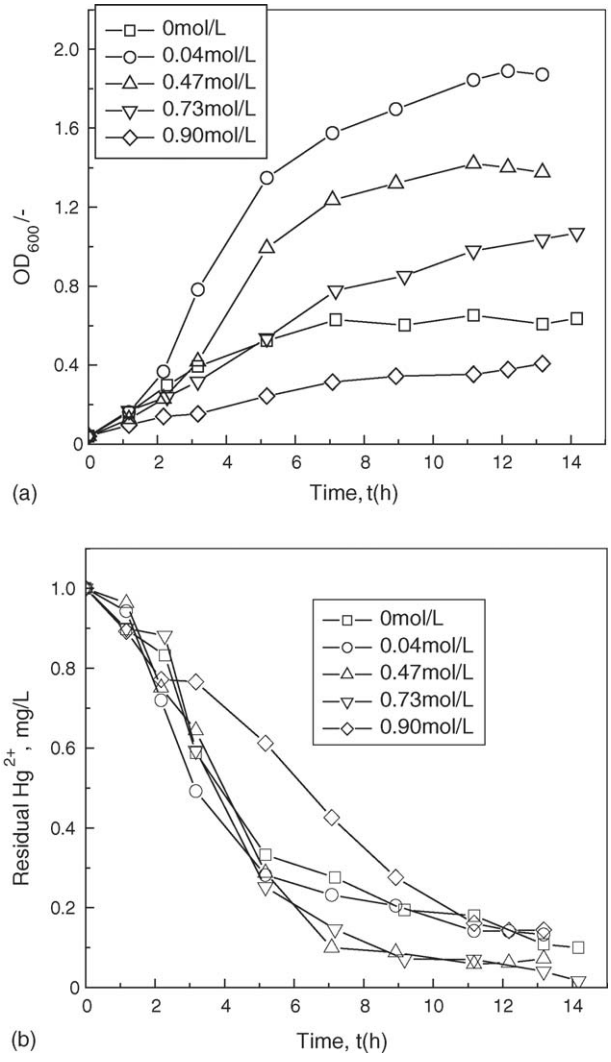

Fig. 2. (a) M1 growth under different concentrations of Na^+ in the solution. (b) Effect of Na^+ concentrations on Hg^{2+} bioaccumulation by M1 in the solution.

concentration rose in the solution (except 0 mol/L Na^+ concentration), the specific Hg^{2+} bioaccumulation capacity increased with the increasing concentration of Na^+ (data no shown). The result can also be explained by metal/biomass ratio mentioned above. From Fig. 2(b), the conclusion that bioaccumulation of Hg^{2+} by growing cells of M1 was not sensitive to Na^+ could be drawn. Considering the growth condition, original 0.5 LB containing 0.04 mol/L Na^+ was used in the following studies.

3.2.2. *Effects of Mg^{2+} and Ca^{2+} on SG&B*

Four different concentrations, e.g. 0, 0.1, 0.3 and 0.5 mol/L of Ca^{2+} and Mg^{2+} were tested for the effects of the two alkaline-earth metal ions on SG&B, respectively. As shown in Fig. 3(a) and (b), the presence of 0.1 mol/L of Ca^{2+} or Mg^{2+} was good to the growth of M1. Hg^{2+} removal ratios by growing cells of M1 under 0 and 0.1 mol/L of Ca^{2+} or Mg^{2+} were basically the same and reached more than 95%. However, excess Ca^{2+} or Mg^{2+} inhibited the growth of M1 severely, and thereby affected the Hg^{2+} removal. Under the presence of 0.3 mol/L Ca^{2+} or Mg^{2+} concentration, OD_{600} reached only 0.40 and 0.22, respectively.

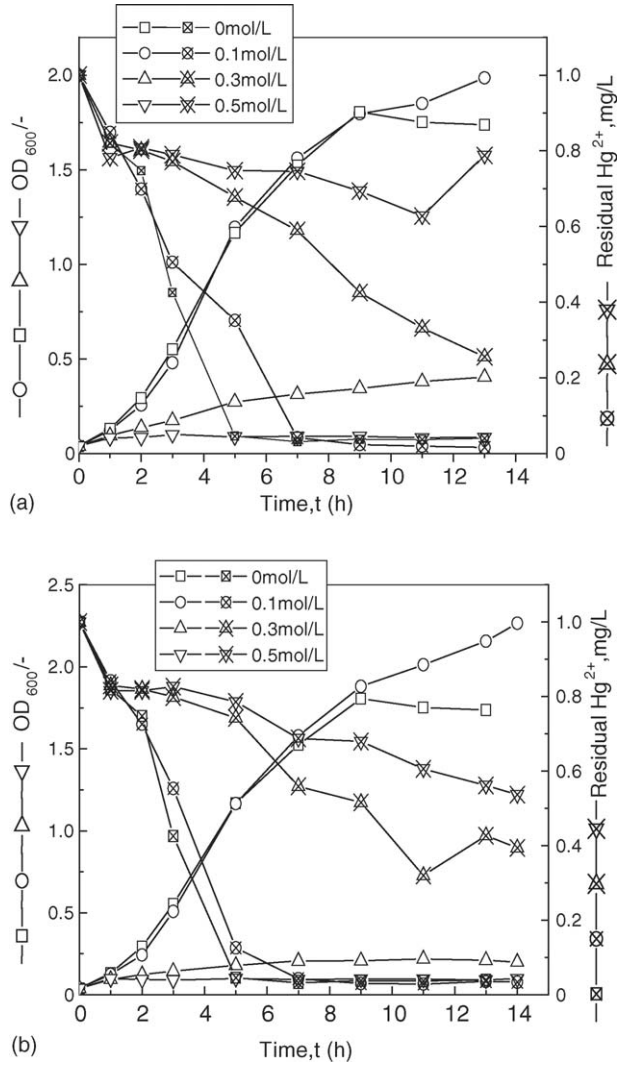

Fig. 3. (a) Effect of Ca^{2+} on SG&B in 0.5 LB. (b) Effect of Mg^{2+} on SG&B in 0.5 LB.

As a result of the inhibition effect on M1 growth, the Hg^{2+} removal ratio decreased from more than 95% when Ca^{2+} or Mg^{2+} concentration was 0.1 mol/L, to about 70% when Ca^{2+} or Mg^{2+} concentration was 0.3 mol/L. Compared Ca^{2+} or Mg^{2+} with Na^+, it was seen that the effects of Ca^{2+} or Mg^{2+} on SG&B were stronger than that of Na^+. It was probably because ionic strength caused by bivalent metal ions like Ca^{2+} and Mg^{2+} was higher than that caused by monovalent metal ion like Na^+.

3.3. *Effect of chelators on SG&B*

Metal chelators such as EDTA had been used in a broad range of industrial processes and found to interfere with metal cleanup processes. EDTA and citrate sodium were usually used as desorbents of biosorption because of its high chelating ability towards metals [16,17]. Previous studies [4] showed that EDTA and citrate did not affect Hg^{2+} bioaccumulation by genetically engineered *E. coli*. EDTA was even found to stimulate the rate of Hg^{2+} uptake [10]. In order to apply SG&B to industrial use, effects of EDTA and citrate sodium on SG&B were tested. From Fig. 4(a), EDTA showed strong inhibition on the growth of M1 at the concentration of 1.4 mmol/L. Although the growth was inhibited severely, M1 cells with low density still exhibited Hg^{2+} bioaccumulation capacity. 0.13 of OD_{600} was achieved and the Hg^{2+} removal ratio reached about 65% when the concentration of EDTA was 1.4 mmol/L. In contrast to EDTA, citrate had little influence on SG&B. As shown in Fig. 4(b) the growth of M1 at 0 and 17 mmol/L of citrate were almost the same, while the growth under 34 and 51 mmol/L were only inhibited slightly. Bioaccumulation of Hg^{2+} was not affected by the presence of citrate, and the Hg^{2+} removal ratios were all more than 90%. The difference between the effects of EDTA and citrate on the growth of M1 may be attributable to the fact that the chelating ability of EDTA was much stronger than that of citrate. As a strong chelator EDTA maybe chelated some essential metals for the growth of M1, or EDTA itself was harmful to cell growth. Citrate possessed a relative weak chelating ability, and on the other side, it was one of the metabolites of microorganisms.

3.4. *Effect of coexisting ions on SG&B*

Some metals are essential to microorganisms at low concentrations, but most of heavy metals are toxic to cells. Falih [18] found the growth of some yeasts was stimulated by 100 mg/L of Cu, Mn and Co, respectively, but was inhibited by these metals at 200 mg/L. The inhibition effect increased with the increasing heavy metals concentrations in the media.

The presence of heavy metals may also affect biosorption for its competing effect. A study of Tsezos et al. [19] showed that significant ionic competition effects could be observed for metals belonging to the same class. According to Pearson's reasoning, metals can be assorted into three classes: soft, hard and borderline. Elements exhibited competition effects among members of their class, but borderline elements were affected by the presence of either hard or soft elements. In previous studies [10], coexisting metals did not affect the bioaccumulation of

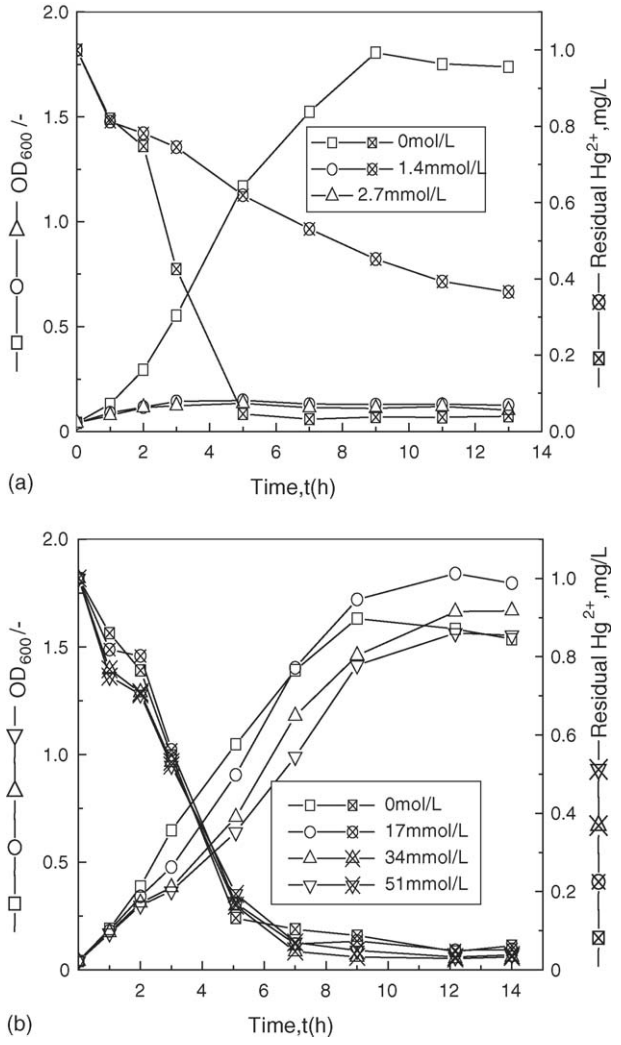

Fig. 4. (a) Effect of EDTA on SG&B in 0.5 LB. (b) Effect of citrate on SG&B in 0.5 LB.

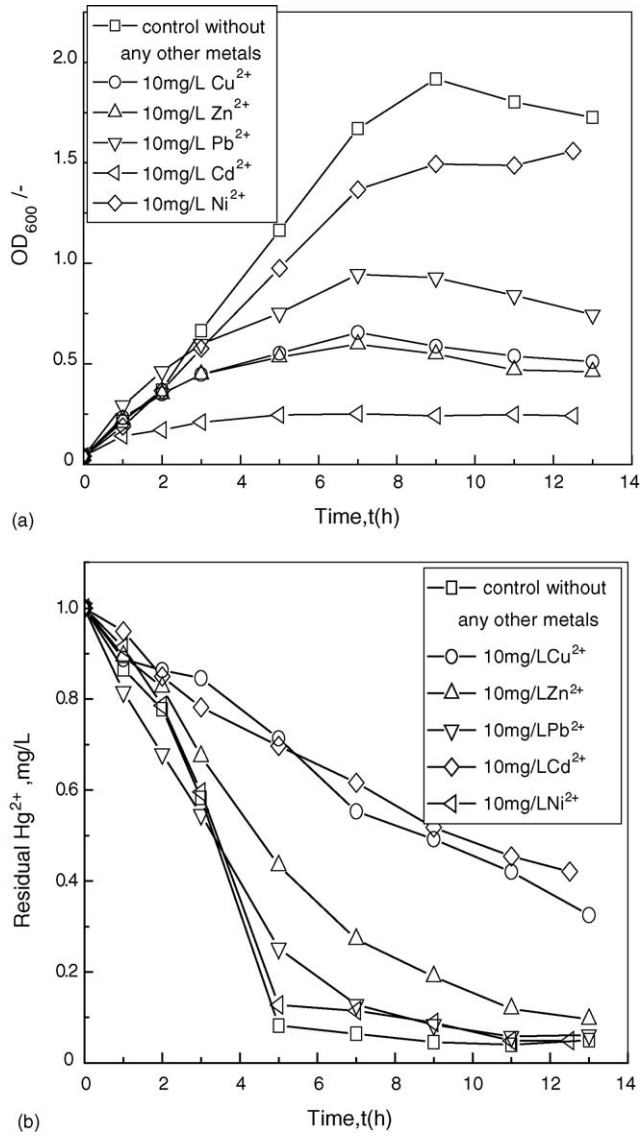

Fig. 5. (a) Effect of co-ions with initial concentration of 10 mg/L on the growth of M1 in 0.5 LB. (b) Effects of co-ions with initial concentration of 10 mg/L on the Hg^{2+} bioaccumulation in 0.5 LB.

Hg^{2+} by genetically engineered *E. coli* due to its high specificity and intracellular uptake process. In this study, five coexisting metal ions, Cu^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+} and Ni^{2+} were tested for their effects on SG&B, respectively. As shown in Fig. 5(a), the presence of tested metals inhibited the growth of M1. Of all tested metals, Cd^{2+} inhibited M1 growth most strongly while Ni^{2+} did least, with 0.25 and 1.55 of OD_{600} in 0.5 LB achieved, respectively. The inhibition order under the same concentration of 10 mg/L was as follows: $Cd^{2+} > Zn^{2+} \geq Cu^{2+} > Pb^{2+} > Ni^{2+}$. The result was consistent with conclusions of previous studies that the toxicity varied with metals and microorganisms [18,20]. From Fig. 5(b), it was seen that, except Cu^{2+} and Cd^{2+}, the presence of other coexisting metal ions had no significant effect on bioaccumulation and Hg^{2+} removal ratio reached above 90%. The differences of Hg^{2+} removal ratio under the presence of coexisting Cu^{2+} and Zn^{2+} need to be further explored because their effects on the growth of M1 were basically the same. It might partly be explained by the different affinities of MT to heavy metals, whose order was Hg > Cu > Cd > Zn > Ni [21]. Furthermore, from the trend of bioaccumulation of Hg^{2+} under the presence of Cd^{2+} and Cu^{2+} shown in Fig. 5(b), the Hg^{2+} removal ratio by M1 might be higher if operation time were longer enough.

4. Conclusions

From the study above, conclusions could be drawn as follows:

(1) Hg^{2+} bioaccumulation of genetically engineered *E. coli JM109* (M1) could be well coupled with its growth. Stronger inhibition on M1 growth occurred with the increasing Hg^{2+} concentration, but the specific bioaccumulation capacity of Hg^{2+} increased with increasing metal/biomass ratio and even reached 25 mg Hg^{2+}/g dry cell at 4 mg/L Hg^{2+} concentration.

(2) Na^{+} was essential to M1 growth. 0.04 mol/L Na^{+} was helpful to M1 growth, while stronger inhibition on M1 growth occurred with the increase of Na^{+} concentration. However,

Hg^{2+} bioaccumulation by growing cells of M1 was not so sensitive to Na^{+}. The presence of 0.1 mol/L Mg^{2+}or Ca^{2+} was good to M1 growth, while 0.3 mol/L Mg^{2+}or Ca^{2+} inhibited M1 growth strongly. The Hg^{2+} removal ratio dropped slightly and still reached about 70% even when M1 growth was inhibited strongly by the presence of 0.3 mol/L Mg^{2+}or Ca^{2+}.

(3) EDTA had a significant effect on M1 growth, but M1 cells with low density still exhibited good Hg^{2+} bioaccumulation capacity. Citrate had little effect on M1 growth between the citrate concentrations of 0–51 mmol/L. Bioaccumulation of Hg^{2+} was not affected by the presence of citrate, and the Hg^{2+} removal ratios were all more than 90%.

(4) Coexisting metal ions affected M1 growth. The inhibition order under the same concentration of 10 mg/L was as follows: $Cd^{2+} > Zn^{2+} \geq Cu^{2+} > Pb^{2+} > Ni^{2+}$. Except Cu^{2+} and Cd^{2+}, the presence of other coexisting metal ions had no significant effect on bioaccumulation and Hg^{2+} removal ratio reached above 90%.

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